Developmental Decisions in *Aspergillus nidulans*

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I. Introduction

Filamentous fungi are ubiquitous eukaryotic microorganisms in nature. Fungi are the main decomposers of organic materials, important environmental nutrient recyclers, and key industrial producers, providing benefits to humankind (Nevalainen and Peterson 2014; Park et al. 2017; Treseder and Lennonb 2015). Conversely, several fungi, such as *Aspergillus flavus* and *Fusarium graminearum*, are key mycotoxin producers that cause a global loss of agricultural commodities (Gugnani 2003; Keller et al. 2005). In addition, a variety of filamentous fungi can cause plant, animal, and human diseases that impact human health and food supplies (Fisher et al. 2012; van Burik and Magee 2001). Owing to the importance of filamentous fungi for humanity, understanding the fungal growth and development would help us to minimize damage and maximize benefits.

Hyphae, long and branching vegetative structures, are the main morphological forms of filamentous fungi in nature (Harris 2011; Riquelme 2013). Fungal cells can undergo reproduction asexually and/or sexually in response to environmental as well as endogenous genetic cues, and these abilities are called **developmental competence** (Axelrod et al. 1973; Noble and Andrianopoulos 2013). In many filamentous fungi, aerial hyphae generally form asexual reproductive structures, the most common reproductive form (Adams et al. 1998). All *Aspergillus* fungi form asexual spores (**conidia**) as the main propagules and infectious particles (Ebbole 2010). Along with the asexual development, some fungi can also reproduce by sexual means and forms sexual structures (Dyer and O'Gorman 2012; Schoustra et al. 2010). These asexual and/or sexual structures (size, shape, color, and arrangement of asexual spores) of filamentous fungi are used for classification (Samson et al. 2014). The formation of asexual and sexual structures is highly sophisticated and regulated by various positive and negative genetic elements that act in several differential stages (Adams et al. 1998; Dyer and O'Gorman 2012). Among filamentous fungi, *Aspergillus*

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nidulans has been used a model organism to understand developmental biology including fungal growth, conidiation, and sexual differentiation (Casselton and Zolan 2002). This chapter summarizes up-to-date information about regulatory elements and decisions of asexual and sexual development in *A. nidulans*.

II. Developmental Morphology of *Aspergillus nidulans*

Life cycle of *A. nidulans* begins with germination of asexual or sexual spores (Noble and Andrianopoulos 2013). Fungal spores are the most widespread fungal structures in the air (Ebbole 2010) and start to germinate in response to appropriate cues forming the germ tubes (d'Enfert 1997). After germination, the germ tube can further extend apically and form the hyphae, the tube-like structures that are the main mode of vegetative growth (Harris 2011). The hyphae consist of several septated cells and the apical **Spitzenkörper**, the supply center for hyphal tip extension (Harris 2006; Steinberg 2007). During hyphal growth, cytokinesis, septum formation, biosynthesis of cell wall components, and extension of the plasma membrane occur, and these processes are tightly regulated (Harris 2008; Lew 2011). The hyphae must acquire the competence to enter developmental processes. The hyphal cells that have acquired developmental competence cease growth and turn on the developmental programs depending on various environmental stimuli including light, nutrients, oxygen supply, fungal pheromones, and stress conditions (Axelrod et al. 1973; Yager et al. 1982). To obtain the developmental competence from a single spore, approximately 18 and 24 hours of vegetative growth are required for asexual and sexual development, respectively (Axelrod et al. 1973; Noble and Andrianopoulos 2013). Formation of asexual and sexual structures will be completed in about 12 and 27 hours after developmental induction (Fig. 1).

Conidiophores are the asexual developmental structures that bear conidia (Adams et al. 1998; Yu 2010). Development of conidiophores starts with the formation of thick-walled foot cells. The foot cells with developmental competence branch to form aerial stalks under air-exposed conditions. The stalk tip then begins to swell and forms a multinucleate structure called

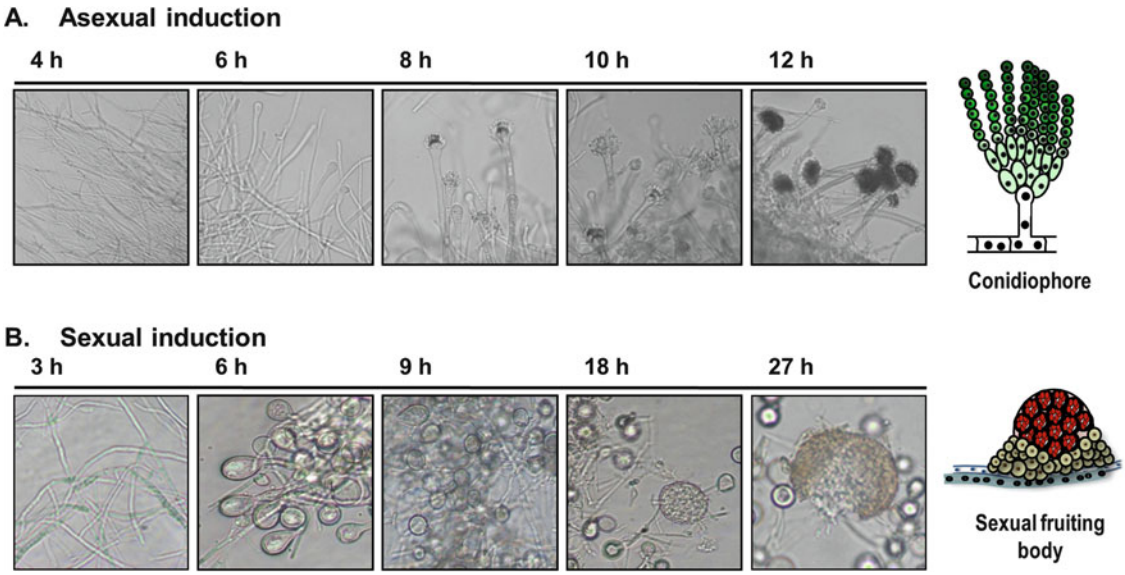

Fig. 1 Developmental processes in *A. nidulans*. (**a**) Conidiophores formation in a wild-type strain after asexual induction. (**b**) Sexual fruiting bodies formation after sexual induction. Dr. Dong-Min Han (Wonkwang University, S. Korea) kindly provided the photomicrographs

vesicle. On the surface of the vesicle, budding-like division occurs, leading to formation of **metulae** and **phialides**, two layers of sterigmata. A secondary layer of sterigmata, termed phialides, generates conidial chains via repeated asymmetric mitotic cell division. After formation of fresh conidia, they undergo maturation processes and finally complete asexual development (Fig. 1a) (Ni et al. 2010; Timberlake 1990; Yu 2010).

Many Aspergilli are able to produce sexual spores with or without mating partners. Some *Aspergillus* species, including *A. nidulans*, are homothallic so they can undergo self-sexual reproduction (Dyer and O'Gorman 2011; Geiser 2009) (Fig. 1b). In *A. nidulans*, sexual reproduction begins with the formation of coiled lumps formed antheridia-like exterior hyphae with core cells (Sohn and Yoon 2002). These coiled lumps are enlarged to form ascogenous hyphae and finally to develop an ascocarp, the fruiting body of ascomycetes, that contains ascospores. In *A. nidulans*, ascocarps are named **cleistothecia** (Krijgsheld et al. 2013). In the early stage of cleistothecia development, the thick-walled globose **Hülle cells** appear, and these cells nurse cleistothecia during sexual development. Young cleistothecia are surrounded by Hülle cells, numerous aerial hyphae, and conidial balls and form a bird nest-like structure; thereby, the species name "*nidulans*" was given (Han 2009; Scherer and Fischer 1998). Core cells of cleistothecia are enlarged and multinucleate, which then form ascogenous cells which then produce the asci (Sohn and Yoon 2002).

III. Environmental Factors Affecting Developmental Fate in *A. nidulans*

The hyphal cells that have acquired developmental competence can undergo asexual or sexual development dependent on the environmental conditions (Riquelme 2013). Various environmental factors, such as nutritional status, air, and culture conditions, control the developmental fate in *A. nidulans* (Bahn et al. 2007; Han et al. 2003; Rai et al. 1967).

Light is one of the most critical factors affecting fungal growth and development (Rodriguez-Romero et al. 2010; Tisch and Schmoll 2010). In the light, *A. nidulans* undergoes asexual development (Mooney and Yager 1990). Light controls expression of certain genes associated with fungal development such as *brlA* and *fluffy* genes (Bayram et al. 2016; Mooney and Yager 1990; Ruger-Herreros et al. 2011; Sarikaya Bayram et al. 2010). *A. nidulans* contains several light sensors that work with the *velvet* regulators and induce mRNA expression of conidiation-specific genes (Bayram et al. 2016; Blumenstein et al. 2005). Three **photoreceptors**, FphA (*f*ungal *ph*ytochrome A), LreA, and LreB (*l*ight *r*esponse A and B), play differential roles in conidiation (Atoui et al. 2010; Blumenstein et al. 2005; Purschwitz et al. 2008, 2009). The deletion of *fphA* encoding a red-light receptor causes reduced *brlA* expression and conidial production, indicating that FphA functions as an activator of conidiation (Atoui et al. 2010; Blumenstein et al. 2005; Ruger-Herreros et al. 2011). Production of conidia in the *lreA* and *lreB* deletion mutants was slightly increased, suggesting that the LreA and LreB complex acts as a repressor of conidiation (Purschwitz et al. 2008; Ruger-Herreros et al. 2011). Light also regulates the localization of the **velvet protein** VeA (*ve*lvet A), a key regulator for development and secondary metabolism in *Aspergillus* spp. (Kim et al. 2002; Stinnett et al. 2007).

Interestingly, VeA interacts with the LreA/LreB/FphA complex and forms the LreA/LreB/FphA/VeA complex, the major light-sensing unit (Bayram et al. 2010; Ruger-Herreros et al. 2011). In general, *A. nidulans* favors sexual development under dark conditions. However, fungal development occurs differentially depending on the light sources. For example, exposure to red or blue light leads to inhibition of sexual development, whereas far-red light can induce sexual development (Bayram et al. 2010; Blumenstein et al. 2005). Light can inhibit sclerotial development in *A. flavus* and *A. parasiticus* (Bennett et al. 1978; Calvo et al. 2004; Duran et al. 2007).

Nutrient sources are also major factors for balancing between asexual and sexual development (Atoui et al. 2010; Han et al. 2003). First, the amount and types of carbon sources affect sexual development (Han et al. 2003). At concentrations of less than 0.5% or higher than 6% glucose, the number of cleistothecia dramatically decreases,

suggesting that a certain level of carbon is required for sexual development (Han et al. 2003). Several carbon sources such as lactose and glycerol favor sexual development, whereas acetate can block formation of cleistothecia. Second, the type of nitrogen sources is important for deciding developmental process, and organic nitrogen sources can induce sexual development in *A. nidulans* (Han et al. 2003). The ratio between carbon and nitrogen is most important for asexual or sexual reproduction (Han et al. 2003). Nutrient starvations can induce fungal development in the submerged culture (Martinelli 1976; Saxena and Sinha 1973). Carbon and nitrogen starvation induces *brlA* expression and causes asexual developmental induction in differential pattern (Skromne et al. 1995). Glucose starvation causes formation spores on abnormal conidiophores that bypass the vesicle and metulae stages, whereas nitrogen starvation induces the production of more elaborate conidiophores (Skromne et al. 1995).

A study proposes that FlbD (*f*luffy *l*ow *b*rlA locus *D*), a cMyb-type transcription factor (TF) necessary for the proper expression of *brlA*, is associated in response to nitrogen starvation (Arratia-Quijada et al. 2012). High concentrations of salts, such as sodium chloride or potassium chloride, activate conidiation (Han et al. 2003; Lee and Adams 1994).

Oxygen also affects fungal development (Grahl et al. 2012). Fungal hyphae grow in submerged culture condition or in restricted exposure to air. After acquired developmental competence, a high oxygen concentration (air exposure) can induce the production of conidiophores (Adams et al. 1998; Axelrod et al. 1973; Morton 1961). Conversely, low concentrations of oxygen can initiate sexual development (Zonneveld 1988). However, the detailed mechanisms of fungal development regulated by air remain to be understood. Osmolarity is also responsible for the preferential development of conidia (Lee and Adams 1994). Addition of 1 M KCl or 1 M NaCl can induce production of asexual spores but decrease sexual development. However, higher concentration of salts can inhibit fungal growth, blocking both asexual and sexual development (Han et al. 2003; Song et al. 2001).

IV. Developmental Decision for Conidiation

Conidiation in *Aspergillus* occurs as an integral part of the life cycle primarily controlled by the intrinsic genetic program. The formation of conidiophore is tightly regulated by multiple genetic elements, and these are extensively studied in *A. nidulans* (Fig. 2) (Adams et al. 1998; Park and Yu 2012). Three TFs BrlA (*br*istle A), AbaA (*aba*cus A), and WetA (*wet*-white A) are central regulators for conidiation that control expression of genes associated with the assembly of the conidiophore (Adams et al. 1998; Yu 2010). To activate central regulators of asexual development, upstream regulators should induce *brlA* expression, and several **repressors** should be removed from the promoter regions of *brlA* (Lee et al. 2016) (Fig. 2c). A recent study has revealed that there are at least three negative regulators of conidiation and that a key event for the acquisition of the asexual developmental competence is to remove the repressive effects imposed by NsdD (*n*ever in *s*exual *d*evelopment locus *D*) and VosA (*v*iability *o*f *s*pores A) (Lee et al. 2016). Importantly, for the first time, this study demonstrated that NsdD physically binds to three different regions in the *brlA*β promoter, further supporting the idea that NsdD directly (rather than indirectly) represses the onset of *brlA*β expression and conidiation. After completion of conidiophore formation, feedback regulators turn off the activities of the central regulators (Ni et al. 2010). The VosA-VelB (*vel*vet-*l*ike B) complex acts as a key feedback regulator that represses *brlA* expression on conidia (Ni and Yu 2007; Park et al. 2012).

A. Upstream Regulators of Conidiation

In response to environmental conditions, hyphal cells that have acquired developmental competence stop vegetative growth and start conidiation (Adams et al. 1998). Various studies have identified several upstream developmental regulators that induce *brlA* expression. Mutations in any of the genes *fluG* (*flu*ffy locus A) and *flbA-E* (*f*luffy *l*ow *b*rlA loci A~E) lead to

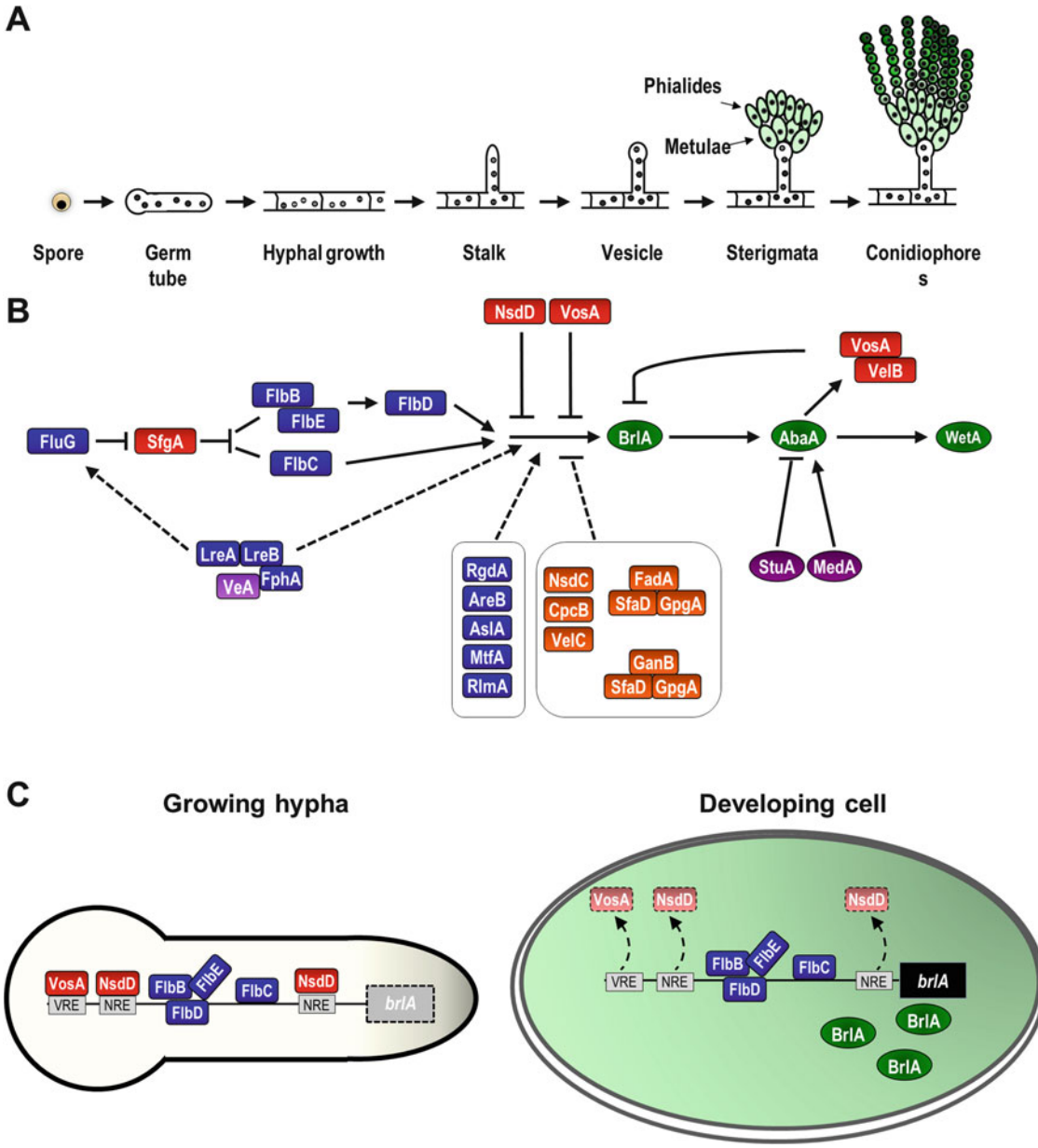

Fig. 2 Asexual development in *A. nidulans*. (**a**) A schematic presentation of morphological changes during conidiophore formation in *A. nidulans*. (**b**) A genetic model for developmental regulation. Several upstream activators are required for initiation of central regulator pathway (BrlA → AbaA → WetA) of asexual development. In contrast, several TFs, including SfgA, VosA, and NsdD, or the G protein signaling pathways, repress initiation of asexual development. (**c**) A model depicting the roles of positive and negative regulators in governing the acquisition of the developmental competence. In hyphae, VosA and/or NsdD bind(s) to the upstream regulator region of *brlA*, which then repress of mRNA expression of *brlA*. In developing cells, NsdD and VosA can be displaced from the *brlA* promoter, and the FlbB-FlbD and FlbC activate *brlA* expression and conidiation

"fluffy," cotton-like phenotypes (Adams et al. 1998; Etxebeste et al. 2010).

FluG is one of the upstream developmental activators which is required for inhibition of vegetative growth and initiation of conidiation

(Lee and Adams 1994). Overexpression of *fluG* causes conidiophore formation and *brlA* activation in liquid submerged cultures, whereas the deletion of *fluG* leads to the fluffy phenotypes (D'Souza et al. 2001; Lee and Adams 1996; Wieser et al. 1994). FluG is essential for synthesis of a diorcinol-dehydroaustinol adduct, an extracellular sporulation inducing factor (known as the **FluG factor**), which signals the activation of conidiophore development (Lee and Adams 1994; Rodriguez-Urra et al. 2012). This adduct can rescue asexual developmental defects caused by absence of *fluG* (Rodriguez-Urra et al. 2012). FluG-mediated signaling regulates proliferation and development via two independent pathways; the cessation of vegetative growth via FlbA activation and the initiation of conidiation via activation of developmental genes (*flbB~E*) (Yu 2010). The FluG-mediated developmental regulation is divided in two independent pathways, FlbE/FlbB/FlbD and FlbC, for initiation of conidiation and activation of *brlA* (Etxebeste et al. 2010; Park and Yu 2012).

Four upstream transcriptional activators including FlbB, FlbC, FlbD, and FlbE regulated by FluG are putative TFs which are needed for cessation for hyphal cell growth and regulation of development (Park and Yu 2012). FlbC contains two C_2H_2 zinc finger DNA-binding domains which are required for interaction of the promoter region of *brlA* and activation of *brlA* (Kwon et al. 2010a). Deletion of *flbC* leads to reduction in conidiation, whereas overexpression of *flbC* causes inhibition of hyphal growth and induction of *brlA*, *abaA*, and *vosA*, suggesting that FlbC is vital for coordinating fungal growth and development (Kwon et al. 2010a). FlbB has a basic leucine zipper (b-zip) domain and localizes at the hyphal tip (Etxebeste et al. 2009). FlbB interacts with FlbE and forms the FlbB-FlbE complex which activates *flbD* expression (Etxebeste et al. 2009; Garzia et al. 2009; Kwon et al. 2010a). Then, FlbD, a cMyb-type TF, also forms a complex with FlbB in the nucleus, and this complex directly binds to the promoter region of *brlA* and activates its expression (Garzia et al. 2010).

In addition to the roles of FlbB~FlbEs for growth and asexual development, several studies demonstrated that FlbB~FlbE are also required for proper sexual development (Arratia-Quijada et al. 2012; Kwon et al. 2010a, b; Oiartzabal-Arano et al. 2015).

Proper and precise control of *brlA* in vegetative cells is extremely crucial for the survival and fitness of *Aspergillus* fungi (Lee et al. 2016). During the early phase of vegetative growth, the Flb proteins can occupy in the promoter region of *brlA*. However, the Flb proteins cannot induce *brlA* transcription, as several repressors directly bind to the *brlA* promoter and interfere with the function of the Flb proteins (Lee et al. 2016). Gain-of-function genetic screens proposed that VosA acts as a key repressor of conidiation (Ni and Yu 2007). In hyphal cells, VosA, a fungal-specific velvet family TF, forms the VosA-VosA homodimer or the VosA-VelB heterodimer and represses *brlA* expression in liquid submerged culture (Park et al. 2012; Sarikaya Bayram et al. 2010). NsdD is another regulator that represses *brlA* expression during vegetative growth (Lee et al. 2014). In the developing cell, the NsdD and VosA proteins may be subject to degradation and removed from the *brlA* promoter, and then the Flb proteins induce *brlA* expression (Lee et al. 2016) (Fig. 2c).

Several TFs have been shown to influence growth in *A. nidulans*. SfgA is a putative TF with a $Zn(II)_2Cys_6$ binuclear DNA-binding domain (Seo et al. 2003, 2006). SfgA plays a role downstream of FluG but upstream of FlbA, FlbB, FlbC, FlbD, and BrlA (Seo et al. 2006). OsaA (*o*rchestrator of *s*ex and *a*sex A) is a functional equivalent of Wor1 in *Candida albicans*. The presence of multiple *osaA* copies in its genome represses conidiation (Alkahyyat et al. 2015; Ni and Yu 2007). RgdA (a putative APSES TF), MtfA (*m*aster *t*ranscription *f*actor A; a C_2H_2 zinc finger TF), RlmA (a major MpkA-dependent TF), AreB (a putative GATA zinc-finger TF), and AslA (*a*sexual differentiation with *l*ow-level conidiation A; a C_2H_2-type zinc finger TF) are also involved in normal growth and development in *A. nidulans* (Kim et al. 2017; Kovacs et al. 2013; Lee et al. 2013; Ramamoorthy et al. 2013; Wong et al. 2009). De Souza and colleagues identified various kinases, such as CkiB, Gsk3, PkaA, NikA, and PlkA, which are essential for proper fungal growth (De Souza et al. 2013).

Heterotrimeric G proteins (G proteins) compose of α, β, and γ subunits that are involved in most biological processes in filamentous fungi (Yu 2006). In *A. nidulans*, two heterotrimeric G protein signaling pathways, FadA (*f*luffy *a*utolytic *d*ominant A)-mediated and GanB (*G* protein *a*lpha subunit in *A. nidulans* B)-mediated signaling pathways, were studied, and these two pathways govern fungal growth, development, and secondary metabolism (Chang et al. 2004; Wieser et al. 1994; Yu 2006; Yu et al. 1996). In response to environmental stresses, two G alpha proteins, FadA and GanB, dissociate from the cognate GPCR (G protein-coupled receptor) and the Gβγ heterocomplex SfaD(Gβ)::GpgA(Gγ), and the dissociated Gα subunit (FadA and/or GanB) and/or the Gβγ hetero-complex cooperatively regulates vegetative growth and represses both asexual and sexual development via the cyclic AMP (cAMP)-dependent protein kinase PkaA (Lafon et al. 2005; Rosen et al. 1999; Seo et al. 2005; Shimizu and Keller 2001). FadA- and GanB-mediated signaling pathways are negatively controlled by the *r*egulators of *G* protein *s*ignaling (RGSs) FlbA and RgsA (*r*egulators of *G* protein *s*ignaling A), respectively (Han et al. 2004b; Hicks et al. 1997; Wieser et al. 1997). In addition, GanB-mediated signaling is in part activated by the putative GDP/GTP exchange factor RicA (an orthologue of *Caenorhabditis elegans* RIC-8) (Kwon et al. 2012). Another G protein component CpcB (*c*ross-*p*athway *c*ontrol B; Gβ-like protein B) is required for proper fungal growth and development in *A. nidulans* (Kong et al. 2013).

In fungi, MAPKs (mitogen-activated protein kinases) are involved in hyphal growth, development, and virulence (Xu 2000). Among four MAPK genes, including *mpkA*, *mpkB*, *mpkC*, and *hogA* in *A. nidulans*, *mpkB* encodes a homolog to Fus3p of the baker's yeast and is required for proper fungal growth, development, and secondary metabolism (Atoui et al. 2008; Bayram et al. 2012; Jun et al. 2011; Kang et al. 2013; Paoletti et al. 2007). Deletion of *mpkB* results in increased expression of *brlA* and decreased VeA phosphorylation and VeA-VelB formation, which function as an activator of sexual development, suggesting that MpkB plays an important role in both asexual and sexual development (Bayram et al. 2012; Kang et al. 2013).

B. Initiation of Conidiation

Under appropriate conditions, some of the vegetative cells cease hyphal growth and initiate conidiation (Adams et al. 1998). The key step for developmental transition from apical growth to conidiation is **activation of *brlA*** (Adams et al. 1988, 1990). *brlA* null mutants show phenotypes including indeterminate structures that resemble conidiophore stalks (thus termed "bristle") and fail to form any asexual structures including vesicles, metulae, phialides, and conidia (Adams et al. 1988). In contrast, overexpression of *brlA* leads to termination of hyphal growth and the formation of viable spores from hyphal apices (Adams et al. 1988). External signals, such as nutrient limitations or several stresses, cannot bypass the BrlA requirement for asexual development, suggesting that *brlA* activation is an essential control step for commencing conidiation.

A recent study has revealed that the abovementioned upstream developmental activators are needed for maximum conidiation, but not for the commencement of development. This is based on the fact that the deletion of *nsdD* could bypass the need for *fluG*, *flbB*, *flbE*, *flbD*, and *flbC*, but not *brlA*, in conidiation (Lee et al. 2016).

Once the negative regulators NsdD and VosA are removed and upstream activators maximize expression of *brlA*, the C_2H_2 zinc finger TF BrlA activates expression of several genes involved in conidiation (Adams et al. 1990). Deletion of *brlA* blocks expression of *abaA* and *wetA*, whereas forced expression of *brlA* leads to activation of developmental regulatory genes (Mirabito et al. 1989). These developmental genes, including *abaA*, *wetA*, *rodA*, and *yA*, contain the BrlA response elements (BREs; 5′-(C/A)(G/A)AGGG(G/A)-3′) in their promoter regions (Chang and Timberlake 1993; Prade and Timberlake 1993). The *brlA* locus consists of two overlapping transcriptional units, designated *brlA*α and *brlA*β. The regulatory mechanisms of *brlA*α and *brlA*β are different. *brlA*α is

controlled via a transcriptional mechanism, while *brlA*β is regulated at both the transcriptional and translation levels (Han and Adams 2001; Han et al. 1993). The *brlA*β mRNA is produced in vegetative cells before developmental induction, but it does not accumulate to substantial levels, likely because translation of the *brlA*β μORF represses BrlAβ translation to block development. Following BrlAβ translation, *brlA*α transcription is activated primarily through the *brlA*-dependent positive feedback loop (Adams et al. 1998). The ultimate result of *brlA* activation is activation of other development-specific genes including *abaA* and *wetA*.

C. Progression and Termination of Conidiation

After activation of *brlA*, BrlA directly induces expression of *abaA* required for formation of phialides during the middle phase of asexual development (Boylan et al. 1987; Sewall et al. 1990a). The *abaA* null mutant forms non-sporulating conidiophores, similar to an abacus-like structure, and does not form phialides, suggesting that *abaA* is required for proper formation of phialides (Clutterbuck 1969; Sewall et al. 1990a). Overexpression of *abaA* leads to cessation of vegetative growth and accentuates cellular vacuolization without spore formation in liquid submerged culture (Mirabito et al. 1989).

AbaA is a TEF1 (transcriptional enhancer factor-1) family member which contains an ATTS (AbaA, TEC1p, TEF-1 sequence)/TEA DNA-binding motif (Andrianopoulos and Timberlake 1991, 1994). AbaA binds to the *cis* consensus sequence 5′-CATTCY-3′ (AbaA response element (ARE), where Y is a T or C) and regulates their expression during phialide differentiation (Andrianopoulos and Timberlake 1994). Previous studies demonstrated that AbaA positively regulates expression of several genes, including the chitin synthase gene *chsC*, a component of the axial bud site marker *axl2*; developmental genes including *yA*, *rodA*, *wA*, *brlA*, *wetA*, *vosA*, and *velB*; and *abaA* itself, which contain AREs in their promoter regions (Aguirre et al. 1990; Aramayo and Timberlake 1993; Ichinomiya et al. 2005; Park et al. 2003, 2012; Si et al. 2012). In addition, AbaA is required for repression of *brlA* during mid-phage of conidiation, without requiring AbaA binding to the *brlA* promoter region, suggesting that AbaA may indirectly repress *brlA* expression (Aguirre 1993; Han and Adams 2001).

During late phase of conidiation, WetA, VosA, and VelB play crucial roles in formation, maturation, integrity, and dormancy of conidia (Marshall and Timberlake 1991; Ni and Yu 2007; Park et al. 2012; Sewall et al. 1990b). WetA is a key regulator for the conidium wall modification which is essential for the stability of mature and dormant conidia (Marshall and Timberlake 1991; Sewall et al. 1990b).

The *wetA* mutant produces colorless and autolytic conidia, described as “wet-white” (Clutterbuck 1969). In addition, the *wetA* mutant conidia lack of both the condensation of the C2 wall layer and the formation of C3 and C4 layers (Sewall et al. 1990b). WetA also acts as a regulator of conidium-specific genes including *wA* (Marshall and Timberlake 1991). With BrlA and AbaA, WetA has been proposed to define a central regulatory pathway that functions in concert with other genes to regulate conidiation-specific gene expression and determine the order of gene activation and repression (Adams et al. 1998; Mirabito et al. 1989).

In conidia, two velvet regulators VosA and VelB interact with each other and form the VosA-VelB complex that plays a crucial role in conidial maturation, conidial trehalose biogenesis (Ni and Yu 2007; Park et al. 2012; Sarikaya Bayram et al. 2010). The deletion of *vosA* or *velB* results in a loss of conidial viability, the lack of trehalose in conidia, and a reduction of conidial tolerance to environmental stresses (Ni and Yu 2007; Park et al. 2012; Sarikaya Bayram et al. 2010). The velvet regulators are fungal-specific TFs which have the DNA-binding *velvet* motif (Ahmed et al. 2013). The VosA-VelB complex positively regulates the expression of conidia-specific genes and represses certain development-associated genes (Ahmed et al. 2013; Park et al. 2015). Overall, the VosA-VelB complex controls the commencement, progression, and completion of sporogenesis.

Two developmental modifiers, StuA (*stu*nted) and MedA (*med*usa), work with central regulatory genes and are necessary for the precise organization of conidiophores (Adams et al. 1998). StuA is a TF containing the APSES motif and is required for proper activation of *brlA* and repression of *abaA* (Dutton et al. 1997). MedA is also required for proper expression of *brlA* and *abaA* and proper formation of conidiophores (Busby et al. 1996).

V. Developmental Decisions for Sexual Development

Due to the complexity of the sexual reproduction, only a few studies have been conducted compared to conidiation (Dyer and O'Gorman 2012). Like conidiation, *A. nidulans* has several advantages in studying sexual reproduction, including the homothallic sexual cycle, early availability of the whole genome data, and various tools for genetic manipulation; thus it has been used to identify and characterize genes associated with sexual fruiting (Archer and Dyer 2004; Galagan et al. 2005; Todd et al. 2007). Less than 100 genes required for proper sexual development in *Aspergillus* spp. have been identified (Fig. 3), and their roles have been described in the other excellent reviews (Dyer and O'Gorman 2012; Dyer et al. 2003). In this section, functions of select genes are summarized.

Most **homothallic fungi** contain mating-type genes which are crucial for mating processes. In *A. nidulans*, two genes MAT-1 (*matB*) and MAT-2 (*matA*) were the first to be described. They were distinct from other fungi in that they are not localized on the same chromosome (Paoletti et al. 2007). Deletion of *MAT1* or *MAT2* results in a significantly decreased number of abnormal cleistothecia. Overexpression of the mating-type genes causes cleistothecia production on submerged liquid culture which represents unfavorable conditions for sexual development, suggesting that there are key genes for sexual development (Paoletti et al. 2007).

As mentioned above, the velvet family proteins are multifunctional coordinators of fungal growth, conidiation, sexual development, and secondary metabolism in filamentous fungi (Bayram and Braus 2012). The roles of the

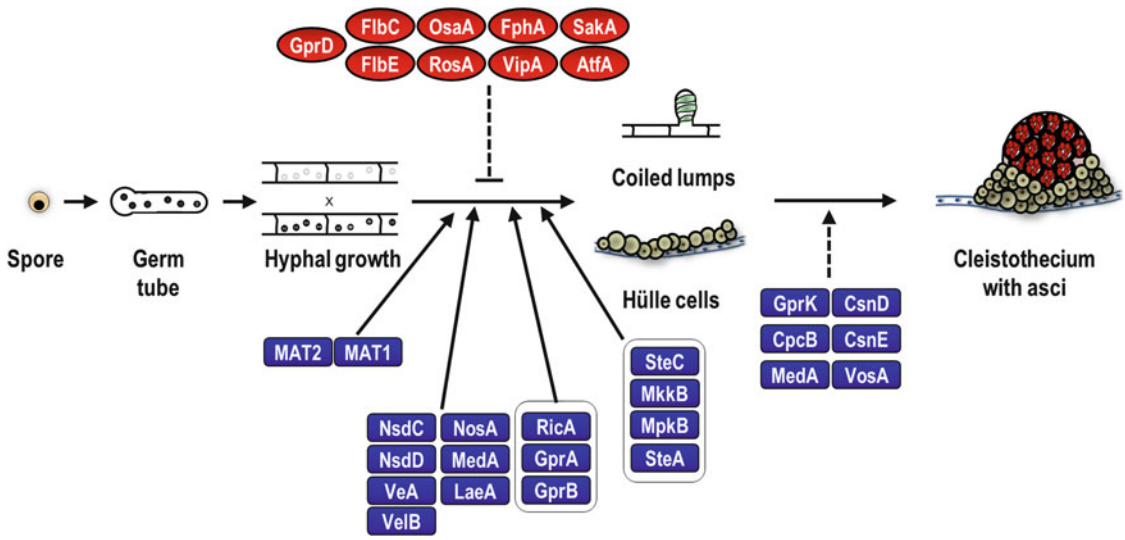

Fig. 3 Sexual development of *A. nidulans*. A schematic presentation of development of sexual fruiting bodies and associated developmental genes in *A. nidulans*. Proteins in the red circle can act as repressors during sexual development. Whereas, proteins in the blue rectangle can induce formation of sexual fruiting bodies. (See main text for details)

velvet proteins in sexual development were described by Käfer (Kafer 1965). The *veA1* mutant produced decreased numbers of sexual fruiting bodies with increased conidiation, suggesting that VeA acts as a balancer between asexual and sexual development (Kafer 1965; Mooney and Yager 1990). Follow-up studies extensively characterized the roles of the velvet proteins. Importantly, Kim et al. identified the *veA* gene and showed that VeA was crucial for sexual development and sterigmatocystin production (Kim et al. 2002). Later studies have revealed the molecular mechanisms of VeA action, which showed that the binding partners and localization of VeA were important for the roles of VeA in sexual development, especially the light-dependent condition (Bayram et al. 2008; Stinnett et al. 2007). In the light, VeA is mainly localized in the cytoplasm; therefore it cannot induce sexual development. The nuclear localization of VeA is regulated by the light complex components FphA, LreA, and LreB (Purschwitz et al. 2008, 2009). Under dark conditions, however, VeA interacts with VelB in the cytoplasm and translocates in the nucleus, leading to the formation of the VeA-VelB or VelB-VelB-LaeA complexes that control cleistothecia production and sterigmatocystin biosynthesis (Bayram et al. 2008). Deletion of either *veA* or *velB* results in the absence of sexual fruiting bodies under sexually favorable conditions (Kim et al. 2002; Park et al. 2012). The *laeA* deletion mutant also produced abnormal cleistothecia (Sarikaya Bayram et al. 2010). Another VeA interacting protein, VipA (*ve*A-*i*nteracting *p*rotein A), is also involved in the light-dependent developmental process. The phenotype of the *vipA* null mutant is similar to that of the *fphA* null mutant (Rohrig et al. 2017). Recently, Rauscher et al. demonstrated that phosphorylation of VeA affects their roles in sexual development (Rauscher et al. 2016). With VeA, VelB is also required for the initiation of sexual development.

VelB exists in both the VeA-VelB and VelB-VosA complexes that play different roles in hyphae. Previously, we proposed the ratio of VeA-VelB and VelB-VosA is crucial for initiation of sexual reproduction, and this ratio can be regulated by VelC (*ve*lvet-*l*ike C). After acquisition of the sexual developmental competence, VelC is produced and forms the VosA-VelC complex, leading to decreased formation of the VelB-VosA hetero-complex whereas increased formation of VelB-VeA (Park et al. 2014).

Classical genetic approaches are a useful way to identify genes that play crucial roles in developmental stages (Han 2009). Han and colleagues screened massive mutants showing defective sexual reproduction and classified them into three groups: **NSD mutants** (never in sexual development), **BSD mutants** (block in sexual development), and **ASD mutants** (abnormal in sexual development) (Han et al. 1990). NSD mutants exhibited common phenotypes including the absence of sexual fruiting bodies, apical growth, and earlier development of conidiospores (Han et al. 1994, 1998). Among them, two genes, *nsdC* and *nsdD*, were further characterized (Han et al. 2001; Kim et al. 2009). NsdC contains a C_2H_2-C_2H_2-C_2HC zinc finger DNA-binding domain and acts as a key positive regulator of sexual development. Overexpression of *nsdC* leads to increased formation of sexual fruiting bodies and overcome environmental factors which inhibit cleistothecial development (Kim et al. 2009). NsdC is also required for repression of asexual development and *brlA* expression, suggesting that NsdC can regulate balance between asexual and sexual development. NsdD is a GATA-type TF that functions as activator of sexual reproduction (Han et al. 2001). Similar to the *nsdC* mutants, strains overexpressing *nsdD* can produce more sexual fruiting bodies compared to the wild-type strain (Han et al. 2001).

Signal transduction pathways, including G protein signaling pathways and **mitogen-activated protein kinase** (MAPK) cascades, play multifunctional roles in biological processes in most organisms (Lengeler et al. 2000). In *A. nidulans*, several G protein-coupled receptors have been shown to be required for self-fertilization, sexual reproduction, and/or secondary metabolism (Han et al. 2004a; Seo et al. 2005). Three G protein-coupled receptors (Gpr), GprA, GprB, and GprD, are required for proper sexual development (Han et al. 2004a; Seo et al. 2004). Especially, GprD signaling

pathway may act as an upstream negative regulator for GprA- and GprB-mediated sexual reproduction and the formation of sexual fruiting bodies (Han et al. 2004a; Seo et al. 2004). Another G protein-coupled receptor, GprK, might be required for the maturation of cleistothecia. The *gprK* deletion mutant can produce Hülle cells but is blocked in formation cleistothecia (Dyer and O'Gorman 2012). The Gβ-like protein CpcB is involved in middle of end phase of sexual development (Hoffmann et al. 2000; Kong et al. 2013; Palmer et al. 2006). Deletion of *cpcB* causes decreased formation of cleistothecia and the absence of ascospores in cleistothecia (Kong et al. 2013). The guanine nucleotide exchange factor RicA regulates the G protein signaling pathway required for fungal growth and sexual development. The absence of *ricA* resulted in the lack of Hülle cells or cleistothecia formation (Kwon et al. 2012). Overall, these results demonstrate that several G protein signaling pathways play a crucial role in the sexual development in *A. nidulans*.

As mentioned above, the functions of MAPK pathways were well characterized in *Saccharomyces cerevisiae* and other filamentous fungi, and several kinases in the MAPK signaling cascade are involved in pheromone response, pathogenesis, and stress responses (Xu 2000). SteC (equivalent of yeast Ste11p, MAPKKK, or MAPKK kinase), MkkB (homolog of yeast Ste11p, MAPKK, or MAPK kinase), MpkB (homolog of yeast Fus3p, MAPK, or MAP kinase), and SteA (homolog of yeast Ste12p) are components of MAPK cascade in *A. nidulans*, and these proteins work together to regulate sexual development (Bayram et al. 2012; Paoletti et al. 2007; Vallim et al. 2000; Wei et al. 2003). The absence of any of these genes results in failure to form ascogenous hyphae and cleistothecia. The **HOG pathway** (*h*igh-*o*smolarity *g*lycerol) is mainly involved in stress response in many yeast and fungi. However, this pathway is also involved in controlling sexual development in *A. nidulans* (Kawasaki et al. 2002). Kawasaki and colleagues found that deletion of *sakA* (*hogA*) causes increased production of cleistothecia, suggesting that SakA (*s*tress *a*ctivated *k*inase A) acts as a repressor of sexual development (Kawasaki et al. 2002). The SakA interacting protein AtfA (homolog of *Schizosaccharomyces pombe* Atf1) is also associated with sexual development as the *atfA* deletion mutant produces increased number of cleistothecia (Lara-Rojas et al. 2011).

The balance between asexual and sexual development is regulated by various factors. Three oxylipin biosynthetic genes *ppoA*, *ppoB*, and *ppoC* (psi factor producing oxygenase) are required for proper asexual and sexual development (Brodhun and Feussner 2011; Tsitsigiannis and Keller 2007; Tsitsigiannis et al. 2004, 2005). Deletion of *ppoA* or *ppoB* causes increased conidial production suggesting that PpoA and PpoB negatively affect asexual development (Tsitsigiannis et al. 2004, 2005). However, deletion of *ppoC* leads to decreased asexual sporulation, suggesting that PpoC positively influences conidiation and antagonizes PpoA and PpoB (Tsitsigiannis et al. 2004, 2005). As mentioned above, OsaA functions as a key orchestrator of sexual and asexual development (Alkahyyat et al. 2015). The deletion of *osaA* causes enhanced sexual fruiting with reduced conidiation, suggesting that OsaA acts as a repressor of sexual development and indirectly affects asexual development in a positive way. The deletion of *osaA* could suppress the *veA1* mutant allele leading to the sexual developmental phenotype similar to that of *veA*+ wild type. This indicates that OsaA acts downstream of VeA as a key negative regulator of sexual development (Alkahyyat et al. 2015). Thus, one key event to achieve the sexual developmental competence is to remove the repressive effects imposed by OsaA.

Some asexual regulators are involved in both asexual and sexual development. StuA and MedA are asexual developmental modifiers that are necessary for formation of cleistothecia (Clutterbuck 1969; Martinelli 1976). While the *stuA* deletion mutant cannot produce Hülle cells, the *med1* mutant is able to produce Hülle cells, suggesting that these two modulators play different roles in sexual development (Busby et al. 1996; Wu and Miller 1997). Upstream activators of asexual development FlbC and FlbE repress sexual development. Deletion of either *flbC* or *flbE* resulted in increased formation of cleistothecia (Kwon et al. 2010a, b).

CSN, the **constitutive photomorphogenesis complex 9** (COP9) **signalosome**, is a multisubunit complex that is involved in multiple fungal developmental processes (Braus et al. 2010). In *A. nidulans*, eight subunits were identified, and these subunits play diverse roles in sexual development. For example, the *csnD* deletion mutant cannot enter the primordial stage in sexual development, but this mutant can produce primordia under light conditions (Busch et al. 2003). CsnE is required for expression of cell wall-degrading enzymes and maturation of sexual fruiting bodies (Nahlik et al. 2010). Unlike CsnD and CsnE, three subunits including CsnA, CsnB, and CsnG act as activators of sexual development (Busch et al. 2003, 2007; Nahlik et al. 2010). Overall, CSN is a major contributor to regulate sexual developmental processes (Braus et al. 2010).

Two orthologues of *Sordaria macrospora* Pro1, RosA (*r*epressor *o*f *s*exual development) and NosA (*n*umber *o*f *s*exual spores), were found in the *Aspergillus* genome (Vienken and Fischer 2006; Vienken et al. 2005). Genetic analysis proposed that NosA might be an NsdD downstream activator for sexual development and be associated with a completion of sexual reproduction (Vienken and Fischer 2006). Unlike NosA, RosA regulates expression of genes involved in sexual primordia and, hence, functions in an early stage of sexual development (Vienken et al. 2005).

VI. Conclusions

Fungal development is a very complex process which is influenced by various internal and/or external factors. To enter development stages, fungi must acquire the developmental competence. Increasing evidence from numerous recent investigations suggest that the key event for the acquisition of the developmental competence is to remove the repressive effects imposed by multiple negative regulators of asexual or sexual development. It appears that, even in the presence of developmental activators, the initiation of developmental processes would not occur, as long as developmental repressors or growth inducers are prevailing. During developmental stages, several TFs or signal cascades control developmental processes. In this chapter, we have summarized key regulators for making the developmental decisions and their roles in sexual and asexual development in *A. nidulans*. Further studies aimed at revealing the detailed molecular mechanisms of sexual or asexual reproduction in diverse fungal species will illuminate the common and distinct regulators and signaling cascades governing growth and development in fungi.

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