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REVIEW

Functional genes associated with the occurrence of mycotoxins produced by *Aspergillus* in foods

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Highlights

- This review provides a comprehensive summary of the genes involved in aflatoxin biosynthesis and constructs the regulatory network governing toxin production, offering new insights for the development of aflatoxin control strategies.
- It also systematically summarizes the functions of key genes regulating the growth and development of *Aspergillus* species, thereby laying a theoretical foundation for the development of source-level control technologies against *Aspergillus*.

Abstract

Aspergillus species are ubiquitous fungi that produce mycotoxins (secondary metabolites) known as sterigmatocystin and aflatoxins in many different kinds of foods, which leads to serious contamination in agricultural products, thereby endangering human health. Extensive studies on *Aspergillus* fungi have been conducted on growth and development, aflatoxin biosynthesis, and their interactions with environment. Here, we summarized a series of functional genes of the main *Aspergillus* fungi relative to toxins occurrence in foods, which revealed the signal transduction mechanisms of their involvement in growth and development, toxin production, and response to light, anticipating providing theoretical guidance on developing control and prevention technologies for mycotoxin contamination in agricultural products to ensure food safety.

Keywords: functional genes, *Aspergillus*, aflatoxin, development

1. Introduction

Aspergillus fungi are known to produce a range of biologically active secondary metabolites, including antibiotics and mycotoxins, the latter of which are toxic secondary metabolites produced by certain fungi (Kato *et al.* 2003). Due to their high incidence and the severe threats they pose to human and animal health, mycotoxins have long been a global concern (Jia *et al.* 2021). Mycotoxin contamination may occur in different matrices such as field cereals (Haque *et al.* 2020), feeds (Weaver *et al.* 2020), and traditional Chinese medicines (Zhang *et al.* 2018). Globally, the rate of mycotoxin contamination in agricultural and feed products ranges from 30 to 70%, among which cereals have received special attention due to their high incidence of contamination and large consumption by humans and animals (Rodrigues and Naehrer 2012; Streit *et al.* 2013).

So far, hundreds of mycotoxins of different categories have been discovered, among mycotoxins, sterigmatocystin (ST) is mainly the final metabolite of heterotrophic *Aspergillus* is also an intermediate product in the late synthesis of aflatoxin. ST can cause DNA damage, leading to DNA breakage and chromosome aberration. Eating grains contaminated with ST can cause loss of appetite, depression, fatigue, and even death in humans and animals. In addition, ST has been to induce hepatocellular carcinoma and squamous cell carcinoma (Xie *et al.* 2000).

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Aflatoxins are a group of polyketide-derived furanocoumarins widely found in various food crops, including cereals, nuts, dairy products, and other agricultural products. They are among the most toxic and carcinogenic mycotoxins known. Although *A. flavus* and *A. parasiticus* are the primary producers of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), several other, less common species — such as *Aspergillus nomius*, *Aspergillus pseudotamarii* (*Aspergillus pseudocaelatus*), *Aspergillus ochraceoroseus*, *Emericella venezuelensis*, and *Aspergillus pseudonomius* also produce aflatoxins (Varga *et al.* 2011; Roze *et al.* 2013). Other members of the aflatoxin family, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2), are oxidized forms of AFB_1 isolated from milk, urine, and feces of certain animals (Yu *et al.* 2004). Of these, the group B, group G, and group M aflatoxins (particularly AFM_1) are considered the most critical from a food-safety perspective (Jia *et al.* 2021).

Aspergillus nidulans is widely recognized as a powerful model system for dissecting eukaryotic genetics, secondary metabolism, and the interactions between secondary metabolism and environmental factors (Bayram *et al.* 2008b, 2016). As knowledge in the field has advanced, numerous functional genes originally identified in *A. nidulans* have also been explored in other *Aspergillus species*, such as *A. flavus*, *A. parasiticus*, and *A. fumigatus*. Cross-species investigations of homologous functional genes not only clarify their evolutionary conservation and uncover additional roles but also reveal species-specific differences and offer promising directions for practical applications. Understanding the roles of genes that regulate growth, reproduction, and toxin biosynthesis in *Aspergillus* is essential for devising innovative approaches to mitigate mycotoxin contamination. Such knowledge enables the inhibition of toxin production at its origin, thereby reducing overall contamination risks. Nevertheless, most current reviews focus on isolated biological processes and fail to provide a systematic and integrated summary of functional genes across the major *Aspergillus* species.

This review provides an integrated overview of key functional genes implicated in the biosynthesis of major secondary metabolites, such as ST and aflatoxins, as well as genes governing growth, development, and environmental responses (e.g., light) in *A. nidulans*, *A. flavus*, *A. parasiticus*, and *A. fumigatus*.

2. The genes involved in sterigmatocystin and aflatoxin biosynthesis pathway

The sterigmatocystin and aflatoxin biosynthesis pathways have been characterized, in which ST serves as a representative late-stage intermediate preceding aflatoxin formation (Yabe *et al.* 1989). Previous studies have revealed that the ST biosynthesis gene cluster in *A. nidulans*, which comprises at least 25 genes, is highly homologous to the aflatoxin synthetic gene cluster in *A. flavus* and *A. parasiticus* (Keller *et al.* 1995a; Brown *et al.* 1996). In *Aspergillus* species, the genome is organized into eight chromosomes, with the aflatoxin biosynthetic cluster located in cluster 54 on chromosome 3. Approximately 30 genes within this cluster are under the control of the transcription factors *aflR* and *aflS* (Chang 2003).

To clarify the roles of genes at each enzymatic step of the ST and AF biosynthetic pathways, the discussion is organized around several key stable intermediates. These include norsolorinic acid (NOR), versicolorin B (VER B), demethylsterigmatocystin (DMST) and demethyldihydrosterigmatocystin (DHDMST), along with the enzymatic conversions of DMST to ST, AFB_1 and AFG_1, and the conversion of DHDMST to AFB_2 and AFG_2.

2.1. Conversion of acetate into NOR

*Afl*A (*fas-2*), *afl*B (*fas-1*), and *aflC* (*pksA*) encode a fatty acid synthase alpha, a fatty acid synthase beta, and polyketide synthase (PksA) respectively. These enzymes catalyze the primary metabolite acetate in AF-producing pathway to form polyketide backbone of AFB_1. This step is required for the early steps of aflatoxin biosynthesis (Chang *et al.* 1995; Brown *et al.* 1996; Payne and Brown 1998).

HypC encodes a 17 kDa oxidase that is similar to monooxygenase in bacteria and catalyzes the oxidation of nor-anthronic acid (NAA). Therefore, this enzyme catalyzes the conversion of norsolorinic acid anthrone to NOR in the aflatoxin biosynthesis pathway. *HypC* is expressed only under conditions that favor AF production and is located between the *pksA* (*aflC*) and *nor-1* (*aflD*) genes in the aflatoxin biosynthesis gene cluster. This enzyme contains a domain of unknown function (DUF 1772) that may mediate protein-protein interactions. In addition, there is a typical binding sequence for the AF pathway-specific transcription factor AflR 168 bp upstream of the *hypC* transcription start site. However, the loss of *hypC* does not affect aflatoxin production (Ehrlich *et al.* 2010b), suggesting that the possibility of alternative, enzyme-independent mechanisms compensating for its loss.

2.2. Conversion of NOR into versicolorin B

AflD (*nor-1*) encoding a norsolorinic acid ketoreductase catalyzes the transformation from the 1′-ketogroup of NOR to the 1′-hydroxyl group of averantin (AVN) (Zhou and Linz 1999).

AflE (*norA*) is presumed to catalyze the conversion of NOR to AVN in the biosynthesis pathway of aflatoxin (Cary *et al.* 1996). They isolated a 43 kDa enzyme named *norA*. This enzyme has norsolorinic acid reductase activity and is located upstream of *ver-1* in the aflatoxin biosynthesis gene cluster. This study also identified a homologous gene of *norA* in *A. flavus*, which also illustrates the high conservation of *norA* in the toxin-producing fungi *A. flavus* and *A. parasiticus*. The expression of norA is tightly associated with that of the aflatoxin regulatory gene aflR. In *A. flavus*, deletion of aflR abolishes norA expression, and non-toxigenic A. parasiticus strains likewise fail to express either aflR or norA. (Cary *et al.* 1996).

AflG (*avnA*) encodes a fungal cytochrome P450 enzyme that is involved in the conversion of averantin (AVN) to averufin (AVF) in the aflatoxin biosynthetic pathway in *A. parasiticus*. *AflG* encodes a protein consisting of 495

amino acids, with an approximate molecular weight of 56.3 kDa. And it is located between *ver-1* and *omtA* in the aflatoxin biosynthetic gene cluster. In *A. parasiticus*, the deletion of *avnA* resulted in the accumulation of AVN, which fails to be converted to aflatoxins. This was the first report of a gene encoding a cytochrome P450 enzyme involved in aflatoxin biosynthesis among several putative fungal genes (Yu *et al.* 1997) .

The dehydrogenase encoded by *aflH* (*adhA*) is involved in catalyzing the conversion of 5´-hydroxyaverantin (HAVN) to AVF in the biosynthesis of aflatoxin in *A. parasiticus* (Chang *et al.* 2000). Further studies have found that the conversion of HAVN to AVF consists of two enzymatic reactions: The first is the conversion of HAVN to a novel intermediate 5´-oxoaverantin (OAVN) catalyzed by HAVN dehydrogenase (*adhA*), and the second is the conversion of this intermediate to AVF catalyzed by *aflK* (*vbs*) encoding verb synthase (Sakuno *et al.* 2003, 2005). *AdhA* is a homodimer composed of 28 kDa subunits and requires NAD rather than NADP as a cofactor to function (Sakuno *et al.* 2003).

AflI (*avfA*) was found to be involved in the conversion of AVF to versiconal hemiacetal acetate (VHA) in *A. parasiticus* and *A. flavus*. Studies have shown that *aflI* encodes a protein containing 285 amino acids and a molecular weight of 30.9 kDa in *A. flavus*, *A. sojae*, and *A. parasiticus*. The amino acid sequence of *aflI* in *A. parasiticus* is 89% homologous to *aflI* in *A. flavus* and 98% homologous to *aflI* in *A. sojae*. In the aflatoxin biosynthesis gene cluster, the transcription terminator of *omtB* is located only 173 bp away from the start codon of *aflI* (Yu *et al.* 2000). In addition, *aflV* (*cypX*) and *aflW* (*moxY*) have also been shown to be involved in this step. Specifically, *aflV* encodes averufin monooxygenase, which catalyzes the transformation of AVF into hydroxyversicolorone (HVN), and *aflW* (*moxY*) encodes HVN monooxygenase, which catalyzes the conversion of HVN to VHA through the Baeyer-Villiger reaction (Wen *et al.* 2005).

AflJ (*estA*) encoding an esterase is involved in catalyzing VHA to versiconal (VAL). *estA* is located between *adhA* and *norA* in the aflatoxin biosynthesis gene cluster. The expression pattern of *EstA* is different from that of other genes in the aflatoxin biosynthesis gene cluster (*nor-1*, *ver1*, *omtA*, *ordA*), and it is expressed even under conditions that are unfavorable for aflatoxin production. The promoter region of *estA* contains two AFLR binding motifs ($TCGN_5CGA$) (Yu *et al.* 2003). Further studies have shown that *estA* catalyzes the transformation of VHA to versiconal (VHOH) and the transformation of versiconol acetate (VOAc) to versiconol (VOH) (Chang *et al.* 2004a).

AflK catalyzes the conversion of VHOH to versicolorin B (VerB). However, high concentrations of VHOH can inhibit *aflK*-catalyzed conversion of OAVN to AVF, but can catalyze the formation of versicolorin B (Sakuno *et al.* 2005).

2.3. Conversion of VER B into DMST and DHDMST

A desaturase encoded by *aflL (verB)* is presumed to catalyze the conversion of VER B into versicolorin A (VERA) in *A. parasiticus*, *A flavus*, and *A. nidulans* (Yabe and Hamasaki 1993; Kelkar *et al.* 1997). A study showed that the deletion of *aflL* in *A. nidulans* leads to the accumulation of VER A (Keller *et al.* 1995b). Later, further study reported that *aflL* may be involved in the conversion of VER B to DHDMST (Yu *et al.* 2004).

AflM (*ver-1*) encoding a NADPH-dependent oxidoreductase and *aflN* (*verA*) encoding a cytochrome P450 catalyze the conversion of VERA to DMST (Keller *et al.* 1994; Henry and Townsend 2005). *AflY* (*hypA*) encoding a Baeyer-Villiger oxidase is also predicted to be involved in this process. This enzyme is required for the formation of the flavonoid ring of the aflatoxin precursor DMST. Deletion of *aflY* leads to the accumulation of ST in the mutant (Ehrlich *et al.* 2005). *AflX* encoding a NADH-dependent oxidoreductase has also been shown to be involved in this process. Deletion of *aflX* leads to a more than four-fold increase in VERA and a more than four-fold decrease in aflatoxin in the mutant compared to the wild-type *A. flavus* strain. *AflX* is predicted to have a typical domain structure for an NADH-dependent oxidoreductase and has 27% homology at the amino acid level with the protein encoded by the aflatoxin cluster gene *avfA* (Cary *et al.* 2006).

2.4. Conversion of DMST to ST, AFB_1 and AFG_1 and DHDMST to AFB_2 and AFG_2

AflO (*omtB*) encodes an O-methyltransferase and is responsible for catalyzing the conversion of DMST to ST and the conversion of DHDMST to dihydrosterigmatocystin (DHST) in the aflatoxin biosynthesis pathway. This gene is highly homologous to *stcP* in *A. nidulans* and is expressed only under culture conditions that favor aflatoxin production (Yu *et al.* 2000).

AflP (*omtA*) is involved in the conversion of ST to O-methylsterigmatocystin (OMST) and DHST to DHOMST during aflatoxin biosynthesis in *A. parasiticus* (Yu *et al.* 1993). *AflP* (*omtA*) has 97% sequence homology between *A. parasiticus* and *A. flavus*. The substrate binding motif of the *omtA* gene is identical in *A. parasiticus* and *A. flavus* (Yu *et al.* 1995)

AflQ (*ordA*) encodes a cytochrome P450 monooxygenase involved in the conversion of OMST to aflatoxin B_1 (Yu *et al.* 1998). It encodes a 60.2 kDa protein containing 528 amino acids (Prieto and Woloshuk 1997), and is adjacent to *omtA* in the aflatoxin biosynthesis gene cluster. The conserved heme binding and redox domains encoded by *aflQ* (*ordA*) are critical for cytochrome P450 catalytic activity. The *ordA* has been classified into a newly identified cytochrome P450 gene family, CYP64 (Yu *et al.* 1998).

HypB (*hypB2*) is predicted to encode a 17 kDa protein located between *verB* and *avfA*. It is another hypothetical gene found in the aflatoxin cluster in *A. parasiticus* and *A. flavus*. *HypB* is expressed under conditions that induce aflatoxin production (Ehrlich 2009; Roze *et al.* 2013). The putative promoter region of *hypB* has a TCGN5CGA motif, which is a typical binding sequence for the transcriptional regulator *aflR* in the aflatoxin biosynthesis pathway. *HypB* is involved in the second oxidation step of OMST to aflatoxin, participating in the introduction of oxygen into the

keto tautomer of HOMST and rearrangement to a 370 Da intermediate (Ehrlich 2009).

In *A. flavus*, the *aflE* (*norA*) deletion mutant produces more NorA substrate AFOH, which undergoes acid-catalyzed dehydration under acidic conditions to produce deoxy. These recent studies support the hypothesis that *aflE* is involved in the final two steps of group B aflatoxin biosynthesis (Ehrlich *et al.* 2010a).

HypE is part of the aflatoxin gene cluster and is predicted to contain an EthD domain that is required for the oxidative degradation of ethyl tert-butyl ether in the presence of cytochrome P450 monooxygenases (Holmes 2008; Ehrlich 2009). Orthologs of *hypE* exist in many fungi (Ehrlich 2009). *HypE* deletion mutants produce fewer AFB_1 and AFB_2 than wild-type *A. flavus* strains (Holmes 2008). Further studies have shown that *hypE* may cooperate with one of the five AF biosynthetic cluster cytochrome P450 monooxygenases (probably CypX or OrdA) to hydroxylate the methyl ether, initially forming an acetal intermediate, which subsequently yields AFB_1 through the loss of formaldehyde (Ehrlich 2009).

Further studies have shown that the biosynthesis of group G aflatoxins requires additional enzymes. For example, *aflU* (*cypA*) encodes a cytochrome P450 monooxygenase. When *aflU* is disrupted in *A. parasiticus*, the mutant cannot produce G-group aflatoxins, but can produce B-group aflatoxins (Ehrlich *et al.* 2004). The *nadA* gene in the aflatoxin gene cluster encodes a cytosolic enzyme that is involved in the last step in aflatoxin biosynthesis, catalyzing the conversion of NADA (a new yellow fluorescent pigment and a precursor of AFG_1) to AFG_1. It is located at the end of the aflatoxin gene cluster and is only expressed in a culture medium that is conducive to aflatoxin production and is not expressed in *aflR* deletion mutants (Cai *et al.* 2008). *AflF (norB)* encoding an aryl alcohol dehydrogenase is responsible for the final oxidation step in the formation of AFG_1 (Ehrlich *et al.* 2008).

2.5. Genes with uncharacterized role in the aflatoxin biosynthesis pathway

In the aflatoxin pathway gene cluster of *A. parasiticus*, *aflT* encodes a transmembrane protein of 514 amino acids with 14 putative transmembrane domains. *AflT* is located between *cypA* and *pksA* and has homology with multiple genes encoding multidrug resistance proteins or toxin transporters. In addition, *aflT* in *A. parasiticus* has 98% homology with *aflT* in *A. flavus* and is located in the same position as in *A. parasiticus* in the *A. flavus* aflatoxin gene cluster. The promoter region of *aflT* lacks the binding site of AflR ($TCGN_5CGA$), therefore *aflT* is not regulated by *aflR* and *aflS*, and the loss of *aflT* does not affect toxin formation and secretion (Chang *et al.* 2004b).

In the aflatoxin cluster, a 129 Da integrated membrane-binding protein with a DUF6 domain was discovered, designated as HypD, which affects fungal development and reduces aflatoxin production, and is found in *A. ochraceoroseus*, *A. nidulans*, *A. fumigatus*. It is highly conserved among fungi such as *Aspergillus*. In *A. parasiticus*, deletion of *hypD* resulted in significantly enhanced sporulation ability and reduced production of AFB_1 and AFG_1. This result indicates that *hypD* affects development and secondary metabolism. This may reflect an inverse relationship between fungal development and secondary metabolism. and the inner membrane protein HypD acts as a permease, loss of *hypD* leads to feedback inhibition of aflatoxin biosynthesis, which simultaneously leads to an increase in conidia formation and a decrease in aflatoxin (Ehrlich 2009).

Recently, a protein named PO8, identified in *A. flavus* and *A. parasiticus* was reported as a biomarker for predicting aflatoxin production (Liang *et al.* 2022; Yan *et al.* 2022). Subsequently, another protein with a similar function, designated PAB01, was identified (Fu *et al.* 2023). These findings suggested that the genes encoding PO8 and PAB01 are involved in aflatoxin occurrence. However, their specific roles in aflatoxin biosynthesis remain unclear.

2.6. Aflatoxin cluster-specific regulators

AflR encodes a sequence-specific Gal4-type 47 kDa polypeptide containing a cysteine-rich region (Cys-2-Cys-6-Cys-6-Cys-2-Cys-6-Cys) that is a DNA-binding binuclear zinc cluster (Zn(II)2Cys6) protein in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Yu *et al.* 1996a; Yu and Keller 2005). Zinc finger binding proteins with conserved DNA binding specificity are a superfamily of regulatory factors unique to fungal proteins and play key roles in transcription regulation (MacPherson *et al.* 2006). The AflR protein is highly homologous in *A. flavus* and *A. parasiticus*. The AflR protein of *A. nidulans* has only 33% homology between these two fungi, but the zinc finger domain is highly homologous among three *Aspergillus* species. Induced expression of the *A. flavus aflR* gene in *A. nidulans* led to activation of genes in the ST biosynthesis pathway. The results confirmed that the function of AflR is conserved among *Aspergillus* species and that *aflR* expression is sufficient to activate the expression of genes in the ST pathway (Yu *et al.* 1996a).

Another study demonstrated that AflR protein can regulate *aflC*, *aflD*, *aflH*, *aflE*, *aflM*, *aflP*, *aflQ*, and *aflK* genes within the ST or aflatoxin biosynthesis gene cluster. This regulatory activity is likely due to the ability of AFLR protein in *A. parasiticus*, *A. flavus*, and *A. nidulans* to recognize and bind a conserved motif 5′-$TCGN_5CGA$-3′ in the promoter regions of these genes (Ehrlich *et al.* 1999). Correspondingly, the knockout of *aflR* in *A. nidulans* resulted in the inability to produce ST or AF, and other genes in the ST biosynthesis pathway were not expressed (Yu *et al.* 1996a). These results indicate that *aflR* is a specific positive regulatory activator of the ST/AF biosynthesis pathway.

AflS (*AflJ*) belongs to a protein family that appears to be restricted to fungi. The protein is predicted to have three transmembrane domains (Ehrlich *et al.* 2012). *AflS* is located in the aflatoxin synthesis gene cluster adjacent to the regulatory gene *aflR*, but the mode of transcription is different from that of *aflR*. The deletion of *aflS* in *A. flavus* resulted in the inability to produce aflatoxins, and the pathway intermediates NOR, ST, and OMST cannot be converted into aflatoxins (Meyers *et al.* 1998). Overexpression of *aflJ* in *A. flavus* led to an increase in aflatoxins. Overexpression of *aflR* increased the transcription level of *aflJ*, and *aflR*

regulated the transcription level of *aflJ* (Du *et al.* 2007). In *A. parasiticus*, disruption of *aflJ* resulted in a pigment-free mutant that was unable to synthesize aflatoxin intermediates. In the absence of *aflJ*, gene transcript levels in the early (*pksA* and *nor1*), middle (*ver1*), and late (*omtA*) steps of the aflatoxin biosynthetic pathway were significantly reduced, up to 20-fold. However, while deletion of *aflJ* does not alter *aflR* transcript levels, *aflJ* interacts with *aflR* but shows no interaction with *nor1*, *ver1*, *omtA*, or *ordA* (Chang 2003). Therefore, *aflJ* was considered to be a transcriptional co-activator. The genes mainly involved in the ST and aflatoxin biosynthesis pathway are shown in Fig. 1.

3. The genes involved in the reproductive processes of *Aspergillus*

Development is associated with secondary metabolism in filamentous fungi (Calvo *et al.* 2004). A deeper understanding of these processes will provide us new insights for developing targeted strategies to prevent and control fungal growth and the mycotoxin production, thereby reducing mycotoxin contamination in food and agricultural products and ensuring the safety of grain and oil products.

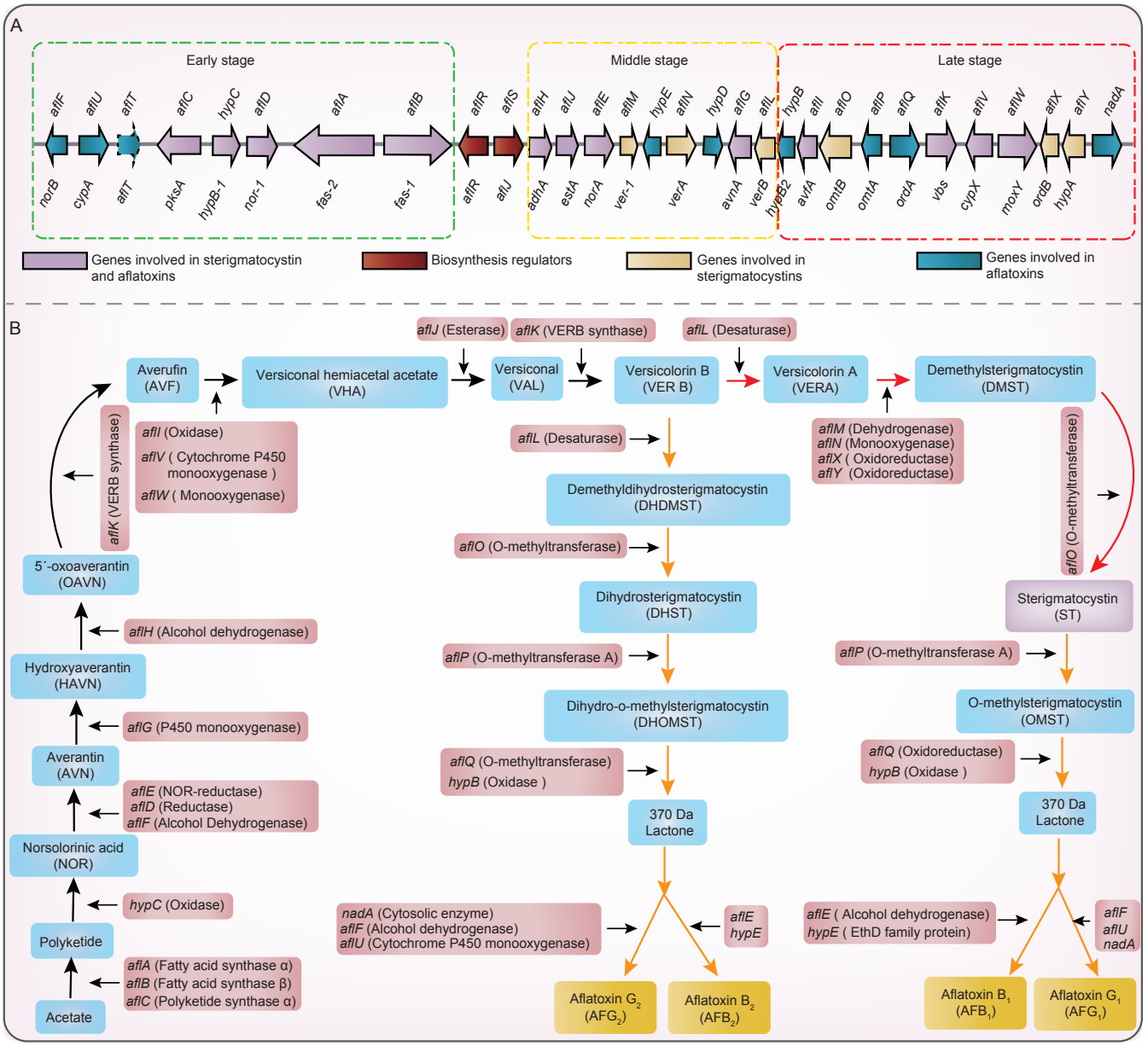


Fig. 1 Genes mainly involved in the biosynthesis pathways of sterigmatocystin and aflatoxin. A, the sterigmatocystin and aflatoxin biosynthetic gene cluster in *Aspergillus parasiticus* and *A. flavus* including the old and new gene nomenclatures. The direction of the arrow represents the direction of gene transcription; the dotted line indicates that the function of this gene is unclear. And the green, yellow and red dashed boxes represent genes expressed in the early, middle and late stages of aflatoxin synthesis, respectively. B, the sterigmatocystin and aflatoxin biosynthetic pathway in *A. parasiticus* and *A. flavus*. The black arrow represents the common biosynthesis pathway of sterigmatocystin and aflatoxin, the red arrow represents the biosynthesis pathway of sterigmatocystin, and the yellow arrow represents the biosynthesis pathway of aflatoxin.

Aspergillus nidulans has long been a model fungus for studying fungal growth and reproduction and mycotoxin production (Chang *et al.* 2017). Taking *A. nidulans* as an example, this section outlines the physiological structures formed by filamentous fungi during sexual and asexual development, emphasizing their functional significance in response to light variability. *Aspergillus nidulans* has two different reproductive development processes, one is asexual development that preferentially differentiates under light conditions, and the other is sexual development that preferentially differentiates under dark conditions (Bayram *et al.* 2016). First, under light conditions, *A. nidulans* preferentially undergoes asexual development, which consists of growth, initiation, and formation stages (Adams *et al.* 1998). The stems extending from the thick-walled foot cells form conidiophores, which swell to form small vesicles, and conidiophores form in the small vesicles. And then, chain conidia are derived from them, and these spores released into the air, which is the main source of the spread of fungus (Fischer and Kües 2006).

In the absence of a light signal, *A. nidulans* undergoes primarily the sexual cycle because asexual development is delayed. During sexual development, *A. nidulans* produces closed spherical fruiting bodies, cleistothecia, in which sexual spores undergo meiosis and are surrounded by Hülle cells, which are thought to provide them with protection and nutrition (Adams *et al.* 1998; Sarikaya Bayram *et al.* 2010). However, when the signals for sexual development may come remains to be determined, it is usually accepted that it does not appear earlier than the signals for asexual development (Kim *et al.* 2002).

In brief, both asexual and sexual development processes are subject to temporal and spatial regulation of gene expression, thereby forming specialized reproductive structures (Adams *et al.* 1998; Calvo *et al.* 2002; Fischer and Kües 2006). To date, many functional genes involved in sexual and asexual development have been identified and characterized (Wu and Miller 1997; Adams *et al.* 1998; Kim *et al.* 2002). However, the relationship between these genes and signal cascades still needs further research and confirmation.

3.1. Sexual development

Previous studies have identified several functional genes associated with sexual development in *Aspergillus*, including *nsdD*, *esdC*, *steA*, and *stuA*. The following section provides a detailed description of each of these genes.

NsdD encoding a GATA-type zinc-finger domain protein contains 461 amino acid residues, and its expression is affected by MpkB (a MAPK gene) and *flbA*, a regulator of G protein signaling (RGS) protein. It antagonizes the signal transmitted by G proteins consisting of FadA, SfaD, and GpgA. In *A. nidulans*, *nsdD* is expressed during the early stage of vegetative growth, and its transcript level progressively increases as sexual development processes. During asexual development, the expression level of *nsdD* decreases during sporulation and becomes silenced during conidia formation. Even under conditions favorable for sexual development, *nsdD* deletion mutants do not form cleistothecia (Han *et al.* 2001). Conversely, overexpression of *nsdD* led to the formation of sexual development-specific structures (Hülle cells) even under conditions unfavorable to sexual development, indicating that *nsdD* plays a critical role in activating sexual development (Han *et al.* 2003).

NsdC encodes a novel C_2H_2-type transcription factor with zinc-finger DNA-binding domain that is highly conserved among most fungi species. Deletion of *nsdC* resulted in the absence of cleistothecia and a reduced growth rate. In contrast, overexpression of *nsdC* accelerated cleistothecia formation, and enabled the fungus overcome adverse external environmental conditions (such as high concentrations of glucose or hypoxia), thereby promoting the formation of fruiting bodies. In the same study, it was also found that neither knockout nor overexpression of *nsdD* or *veA* affected the expression level of *nsdC*, indicating that *nsdD* or *veA* does not directly regulate the expression of *nsdC* (Kim *et al.* 2009). These results indicate that *nsdC* functions as a positive regulator of sexual development and a negative regulator of asexual development.

EsdC encodes a 29.4 kDa polypeptide consisting of 266 amino acids and contains a putative glycogen binding domain in the β subunit of the AMP-activated protein kinase (AMPK) complex. However, the glycogen binding domain in the AMPK β subunithas no homology with the glycogen binding domain in EsdC protein (Han *et al.* 2008). AMPK is activated by sensing the AMP/ATP ratio in cells through a series of complex and sensitive mechanisms. At the same time, in order to maintain the energy balance in cells, AMPK activates metabolic pathways that can produce ATP while inhibiting cell growth and other life processes that consume ATP (Hardie *et al.* 2006). In addition, *esdC* is highly conserved among fungi species. This suggested that *esdC* may contain a glycogen binding domain, which involves in regulating developmental processes at different growth stages. The Δ*esdC* mutants formed more conidiophores than the wild-type strain, but did not produce sexual structures under any conditions. Overexpression of *esdC* also did not induce sexual development. Furthermore, FlbA negatively regulates *esdC* and *nsdD* expression by inactivating the FadA and SfaD G protein subunits (Han *et al.* 2008).

The first protein Ste12 was isolated and identified in the filamentous fungus *A. nidulans*, named SteA (Vallim *et al.* 2000). They found that the protein encoded by *steA* has 63–75% similarity to the homologous domains of other Ste12 proteins, with the highest similarity to FnSte12ap. SteA carries a double C_2H_2 zinc finger domain at its C-terminus, which is a potential target of the mitogen-activated protein kinase (MAPK) signaling pathway and plays a central role in determining cell morphogenesis. The Δ*steA* deletion mutants failed to produce cleistothecia and ascogenous tissue, ascospores, but produced Hülle cells. Deletion of *steA* did not affect the expression of *stuA*, *brlAα*, and *brlAβ*, delayed the accumulation of *abaA* RNA, and suppressed the expression level of *medA*. These results indicated that *stuA* acts upstream of *medA* in the regulatory pathway of sexual development. In *A. oryzae*, the noticeable phenotypic changes were observed when *steA* was disrupted. However,

in the *steA* overexpression strain, vegetative hyphae and conidia showed restricted growth, formed balloon-like structures. Additionally, secretory cell wall degrading enzymes, such as 1,3-β-glucanase, were increased. The phenotypic changes observed in the *steA* overexpression strain may result from the rearrangement of cell wall components, indicating that *steA* may be involved in cell wall metabolism (Morita *et al.* 2007).

During the growth and development of *A. nidulans*, developmental modifier genes, such as *stuA* and *medA*, in addition to the core regulatory pathways, are required for establishing the normal pattern of cell differentiation (Busby *et al.* 1996).

In *A. nidulans*, the expression level of *stuA* increases sharply and remains high during conidiophore formation, indicating that *stuA* is required from the onset of asexual reproduction through to the completion of conidiophore development. However, it does not appear to be essential for conidial differentiation, and the expression of *stuA* transcripts is not affected by other known developmental mutations (Miller *et al.* 1991). When *stuA* expression decreases, the morphology of conidiophores changes and the number of conidia decreases, but the density of conidiophores remains unaffected (Wu and Miller 1997). In *A. fumigatus*, *stuA* deletion mutants were impaired in asexual reproduction, with conidiophores showing pronounced morphological abnormalities, producing only a small number of deformed conidia, and exhibiting premature germination (Sheppard *et al.* 2005). Therefore, *stuA* is required for the proper spatial organization of conidiophores, and regulates the correct cell pattern formation and initiates the sexual reproduction cycle in *A. nidulans*, thereby contributing to overall fungal development and cell cycle progression.

In *A. nidulans*, the loss of *medA* delayed the differentiation of phialides and conidia and the proliferation of the branching chains of sterigmata. The results of the study confirmed that *medA* affects the expression of core regulatory pathways at key points in the cell cycle. First, during the early stage of development, *medA* inhibits the premature expression of two *brlA* transcripts, *brlAα* and *brlAβ*, thereby blocking the activation of *abaA* expression. In the late stage of development, *brlAβ* expression is downregulated. In addition, *medA* functions as auxiliary activator of *abaA* expression. These observations suggest that the delayed differentiation of phialides in the *medA* deletion mutant is likely due to the failure to activate *abaA* expression in the absence of *medA* (Busby *et al.* 1996).

NosA and *rosA* were characterized as the putative Zn(II)2Cys6 transcription factor. *nosA* encodes a protein of 675 amino acids and has 44% sequence homology with Pro1 in *Sordaria macrospora*. *nosA* is stably and continuously expressed throughout the life cycle of *A. nidulans*, although its expression is upregulated during the late stages of asexual development and under conditions of carbon source deficiency. The transcript level of *rosA* is generally low, but it becomes upregulated under conditions of carbon source deficiency and at 12 h of asexual development (Vienken and Fischer 2006).

RosA inhibits sexual development and plays a key role in determining whether a strain enters sexual development or asexual development at an early stage. Under standard culture conditions, deletion of the rosA gene slightly induces the formation of ascospore structures and upregulates the expression of *nsdD*, *veA*, and *stuA*. Under low glucose and high osmotic pressure conditions, the mutants can initiate sexual development, and even Hülle cells formed under submerged growth conditions in liquid medium. In addition, overexpression of *rosA* resulted in reduced hyphal growth and a fluffy phenotype (Vienken *et al.* 2005). *NosA* shares 43% sequence identity with *rosA* and both of them regulate sexual development. *RosA* inhibits the expression of *nosA*, indicating the genetic correlation of these two genes (Vienken and Fischer 2006).

3.2. Asexual development

In darkness, *A. nidulans* will preferentially undergo asexual development. During this progress, the fungus transitions from vegetative growth to the formation of specific asexual structures known as conidiophores. These structures consist of foot cell, stalk, vesicle, subsequent layers of primary sterigmata (metulae), subsequent layers of secondary sterigmata (phialides), and long chains of unicellular mitospores (conidia). The transition from vegetative growth to development requires the coordinated completion of multiple regulators, including FLB proteins, BrlA, AbaA and WetA. The maturation of conidia also requires the coordinated completion of multiple regulators such as VosA and VelB. Here, we mainly introduce the role of the main functional genes in the process of asexual development.

The study of G protein-mediated signaling pathway in a variety of filamentous fungi has contributed to understanding the regulation of fungal development and mycotoxin production. Heterotrimeric G proteins are members of the GTPases superfamily and are composed of α, β and γ subunits. As secondary messenger, G proteins play critical roles in cell regulation. It initiates two downstream cascade pathways: one that affects intracellular cAMP levels by regulating adenylyl cyclase, and the other one being mitogen-activated protein kinase cascade (Morris and Malbon 1999; Deepika *et al.* 2016). In addition, heterotrimeric G proteins not only produce effectors involved in initiating and amplifying cellular responses but also serve as structural unit of the heterotrimeric G proteins signaling complex (Morris and Malbon 1999). In fungi, G proteins regulate major biological processes, including cell division, cell–cell fusion, morphogenesis, secondary metabolism, spore formation and even pathogenicity (Tilburn *et al.* 1995; Bölker 1998; Lengeler *et al.* 2000).

FadA (fluffy autolytic dominant A) encodes the α-subunit of a heterotrimeric G-protein in *A. nidulans* (Wieser *et al.* 1997). The active form of FadA (FadA-GTP) stimulates growth, represses differentiation and ST production (Yu *et al.* 1999). Point mutations in this gene led to the loss of GTPase activity, activation of the proliferation and differentiation pathway, and the obstruction of sporulation. The deletion of *fadA* resulted in overproduction of spores (Yu *et al.* 1996b; Wieser *et al.* 1997). In *A. nidulans*, *flbA* and *fadA* control

the transition of the strain from growth to sporulation and mycotoxin production. FlbA is a G protein signaling regulator that inhibits growth signaling through *fadA*. FadA and PkaA, which is the cAMP-dependent protein kinase catalytic subunit, were found to negatively regulate the synthesis of AF and ST, but *fadA* can positively regulate penicillin production (Deepika *et al.* 2016). PkaA mainly regulates ST biosynthesis by controlling *aflR* and its gene product both transcriptionally and post-transcriptionally (Shimizu and Keller 2001).

Asexual spores are one of the most critical factors in the fecundity and adaptability of filamentous fungi (Wieser *et al.* 1994). Previous studies have demonstrated that the *fluG* gene is a member of a gene family that are required for activation of conidia formation and ST production in *A. nidulans* and functions upstream of *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* (Seo *et al.* 2003). *FluG* acts as a very early regulator in the conidiophore pathway, and activation of *fluG* leads to activation of all genes required for the formation of complete conidiophores, including *flbA*, *flbB*, *flbC*, *flbD*, *flbE* and *brlA*, which locate downstream of *fluG* (Lee and Adams 1996).

In *A. nidulans*, *fluG* has two main functions. First, it removes the inhibitory effect of *sfgA*, which encodes a novel 601 amino acids protein with the Gal4-type Zn(II)2Cys6 binuclear cluster DNA-binding motif at the N terminus and functions as a critical negative regulator of conidiation. This relief leads to activate the expression of *flbA*, which in turn inhibits the FadA-mediated growth signal and indirectly regulates the biosynthesis of ST (Seo *et al.* 2003). Second, *fluG* catalyzes the generation of extracellular signals that activate *brlA* expression and spore development (Wieser *et al.* 1994). This is also confirmed by the loss of *fluG* function blocks conidia development and ST production (Seo *et al.* 2006). In addition, overexpression of *flbA* in a *fluG* null mutant failed to induce conidiophore development or the expression level of *brlA* accumulated. However, overexpression of *fluG* overcame the developmental block in vegetative cells, thereby inducing asexual development and resulting in the formation of normal conidiophore structures (Lee and Adams 1996). The role of *fluG* in the reproductive development and aflatoxin biosynthesis of *A. flavus* has been further characterized. In Δ*fluG* mutants, conidial production was reduced significantly, while the number of sclerotia increased by approximately 30-fold. However, aflatoxin biosynthesis remained unaffected (Chang *et al.* 2012b).

The *flbA* gene (for fluffy low *brlA* expression) encodes an RGS (regulator of G protein signaling) domain protein that plays a critical role in antagonizing vegetative growth signaling, possibly by promoting the intrinsic GTPase activity of FadA. *flbA* is predicted to encode a polypeptide of 717 amino acids and shares 30% sequence identity with the SST2 protein in *Saccharomyces cerevisiae*, which is necessary for yeast cells to identify mating partners and restore growth after long-term exposure to yeast mating pheromone. *FlbA* is not only required for activating conidia development, but also for maintaining normal colony growth, and its expression runs through the entire process of asexual development in *A. nidulans*. Hyphae produced by the Δ*flbA* mutants formed intertwined hyphal masses and eventually lysed instead of activating *brlA* expression and conidia production. Overexpression of *flbA* activates misexpression of *brlA* and also causes hyphal tips to differentiate into spore structures (Lee and Adams 1994). *FlbA* and *fadA*, encoding an RGS domain protein and a heterotrimeric G protein α subunit, respectively, regulate the production of ST and asexual spores, possibly through PkaA, the catalytic subunit of cAMP-dependent protein kinase (Shimizu and Keller 2001). *flbA* expression levels continue to accumulate throughout the life cycle of *A. fumigatus*. When *flbA* was disrupted, conidia reduced, and *brlA* expression was delayed, indicating that the upstream regulator *flbA* contributes to the normal conidial development (Mah and Yu 2006).

In *A. nidulans*, *flbB* is predicted to encode a fungus-specific basic leucine zipper (bZIP-type) transcription factor comprising 426 amino acids, with a molecular weight of 46.8 kDa. *FlbB* is expressed starting in the vegetative growth stage and continues through the early stage of asexual development. When *brlA* transcription begins, *flbB* expression starts to decrease, after 12 hours of asexual development induction, its expression accumulates and is observed in both asexual spores and sexual spores (Etxebeste *et al.* 2008). The absence of *flbB* delayed conidial development, and the Δ*flbB* mutants exhibited defective branching patterns and high autolysis rates under sorbitol or sucrose stress. Overexpression of *flbB* resulted in reduced conidiophore vesicle formation and conidiophore number (Etxebeste *et al.* 2009). Furthermore, *flbB* deficiency resulted in the absence of *brlA* expression, indicating that *flbB* is required for *brlA* expression and is also required for *fluG* to activate conidial development (Etxebeste *et al.* 2008). In *A. fumigatus*, *flbB* is encoded by two transcripts, *flbBα* and *flbBβ*, which are required for asexual development. The shorter transcript *flbBα* is found during conidial development, while the longer transcript *flbBβ* is expressed at a relatively stable level throughout the life cycle. Knockout of *flbB* affected the expression of *brlA* and *abaA*, delayed conidial development, and reduced the number of conidia, and even resulted in premature cell death (Xiao *et al.* 2010).

In *A. nidulans*, *flbC* encodes a 39.3 kDa transcriptional regulator composes of 354 amino acids, which contains two C_2H_2 zinc finger DNA binding domains in the C-terminal region and a potential activation domain in the N-terminal region (Kwon *et al.* 2010b). FlbC locates in the nucleus of hyphae and developmental structures including vesicles, thecae, and phialodes. Deletion of *flbC* led to delayed conidiophore development, fewer conidia, prolonged vegetative growth, delayed expression of *brlA* and *vosA*, and decreased the accumulation of their transcripts. Deletion of *flbC* does not affect the expression and localization of *flbB* and *flbE*. Overexpression of *flbC* led to increased expression levels of *brlA*, *abaA*, and *vosA*, but did not affect the expression level of *wetA*, probably because *flbC* can directly bind to the promoter region of *brlA*, *abaA*, and *vosA*, except *wetA*. Phenotypically, *flbC*-overexpressing strains exhibited restricted-growth and formed abnormal vesicle-like structures, which may indicate that *flbC* overexpression affects multiple genes in different cell types and at different time points. It is noteworthy that the deletion of *brlA* or *abaA* in the *flbC*-

overexpressing strain did not affect the growth inhibition caused by *flbC* overexpression, indicating that *brlA* or *abaA* is not essential for *flbC*-mediated growth inhibition and that *flbC* may have a direct effect on vegetative growth (Kwon *et al.* 2010b).

The *flbD* gene is predicted to encode a polypeptide of 314 amino acids, with its amino terminus showing significant identity with the DNA-binding domain of the Myb transcription factor family. In the wild-type strain, the expression level of *flbD* in vegetative hyphae remains relatively stable following the onset of development (Wieser and Adams 1995). *FlbD* is present in all nuclei of different vegetative hyphae, while *flbB* is only present in most apical nuclei (Garzia *et al.* 2010). Knockdown of *flbD* delayed *brlA* expression and conidiation, and resulted in a fluffy phenotype (Wieser and Adams 1995). In *flbB* and *flbE* deletion mutants, the expression levels of *flbD* and its derived protein were significantly reduced, suggesting that the interaction between *flbB* and *flbE* is a prerequisite for *flbD* expression, and FlbD functions downstream of flbB/FlbE. *FlbB* binds to the promoter of *flbD*. FlbD contains a c-MybDNA-binding domain, and *flbB* and *flbD* bind to the *brlA* promoter region. Therefore, *flbB* activates the expression of *flbD*, and *flbB* and *flbD* jointly activate the transcription of *brlA* (Garzia *et al.* 2010). In addition, overexpression of *flbD* activated conidial development, which required the synergistic action of *brlA*, *flbB* and *flbA*, but did not depend on *flbC* and *flbE*. This result is consistent with the result that *flbC* functions in a different pathway from *flbD* and activates *brlA*. Loss of the *flbC* pathway was partially compensated by the *flbE*, *flbD*, *flbB* pathways, and similarly, the *flbC* pathway partially compensated for the loss of *flbD*, *flbB*, or *flbE* function. In both cases, however, a delayed conidiation phenotype was observed (Wieser and Adams 1995).

FlbE is predicted to encode a protein consisting of 202 amino acids with an estimated molecular weight of 23.1 kDa. The conserved functional domains in *A. nidulans* are unknown (Garzia *et al.* 2009), while in *A. fumigatus flbE* contains 222 amino acids (Kwon *et al.* 2010a). *FlbE* is highly conserved in filamentous fungi such as *A. nidulans*, *A. fumigatus*, *A. niger*, and *A. capsulatus*, *P. nodorum*, and *P. tritici*. In *A. nidulans*, *flbE* is expressed at very low levels in conidia, increases slightly duringvegetative growth, and is barely detectable at other stages. In *A. fumigatus*, its expression remains relatively stable except during the conidial stage and late asexual development (Kwon *et al.* 2010a). FlbB and FlbE are functionally dependent on each other and co-localize at the hyphal tip in an actin cytoskeleton-dependent manner. The stability of FlbB is affected in the absence of FlbE function. FlbB and FlbE act at the same step in the conidian induction pathway (Garzia *et al.* 2009). In *A. nidulans* and *A. fumigatus*, both deletion and overexpression of *flbE* mutants resulted in reduced conidial production, delayed expression of *brlA* and *vosA*, increased autolysis, and premature cell death (Kwon *et al.* 2010a).

It is well known that FluG initiates asexual spore formation at the beginning of the developmental program of *A. nidulans* (Seo *et al.* 2003). Afterwards, the three core regulatory genes *brlA*, *abaA*, and *wetA* that control the development of the asexual reproductive organs conidiophores of *A. nidulans* constitute the core regulatory system of conidia development. In this regulatory cascade, *brlA* is the first gene to be expressed in vegetative cells. Its expression subsequently activates *abaA*, which regulates conidiation. In turn, *abaA* activates *wetA* expression and strengthens *brlA* expression. *WetA* also regulates its own expression (Mirabito *et al.* 1989). This regulatory system is conserved in *A. nidulans* and *A. fumigatus* (Andrianopoulos and Timberlake 1994; Tao and Yu 2011).

In *A. nidulans*, *brlA* encodes a 432 amino acid transcriptional regulatory polypeptide containing two direct repeat motifs and is presumed to be a nucleic acid binding protein that controls the activation of developmental genes during vesicle formation, thereby mediating the budding growth of conidiophores (Adams *et al.* 1988; Calvo *et al.* 2002). Transcripts of *brlA* consist of overlapping α and β transcription units that encode C_2H_2 zinc finger proteins with different functions for proper conidiophore development (Prade and Timberlake 1993).

Disruption of *brlA* in *A. nidulans* resulted in the failure of conidia to form indefinite elongation of the spore stalk, and inactivation of *abaA* and *wetA* (Boylan *et al.* 1987; Adams *et al.* 1988), but did not affect the production of ST (Guzmán-de-Peña *et al.* 1998). Previous studies have shown that the absence of both α and β transcription units resulted in the indefinite growth of conidiophores. Mutant strains lacking only the β transcription unit produced conidiophores but failed to germinate. Similarly, the Δ*brlAα* mutants did not form conidial chains but formed conidiophores. However, conidiophores in these mutants failed to transform into conidiophores (Prade and Timberlake 1993). These results suggest that normal expression of *brlA* is essential for initiating developmental events and that *brlAα* and *brlAβ* are also required for normal conidiophore development. Knocking out *brlA* in *A. fumigatus* resulted in elongation of aerial hyphae, increased hyphal mass, and abolished asexual development (Mah and Yu 2006). These phenotypic characteristics are similar to those of *A. nidulans*. These phenomena suggest that *brlA* is required for conidiophore formation in *A. fumigatus* and is highly conserved in the two *Aspergillus* species.

In *A. nidulans*, *abaA* (abacus) is a regulatory gene controlling phialid differentiation. It is activated by *brlA* during the middle phase of sterigmata differentiation in *A. nidulans* and further mediates the transcriptional activation of *wetA* and the expression of *brlA* (Boylan *et al.* 1987; Chang *et al.* 2012b). The *abaA* deletion mutant produced metulae instead of conidia (Boylan *et al.* 1987; Sewall *et al.* 1990). In *A. fumigatus*, *abaA* is postulated to encode a 797 amino acid protein with an ATTS/TEA DNA domain. *abaA* is highly expressed during conidiation, with its expression level reaching a maximum level 12 h after developmental induction and declining at 48 h. The Δ*abaA* mutants formed defective conidiophores (multilayered cylindrical terminal cells without conidia) and reduced hyphal mass. Overexpression of *abaA* led to growth arrest at the hyphal tip, a decrease in the number of conidia, accelerated hyphal disintegration, and a marked decline in hyphal integrity. In addition, the *abaA* deletion mutant retained a certain level of cell viability compared with the wild-type strain. These results confirmed

that *abaA* has a positive effect on autolysis and cell death, and the normal expression of *abaA* plays an important role in the normal growth and development of *A. fumigatus* (Tao and Yu 2011).

The binding sites of *abaA* (5′-CATTCY-3′, Y is pyrimidine) and *brlA* (5′-(C/A)(G/A)AGGG(G/A)-3′) in *A. nidulans* have been identified (Chang and Timberlake 1993; Andrianopoulos and Timberlake 1994). *AbaA* directly activates *rodA*, *yA*, and *wA*, and its gene products play a crucial role in conidiophores (Andrianopoulos and Timberlake 1994). *AbaA* binds to *wetA*, *vosA*, and velvet proteins (*veA*, *velB*, and *velC*) that are highly conserved in filamentous fungi and affects their expression (Tao and Yu 2011). The velvet protein regulates the development and secondary metabolism of *A. nidulans* (Bayram *et al.* 2008b), suggesting that *abaA* regulates the secondary metabolism of *A. fumigatus* by regulating the expression of genes in the velvet complex. In *A. fumigatus*, *brlA* may regulate the expression of *wetA*, *abaA*, and *vosA*, as these genes are found to contain *brlA* binding sites (Tao and Yu 2011).

WetA is activated by the continuous expression of *brlA* and *abaA* during the final stage of conidial development in *A. nidulans* and *A. fumigatus*. *WetA* is a key component necessary for the synthesis of the cell wall layer, which makes the asexual spores (conidia) mature and impermeable (Marshall and Timberlake 1991; Tao and Yu 2011). *WetA* is speculated to encode a 60 kDa polypeptide transcription factor containing 555 amino acids. *In A. nidulans*, the conidia of the *wetA* mutant failed to reach maturity, and were capable of absorbing water and undergoing autolyse (Marshall and Timberlake 1991). They also found that *wetA* directly or indirectly regulates some genes involved in spore development (*wA*), which are involved in regulating the conidia cell wall layer development.

In *A. fumigatus*, the expression of *wetA* is detectable in conidia 12 h after developmental induction, and reaches its maximum levels at 48 h. Deletion of *wetA* resulted in the production of colorless conidia, a sharp decreased in conidial viability, compromised conidial integrity, decreased trehalose content, and reduced tolerance of conidia to thermal, oxidative and osmotic stress. Furthermore, *wetA*-null mutants exhibited interconnected conidia, incomplete conidial separation and maturation, failure to form hyphal branches, and a loosened mycelial, suggesting that *wetA* plays an important role in conidia maturation (Tao and Yu 2011).

During the conidiophore development stage of *A. nidulans*, there is a class of genes encoding proteins that are special components of the spore wall, such as *rodA*. *rodA* encodes a moderately hydrophobic polypeptide containing eight cysteines, which encodes a fungal cell wall protein that is important for the formation of aerial structures such as conidiophores. In Δ*rodA* mutants, conidia were easily wettable and failed to form an exospore wall layer (Stringer *et al.* 1991).

As *A. nidulans* conidia mature, a dark green pigment accumulates in the spore cell walls, enhancing resistance to ultraviolet radiation. In comparison, yellow and white spores exhibit greater sensitivity to UV light (Aramayo *et al.* 1989). The production of the spore pigment requires the expression of *yA* and *wA* (Mayorga and Timberlake 1992). The *yA* gene, which encodes a p-diphenol laccase and spans 2200 nucleotides, is required for the synthesis of green conidial pigment. Deletion of *yA* resulted in the formation of yellow spores (Clutterbuck 1972; O'Hara and Timberlake 1989). The expression level of *yA* in hyphae is very low or even absent. During the philalide differentiation period and under conidia-inducing conditions, the expression level of *yA* mRNA begins to increase after 10 to 16 h, reaches a peak at 30–36 h. The encoded laccase is subsequently localized to the conidial wall, where it converts a yellow substrate into mature green pigment (Law and Timberlake 1980). Philalide may also produce additional enzymes or substrates that contribute to conidial wall formation (O'Hara and Timberlake 1989).

Wa, which encodes a 250 kDa polyketide synthase, is responsible for synthesizing an intermediate of the yellow spore pigment. This is converted into the mature green pigment through the action of *yA* (Mayorga and Timberlake 1990; Mayorga and Timberlake 1992). The disruption of *wA* led to produce white spores (Pontecorvo *et al.* 1953). *WA* is not expressed in hyphae and vegetative cells but begins to accumulate when conidia begin to develop color. In *brlA*, *abaA*, *wetA* deletion mutants, *wA* is not expressed, and *yA* was not expressed in *brlA*-mutants (Mayorga and Timberlake 1990). Later studies confirmed that *yA* is regulated by *abaA* and *brlA* (Aramayo and Timberlake 1993) as class A, and *wA* is activated by *wetA* as class B (Mayorga and Timberlake 1990; Marshall and Timberlake 1991; Adams *et al.* 1998). The logical relationship of genes that initiate sexual and asexual development of *A. nidulans* is shown in Fig. 2.

4. The genes involved in response to light

Light, as an environmental signal, regulates biological processes in many organisms. In fungi, light contributes to maintaining developmental balance by controlling the formation of reproductive structures. The ability to perceive and respond to light enhances fungal adaptability and survival under changing environmental conditions (Bayram *et al.* 2016). The molecular mechanisms involved in light sensing and signal transduction have been partially elucidated in some aspects (Mooney and Yager 1990).

The activities of fungal-specific velvet family proteins (including VeA, VosA, VelB, and VelC) are closely related to fungal development and secondary metabolism (Park *et al.* 2014). VeA is the founding member of the velvet superfamily of fungal regulatory proteins (Sarikaya Bayram *et al.* 2010). The global regulator, *veA*, participates in a variety of cellular processes under light stimulation, including sexual and asexual development and secondary metabolism. *VeA* has been extensively characterized and has highly conserved functions in *A. nidulans*, *A. flavus* and *A. parasiticus*, and *A. cristatus*. Here, we summarize its roles in several major *Aspergillus* species.

In *A. nidulans*, light mediates regulation of fungal development through *veA*, and *veA* constitutes a genetic link between secondary metabolism and morphological development in *A. nidulans*. Under light stimulation, *veA* can keep growth and development balance by regulating

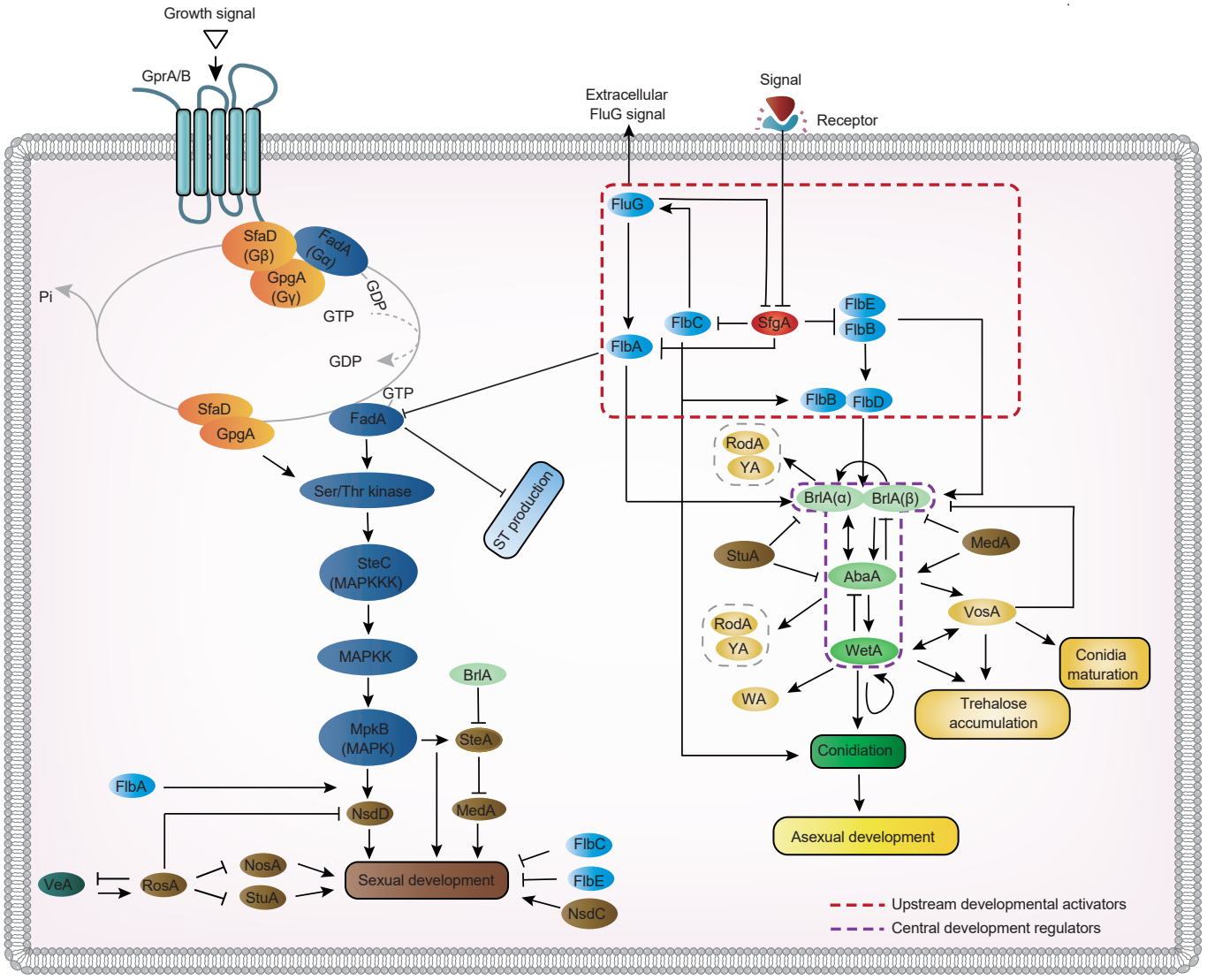


Fig. 2 The central regulatory network initiates both sexual and asexual development of *Aspergillus nidulans*. In sexual development, FadA bound to GTP activates the MAP kinase cascade, which in turn activates the sexual development. During sexual development, FluG is activated by environmental signals and promotes conidial development and secondary metabolism by relieving the SfgA-mediated repression of the flb genes. In asexual development, FluG is activated by environmental signals and promotes conidial development and secondary metabolism by relieving the SfgA-mediated repression of the flb genes.

the developmental ratio between sexual development and asexual development. When *veA* was deleted, the light dependence disappeared, allowing conidia to form in darkness (Mooney and Yager 1990). VeA-deficient mutants cannot form sexual development structures including Hülle cells, cleistothecia, or ascospores, either under light or dark conditions, and the expression of transcription factors *nsdD* and *steA*, which are essential for controlling sexual development, slightly reduced (Kato *et al.* 2003), whereas overexpression of *veA* induces the production of sexual structures, including Hülle cells and cleistothecia, even under stress conditions (Kim *et al.* 2002). These results may indicate that *veA* plays a positive role in sexual development and a negative role in asexual development. In the *rosA* deletion mutant, *veA* transcription level increased, indicating that *rosA* inhibits the expression of *veA*, and is a *veA*-dependent developmental transcription factor (Vienken *et al.* 2005). In the *veA* deletion mutant, *esdC* transcription level significantly decreased, and overexpression of *esdC* could not compensate for the phenotypic changes caused by *veA* functional loss. These results indicate that *esdC* alone is insufficient to activate sexual development and requires *veA* to exert its function (Han *et al.* 2008). Deletion of *veA* abolished *aflR* expression, preventing ST synthesis (Kato *et al.* 2003).

Another study found that *veA* deficiency led to a 25% reduction in colony diameter under light conditions, a 50% reduction in darkness conditions, and a 75% reduction in mycelial mass in liquid culture. The knockout of *veA* led to a 30% reduction in conidia under light conditions and a 46% reduction in darkness conditions. While the wild-type strain produced sclerotia in darkness, the *veA* deletion mutant failed to form them. The deletion of *veA* led to the non-expression of aflatoxin regulatory genes *aflR* and *aflJ*,

and the non-production of versicolorin A, indicating that *veA* regulates aflatoxin biosynthesis and its role in toxin regulation is conserved (Calvo *et al.* 2004).

In *A. flavus*, compared with the wild type strain, the *ΔveA* mutants showed complete loss of AFB_1 and AFB_2, along with a more than 2-fold reduction in CPA yield. In addition, the Δ*veA* mutant was unable to produce aflatrem, indicating that *veA* is involved in the regulation of *atmC*, *atmG*, and *atmM* that located on the aflatrem biosynthesis gene cluster. Furthermore, the Δ*veA* mutants had a slight increase in vegetative growth and abolished the sclerotia formation (Duran *et al.* 2007). In *A. fumigatus*, the spore formation ability of Δ*veA* mutants depends on different nitrogen sources, which indicates that *veA* may be involved in nitrogen metabolism (Krappmann *et al.* 2005). Overexpression of *veA* did not cause obvious phenotypic changes, suggesting that a single gene or a single factor is not sufficient to trigger the development process in *A. fumigatus*.

4.1. velB

In *A. nidulans*, *velB* and *veA* have 18% homology in amino acid sequence. The absence of *velB* prevented sexual fruit bodies formation in the dark, impaired asexual spore formation, and eliminated light-dependent developmental patterns (Bayram *et al.* 2008b). Additionally, disruption of *velB* reduced the expression levels of *brlA*, *abaA*, and *vosA*, as well as the number of spores (Park *et al.* 2012). Overexpression of *velB* did not lead to excessive cleistothecia formation or 2-fold increases in asexual spore productions (Bayram *et al.* 2008b). Trehalose plays a key protective role in the conidia's response to external stress. The expression levels of *tpsA* and *orlA* involved in trehalose biosynthesis were reduced in the *velB* deletion mutants (Sarikaya Bayram *et al.* 2010). These results indicate that *velB* is essential for trehalose biosynthesis and spore viability, and acts a positive role in regulating asexual development. *VelB* exists in the cell nucleus and cytoplasm and is almost not affected by light (Bayram *et al.* 2008b). *VelB* is not only a member of the VelB/VeA/LaeA velvet complex, but also a part of the VelB-VosA complex. In addition, *velB* itself can also form a homodimer in the cell nucleus and cytoplasm (Sarikaya Bayram *et al.* 2010).

4.2. velC

VelC encodes a 57.3 kDa polypeptide consisting of 524 amino acids，and its C-terminus contains a velvet domain that is highly conserved in fungi (Park *et al.* 2014). The transcript level of *velC* accumulates especially in the early stage of sexual development. The deletion of *velC* increased the expression levels of *brlA*, *abaA*, *wetA* and *vosA*, which control sexual spore development. The number of conidia increased and the number of cleistothecia decreased in Δ*velC* mutants. In the *velC* overexpression strains, the number of cleistothecia increased by two-fold, and Hülle cells (specialized structures supporting sexual fruiting) began to appear. These results indicate that *velC* is a positive regulator of sexual development.

4.3. VosA

VosA encodes a transcription factor composed of 430 amino acids and localizes in the nucleus of mature conidia (Ni and Yu 2007). This gene is similar to *veA*, *velB* and *velC* of the velvet family and is highly conserved in both *ascomycetes* and *basidiomycetes*. The Δ*vosA* mutants showed sharp reduction in spore viability, aberrant conidiophores (cleistocerca) and defective sexually fruiting bodies, upregulated the mRNA levels of *brlA* and *rodA*, downregulated *wetA* and *wA* expression, and altered the expression level of *yA*. In addition, the mRNA levels of *tpsA*, *tpsC*, and *orlA* associated with trehalose synthesis decreased, while the mRNA level of *treA*, a gene involved in trehalose degradation increased. The loss of *vosA* led to increased expression of ST biosynthesis genes and elevated ST production in ascospores, and also upregulated the expression of other secondary metabolite biosynthesis genes (Kim *et al.* 2020). The results of the study indicate that *vosA* is involved in regulating conidia development and secondary metabolism, and plays a key role in spore maturation and trehalose biosynthesis.

4.4. LaeA

In *A. nidulans*, *laeA* encodes a 375-amino acid methyltransferase that contains an S-adenosylmethionine (SAM) binding site, which is essential for its function (Bok and Keller 2004). Deletion of *laeA* abolishes the expression of several secondary metabolite gene cluster, including those responsible for sterigmatocystin and penicillin (Bok and Keller 2004). The Δ*laeA* mutants produce more cleistothecia, fewer and smaller Hülle cells, and remained viable ascospores (Sarikaya Bayram *et al.* 2010). Overexpression of *laeA* increased transcription of penicillin biosynthetic genes and penicillin production. However, this regulatory effect did not extend to sterigmatocystin biosynthesis, probably because *laeA* expression is negatively regulated by AflR in a unique feedback loop and modulated by two signal transduction elements protein kinase A and RasA (Bok and Keller 2004).

In *A. flavus*, *laeA* is 75.6% identical to *laeA* in *A. nidulans* and contains an S-adenosylmethionine (SAM) binding site. The Δ*laeA* mutants showed an absence of aflatoxins and a reduction in cyclopiazonic acid, kojic acid, and aspafuran. Overexpression of *laeA* increased aflatoxin production, while reduced the number of sclerotia (Kale *et al.* 2008). On PDA medium, the disruption of *laeA* resulted in delayed conidiophore development, shorter pedicels, abnormal conidial chain length, and increased conidiophore production (Chang *et al.* 2012a). *Aspergillus carbonarius* is considered to be the main fungus that produces OTA in grapes. The absence of *laeA* reduced the number of conidia and OTA production. The nonribosomal peptide synthetase involved in OTA biosynthesis was downregulated. The results showed that *laeA* may regulate the synthesis of OTA by downregulating genes in the OTA synthesis pathway (Crespo-Sempere *et al.* 2013).

4.5. Interactions between Vea, Velb, Laea and Vosa

During spore development, *VelB* initially interacts with *VosA* in the nucleus to form a second light-regulated developmental

complex (Sarikaya Bayram *et al.* 2010; Park *et al.* 2012). The *abaA* gene can directly bind to the promoters of *velB* and *vosA* to regulate the expression levels of *velB* and *vosA* (Park *et al.* 2012). VeA can help VelB transport from the cytoplasm to the nucleus. The interaction between VeA and VelB occurs in the nucleus and cytoplasm, and the VeA-LaeA interaction occurs in the nucleus. In the dark, VeA bridges VelB and LaeA to form a complex, VelB-VeA-LaeA (velvet) complex (Sarikaya Bayram *et al.* 2010). VelB/VeA/LaeA velvet complex control and may support the epigenetic activity of LaeA, which controls the expression of secondary metabolite gene clusters (Bayram *et al.* 2008b). LaeA regulates the formation of velB-vosA and velB-veA-laeA (velvet) complexes, and suppresses the overall protein levels of VeA, VelB and VosA, particularly the post-translational modification level of VeA. However, LaeA does not disrupt the transport of VeA-VelB into the nucleus with the help of importin KapA. However, laeA does not disrupt the transport of VeA-VelB into the nucleus with the help of importin KapA. It should be noted that the importin KapA is unable to be recruited by VosA-VelB and can only be recruited by the VeA-VelB complex (Sarikaya Bayram *et al.* 2010). The interaction between VelB and VeA connects light-dependent development in *A. nidulans* with laeA-regulated secondary metabolism (Bayram *et al.* 2008b).

4.6. FphA, LreA, and LreB

Light is required for asexual sporulation in *A. nidulans*, but conidiation is initiated only when irradiaton occurs during a critical developmental period. Red light stimulates asexual sporulation while inhibiting sexual development. Conidiation in *A. nidulans* is mediated by red photoreceptors that exhibit properties identical to those of phytochromes in higher plants (Mooney and Yager 1990).

The phytochrome FphA of *A. nidulans* binds to the biliverdin chromophore that functions as a red-light sensor and inhibits sexual development under red light conditions. FphA is a 140 kDa protein consisting of 1280 amino acids and is present in the cytoplasm. FphA is required for light sensing in *A. nidulans*, it is not the sole red-light sensor. Deletion of *fphA* partially alleviates the red-light-mediated suppression of sexual development (Blumenstein *et al.* 2005).

LreA and LreB are core components of the blue light sensing system of *Neurospora crassa* (Froehlich *et al.* 2002; He *et al.* 2002). LreA is a protein composed of 836 amino acids and LreB is a protein composed of 417 amino acids. The Δ*lreA* and Δ*lreB* mutants exhibited a slight increase in conidiophores both in light and in the dark. In the dark, cleistothecium formation reduced by 70% and 30%, respectively in Δ*lreA* and Δ*lreB* mutants compared to the wild-type strain. LreA is present in the nucleus, LreB is present in the nucleus and in the cytoplasm, and LreB interacts with LreA. LreA exists in the cell nucleus, LreB exists in the cell nucleus and the cytoplasm, and the interaction between LreB and LreA occurs in the cell nucleus. FphA is part of a protein complex that includes LreA (WC-1) and LreB (WC-2). FphA inhibits sexual development and mycotoxin production, whereas LreA and LreB stimulate both. FphA interacts with LreB, which in turn interacts with VeA, another regulator involved in light response and mycotoxin production, but is dependent on the presence of the linear tetrapyrrole in FphA and this interaction is positive and occurs only in the nucleus. Deletion of *fphA* slightly reduced conidia numbers under both light and dark conditions and did not show any significant decrease in cleistothecium production in darkness. However, in the light, the numbers of cleistothecium produced by Δ*LreA*, Δ*LreB*, and Δ*FphA* mutants were consistent with those in the dark (Purschwitz *et al.* 2008). These results suggest that LreA and LreB act as positive regulators of sexual development, but their activity is inhibited by light through the action of FphA.

4.7. CryA

Cryptochromes are blue light receptors and are presumed to have evolved from the DNA photolyase protein family, which can use photo-repair mechanisms to repair UV-induced DNA damage. One cryptochrome/photolyase-encoding gene was identified in the nucleus of *A. nidulans*, named *cryA*. Transcripts encoding fruiting body formation regulators were upregulated in *cryA*-deficient mutants that produced Hülle cells. This phenotype was also observed in Δ*rosA* mutants and *veA* and *nsdD* overexpressing strains (Han *et al.* 2001; Kim *et al.* 2002; Vienken *et al.* 2005; Vienken and Fischer 2006). This may be because *cryA* acts as a UV/blue light receptor in *A. nidulans*. CryA transmits UVA light signals to the nucleus, thereby inhibits the initiation of the *veA*-mediated feedback loop between *nsdD* and *rosA*, which is essential for the formation of Hülle cells and sexual development (Bayram *et al.* 2008a).

Different wavelengths of light differentially regulate conidial germination (spore germination to produce germ tubes) in *A. nidulans*. Far-red light (740 nm) strongly delays germination (less than 5%), whereas blue light (450 nm) and red light (700 nm) permit approximately 60% germination. The blue-light receptors LreA (WC-1) and LreB (WC-2) are not required for the inhibition of germination, and the absence of CryA does not alleviate the blue-light-mediated blockage. The role of FphA may be independent of light exposure, as Δ*fphA* mutants displayed delayed germination in the dark (Röhrig *et al.* 2013). Here, the key genes involved in light-regulated sexual and asexual development in *A. nidulans* are summarized in Fig. 3.

5. Conclusion and future perspectives

Aspergillus is one of the most abundant and diverse fungi in the world. During the growth of *Aspergillus*, light can affect its growth, reproduction and the production of secondary metabolites. The regulation of secondary metabolite biosynthetic gene clusters largely depends on pathway-specific transcription factors or global regulators, and is also modulated by signaling pathways that link secondary metabolism to spore formation. Exploring these regulatory mechanisms provides deeper insights into fungal-environment interactions and the molecular features underlying mycotoxin production.

This is of great significance for the development and optimization of antifungal agents or biocontrol strains, as it

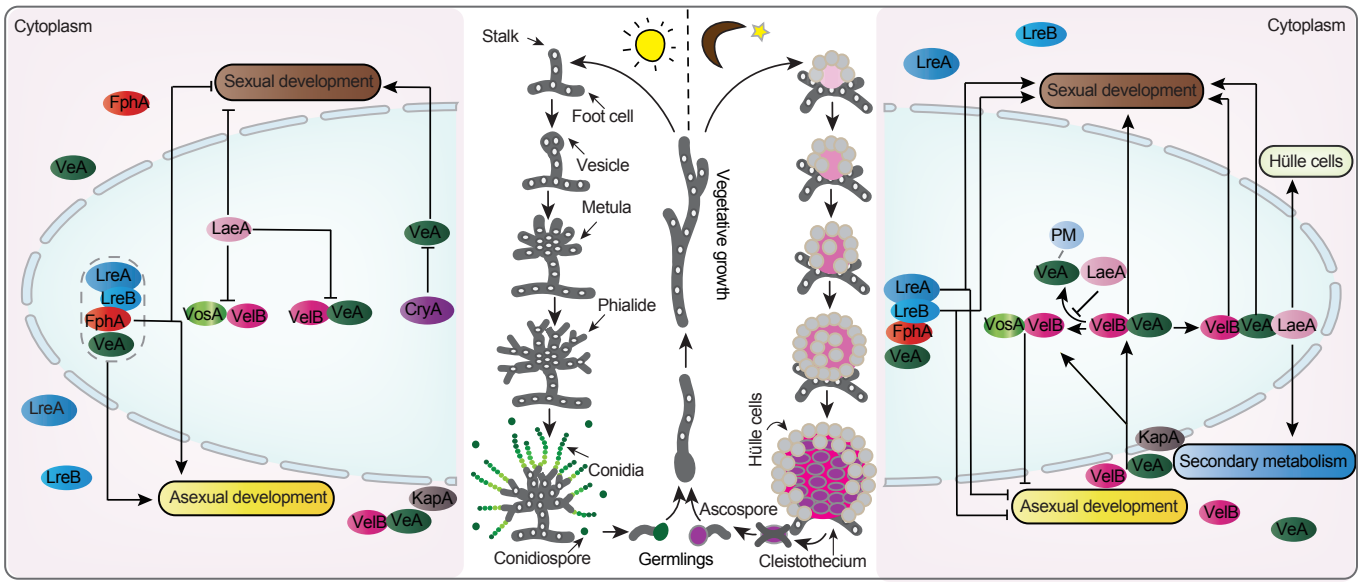


Fig. 3 Light-regulated sexual and asexual development in *Aspergillus nidulans*. In *Aspergillus nidulans*, light activates the asexual developmental pathway through its photoreception system, whereas darkness favors the initiation of sexual development and suppresses asexual sporulation.

enables the suppression of fungal growth and mycotoxin biosynthesis at the source, thereby minimizing mycotoxin contamination, improving crop yield and quality, and safeguarding food safety.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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