Development in *Aspergillus*

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Abstract: The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. Germination of a spore can lead to a vegetative mycelium that colonizes a substrate. The hyphae within the mycelium are highly heterogeneous with respect to gene expression, growth, and secretion. Aspergilli can reproduce both asexually and sexually. To this end, conidiophores and ascocarps are produced that form conidia and ascospores, respectively. This review describes the molecular mechanisms underlying growth and development of *Aspergillus*.

Key words: *Aspergillus*, fungi, asexual reproduction, sexual reproduction, development, conidium, conidiophore, vegetative mycelium, heterogeneity, ascocarp, ascospore, fruiting body.

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INTRODUCTION

Aspergillus is an anamorph genus, which comprises between 260 (Geiser *et al.* 2007, Samson & Varga 2009) and 837 species (Hawksworth 2011). These species are classified in approximately ten different teleomorph genera (Geiser 2009). For instance, *A. nidulans* is part of the teleomorph genus *Emericella*, while *A. fumigatus* and *A. flavus* belong to the genera *Neosartorya* and *Petromyces*, respectively. This shows that *Aspergillus* is a diverse group of fungi. Indeed, comparison of the genomic sequences of *A. nidulans* and *A. fumigatus* (Galagan *et al.* 2005) showed that these fungi are as related to each other as fish to humans. These animals separated about 450 million years ago but diversification in the genus *Aspergillus* is assumed to be restricted to about 200 million years (Galagan *et al.* 2005). The large differences in genomic sequence have been proposed to be caused by an accelerated evolutionary rate (Cai *et al.* 2006).

Aspergillus species are among the most abundant fungi worldwide. They are not very selective with respect to abiotic growth conditions (Table 1). For instance, they can grow over a wide range of temperature (6–55 °C) and at relatively low humidity. In fact, *A. penicilloides* is among the most xerophilic fungi (Williams & Hallsworth 2009). Moreover, *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue. Nonetheless, they are predominantly found on complex plant polymers (Bennett 2010) and are considered to be common food spoilage fungi. The success of *Aspergillus* is also explained by their effective dispersal. Spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances (Bennett 2010). Aspergilli are not only known because of their saprobic life style. *Aspergillus niger* has been reported to be a pathogen of *Zingiber officinale* plants (Pawar *et al.* 2008). Moreover, a wide variety of aspergilli are opportunistic pathogens of animals and humans. They do not infect healthy individuals but do invade individuals with a compromised immune system (Pitt 1994, Brakhage 2005). Aspergilli (*i.e. A. fumigatus*, and to a lesser extent species such *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*) cause invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis (Denning 1998, Stevens *et al.* 2000).

Aspergillus spp secrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For instance, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material. Similarly, elastase is secreted in the human lung to degrade elastin. The capacity to secrete large amounts of proteins (and other metabolites such as organic acids) in combination with established fermentation technology and molecular biology make aspergilli such as *A. niger, A. oryzae, A. awamori, A. sojae,* and *A. terreus* attractive cell factories for the production of homologous and heterologous proteins (Meyer *et al.* 2011). The potential of these fungi is exemplified by strains of *A. niger* that produce more than 30 grams per liter of glucoamylase (Finkelstein *et al.* 1989). Of concern, *Aspergillus spp* can form mycotoxins that are toxic for animals and humans. *Aspergillus flavus* produces aflatoxin, which is one of the most carcinogenic natural molecules (Varga *et al*. 2011). In addition, different aspergilli, including *A. westerdijkiae*, can form ochratoxin on food products such as coffee and grapes (Leong *et al.* 2007).

This review describes the current understanding of development of aspergilli. Germination of spores, formation of a differentiated vegetative mycelium, and formation of asexual and sexual spores are discussed. Table 2 summarises the role of genes in these

Table 1. Conditions for vegetative growth of selected *Aspergilli*.

Species	Optimum Temp (ºC)	Temp range (ºC)	Optimum pH	pH range	Minimal Water activity	Optimum Water activity	Minimum Relative humidity (%)	Optimum Relative humidity (%)	References
A. niger	35–37	6–47	6.0	1.5–9.8	0.77	0.97	88–89	96–98	(Astoreca *et al.* 2007, Ayerst 1969, Leong *et al.* 2006, Mehra & Jaitly 1995, Panasenko 1967, Pitt 1981)
A. oryzae	30–37	7–47	6.0–7.5	4–8		0.99			(Chipeta *et al.* 2008, Gibson *et al.* 1994, Nasseri *et al.* 2002, Panasenko 1967)
A. fumigatus	37	10–55	5.5–6.5	3.0–8.0	0.82	0.97	85	98–99	(Al-Doory 1984, Ayerst 1969, Ogundero 1981, Panasenko 1967, Singh & Sandhu 1982)
A. clavatus	20–25	5–42			0.88		88	98	(Panasenko 1967)
A. terreus	37	15–42	5.0		0.78				(Al-Doory 1984, Mehra & Jaitly 1995, Singh & Sandhu 1982)
N. fischeri	26–45	11–52				0.98			(Beuchat 1986, Nielsen *et al.* 1988, Samson *et al.* 2000, Valik & Pieckova 2001)
A. nidulans	35–37	6–51	7.0	2–12	0.78		80	95	(Agnihotri 1964, Al-Doory 1984, Lacey 1980, Panasenko 1967)

processes. *Aspergillus nidulans*, *A. fumigatus, A. oryzae*, and *A. niger* have been chosen as the lead organisms for this review. The effect of light on the formation of asexual and sexual spores will serve as an example how environmental factors can influence development. The process of meiosis is beyond the scope of this review (for a review see Pöggeler *et al.* 2006), and the relation between primary and secondary metabolism will not be discussed as well. For this we refer to Yu & Keller (2005) and Pöggeler *et al.* (2006). For the effect of other environmental factors than light we refer to Clutterbuck 1977, Skromne *et al.* 1995, Penalva & Arst 2004, and Etxebeste *et al.* 2010b.

VEGETATIVE GROWTH

In nature, aspergilli grow within and on solid substrates. A colony can result from a single sexual or asexual spore but it may also arise after conidia and/or germlings that are in close vicinity to each other have fused. It has been described that fusion in *A. oryzae, A. sojae* and *A. tamarii* most often occurs between conidia (> 80%), while fusions between conidia and germlings and fusion of germlings are much less frequent (Ishitani & Sakaguchi 1956). Fusion is mediated by fusion bridges that are formed by conidia or germ tubes. They may be similar to the conidial anastomosis tubes that are formed by *Colletotrichum* and *Neurospora* (Roca *et al.* 2003, Roca *et al.* 2005a, Roca *et al.* 2005b). These anastomosis tubes are morphologically and physiologically distinct from germ tubes. They are typically short, thin, and unbranched. Fusion of conidia and germlings has been described to occur within *Aspergillus* strains, between *Aspergillus* strains, between different aspergilli and even between *Aspergillus* and *Penicillium* species (Ishitani & Sakaguchi 1956). However, fusion between strains and between species often results in heterokaryon incompatibility. For instance, heterokaryon incompatibility is a widespread phenomenon among *A. niger* strains. The underlying mechanism is, however, not known (van Diepingen *et al.* 2009). Fusion of hyphae was reported to be rare when germlings of *A. oryzae, A. sojae* and *A. tamarii* had formed hyphae (Ishitani & Sakaguchi 1956). Whether this also holds for other aspergilli is not known. At least, fusion of hyphae has been shown to occur in other ascomycetes (for references see Ishitani & Sakaguchi 1956).

Colonies can reach a diameter in the (sub-)milimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. The mycelium of *A. nidulans* (Lee & Adams 1994a) and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively. Colonies can also be grown between porous polycarbonate membranes on an agar medium (Levin *et al.* 2007a, Levin *et al.* 2007b, Masai *et al.* 2006, Wösten *et al.* 1991). Scanning electron microscopy shows that the periphery of a 7 d old sandwiched colony of *A. niger* consists of a single layer of hyphae (Fig. 1A, D). A few millimeters behind the periphery this layer becomes thicker and comprises of up to six layers of hyphae growing on top of each other. Notably, three distinct layers are observed another two millimeters towards the centre (Fig. 1B, E). The upper and lower layer consist of up to five hyphae on top of each other, while the intermediate layer comprises a loose network of thin and thick hyphae, and some non-sporulating conidiophores. Three distinct layers are also observed in the innermost centre of the colony (Fig. 1C, F). In this case, the upper and lower layers consist of up to twenty and six layers of hyphae, respectively. The intermediate layer comprises a dense network of both thin and thick hyphae, and a relatively high number of non-sporulating conidiophores. An *A. niger* colony grows in a similar way when a 0.45 mm thin agarose layer is present in between the polycarbonate membranes.

Mycelium can grow dispersed, as clumps or as micro-colonies, also known as pellets, during submerged growth in liquid medium. Clumps are aggregated hyphae that are considered to be an intermediate state between pelleted and dispersed growth. The

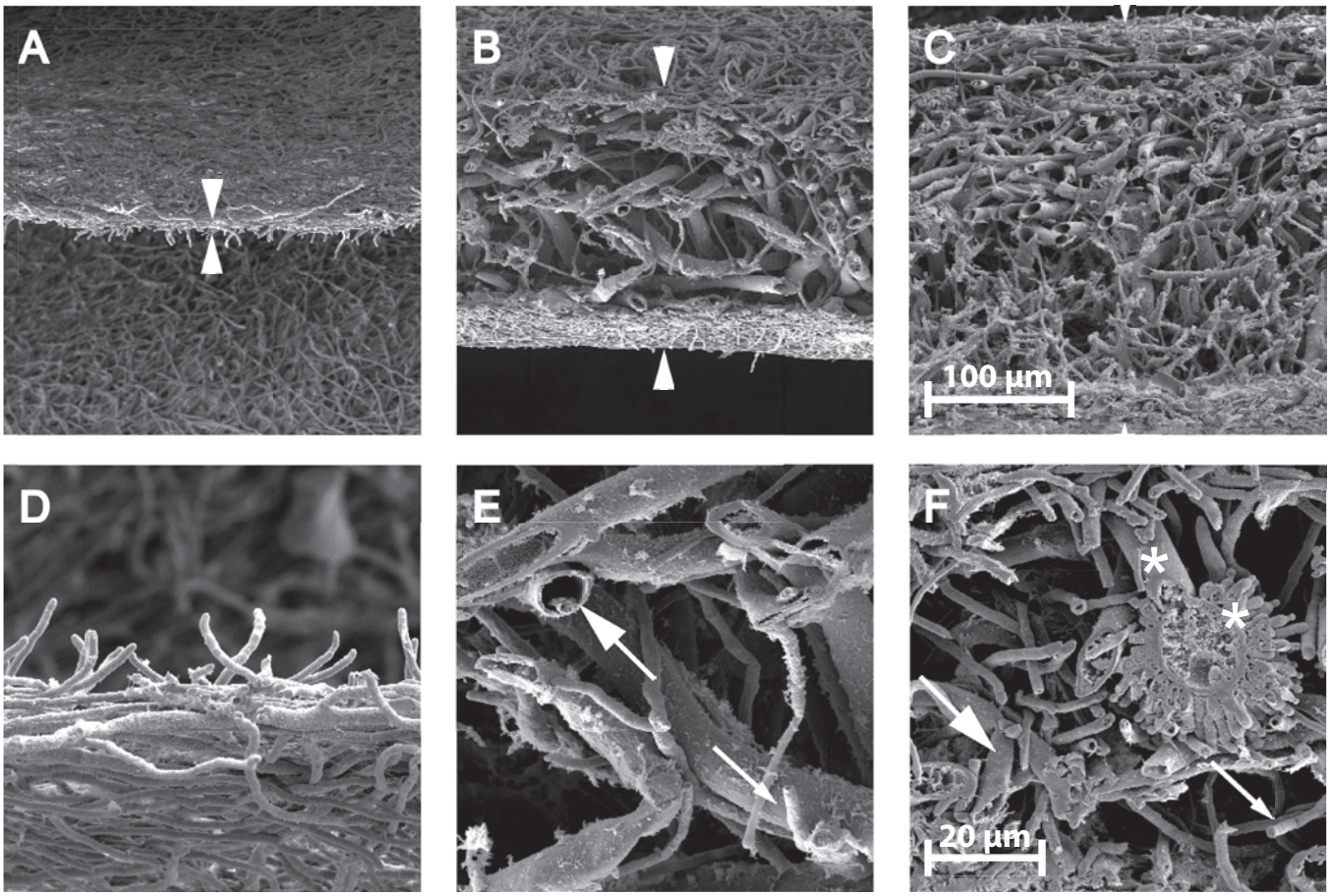

Fig. 1. Scanning electron microscopy of cross sections of a 7 d old sandwiched *A. niger* colony. Cross sections were made at the periphery (A, D), four millimeter behind the periphery (B, E) and at the innermost center (C, F). The thickness of the colony is indicated by the distance between the white triangles. Panels D–F represent higher magnifications of A–C, respectively. Thin and thick arrows point at thin and thick hyphae, respectively. In H asterisks mark a non-sporulating conidiophore. Bars in panel C, for A–C, and F, for D–F, represent 100 and 20 µm.

morphology of the mycelium has an enormous impact on the production of enzymes and primary or secondary metabolites. For instance, micro-colonies are required for the production of citric acid by *A. niger* (Vecht-Lifshitz *et al.* 1990). It is not clear how morphology exactly affects productivity. It has been proposed that this is due to the effect of the fungal morphology on the viscosity of the medium (Bhargava *et al.* 2003). Viscosity correlates with the extent of dispersed growth; large micro-colonies thus result in a low viscosity. The center of large pellets may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium. These conditions may also impact productivity of the pellets.

Pellet formation is caused by coagulation of the conidia in the culture. Parameters that affect coagulation of *A. niger* and *A. orzyae* conidia are initial pH, agitation, and medium composition (Metz & Kossen 1977, Carlsen *et al.* 1996). For instance, the chelating agents EDTA and ferrocyanide lead to small and compact pellets, whereas anionic polymers like carboxypolymethylene and polyacrylate give rise to small but loose pellets. Pellet formation can also be manipulated by changing the surface composition of spores. Formation of micro-colonies was affected in strains of *A. nidulans* in which either or both *dewA* and *rodA* were inactivated (Dynesen & Nielsen 2003). The effect was strongest when both these hydrophobin genes were inactivated, which was accompanied by a huge drop in surface hydrophobicity of the conidia (see below). Pellet formation in *A. niger* was also affected by inactivation of one of the pigmentation genes (van Veluw *et al.* 2013). Conidia were no longer hydrophobic in the case of the Δ*olvA* strain but the other deletion strains affected in pigmentation (*i.e.* the Δ*fwnA* and Δ*brnA* strains) were still hydrophobic. Taken together, surface hydrophobicity of conidia plays a role in pellet morphology but other factors are important as well.

Research in the last two decades has shown that the mycelium of *Aspergillus* is heterogeneous with respect to gene expression, growth, and secretion. Genome wide expression analysis has shown that the RNA composition of central and peripheral zones of colonies of *A. niger* (Levin *et al.* 2007a) and *A. oryzae* (Masai *et al.* 2006) is different. In the case of 7 d old colonies of *A. niger*, 25 % of the active genes show a two-fold or more difference in RNA accumulation between the innermost and outermost zone of the mycelium (Levin *et al.* 2007a). For instance, RNA levels of the glucoamylase gene *glaA* are 3-fold higher at the periphery of maltose-grown colonies when compared to the center. Similarly, accumulation of transcripts of the ferulic acid esterase gene *faeA* is 5-fold higher at the periphery of xylose grown colonies. Notably, 9 % of the genes that are active in a 7 d old colony are expressed in only one of five concentric zones. For instance, genes related to nitrate metabolism are specifically expressed in the outer zone of the colony, whereas mRNA of the hydrophobin *hfbD* is almost exclusively found in a central zone. Half the variation in RNA profiles is explained by differences in the composition of the medium underlying each zone of the colony, whereas the other half of the variation is caused by medium-independent mechanisms (Levin *et al.* 2007a). These findings imply that differentiation occurs within the vegetative mycelium of *Aspergillus.*

The heterogeneity of the mycelium of *A. niger* is also indicated by the fact that distinct zones of the colony grow and secrete

Table 2. Overview of *Aspergillus* genes involved in the different developmental stages. Functions of genes refer to *A. nidulans*, unless otherwise indicated.

Name	Description	Developmental stage	Function	Page number
abaA	ATTS Transcription factor	Asexual development	Regulation of sterigmata formation during conidiophore development	9, 10, 12, 14, 19, 24
abr1	Vermelone dehydratase	Spore protection	Melanin biosynthesis in *A. fumigatus*	21, 24
abr2	Laccase (with homology to *yA*)	Spore protection	Melanin biosynthesis in *A. fumigatus*	21
alb1	Polyketide synthase	Spore protection	Melanin biosynthesis in *A. fumigatus*	21
arp1	Scytalone dehydratase	Spore protection	Melanin biosynthesis in *A. fumigatus*	21
arp2	Hydroxynaphthalene (HN) reductase	Spore protection	Melanin biosynthesis in *A. fumigatus*	21
ayg1	Polyketide carbon backbone modification	Spore protection	Melanin biosynthesis in *A. fumigatus*	21
brlA	C_2H_2 zinc finger transcription factor	Asexual development	Regulation of stalk development	9–14, 17–19, 21, 24
brnA	Multicopper oxidase	Spore protection	Melanin biosynthesis in *A. niger*	3, 9, 21
chiB	Class V endochitinase B	Vegetative growth	Autolysis	10
chsA	Chitin synthase	Asexual development	Septum formation in conidiophores	10
chsC	Chitin synthase	Asexual development	Septum formation in conidiophores	10
cryA	Cryptochrome/photolyase	Blue light response	Inhibition of sexual development in the light	19–21
cyaA	Adenylate cyclase	Germination	GanB mediated germination	24, 25
dewA	Hydrophobin	Asexual development	Coating of conidia	3, 9, 14
fadA	Gα-subunit heterotrimeric G-protein complex	Vegetative growth	Inhibition sexual and asexual development	10–13, 16
		Sexual development	Homothallic cleistothecia and Hülle cell formation	
flbA	Regulator of G-protein signaling	Asexual development	Inhibition of vegetative growth enabling asexual development	10–13, 16
		Sexual development	Homothallic cleistothecia formation	
flbB	bZIP-type transcription factor	Asexual development	Regulation of conidiophore formation	7, 12, 13
flbC	C_2H_2 zinc finger transcription factor	Asexual development	Regulation of conidiophore formation	12, 13, 16, 17, 24
		Sexual development	Repression sexual development	
		Germination	Germination	
flbD	c-Myb transcription factor	Asexual development	Regulation of conidiophore formation	12–13
flbE	Protein involved in conidiophore formation	Asexual development	Regulation of conidiophore formation	12, 13, 16, 17
		Sexual development	Repression sexual development	
fluG	Homology to bacterial glutamine syntetase	Asexual development	Production of extracellular signaling molecule involved in conidiophore development	8, 9, 11–13, 24
		Germination	Germination	
fphA	Phytochrome	Red Light response	Stimulation asexual development and repression of sexual development in the light	19–21
fwnA	Polyketyde synthase	Spore protection	Melanin biosynthesis	3, 9, 21
ganA	Gα-subunit of heterotrimeric G-protein complex of *A. nidulans* and *A. oryzea*	Not known		12, 16
ganB	Gα-subunit of heterotrimeric G-protein complex of *A. nidulans* and *A. oryzea*	Vegetative growth	Repression asexual development	11, 12, 16, 23, 24
		Germination	cAMP dependent Germination	
gaoC	Gα-subunit of heterotrimeric G-protein complex of *A. oryzae*	Not known		12
gpaA	Gα-subunit of heterotrimeric G-protein complex of *A. fumigatus*	Vegetative growth	Promotion vegetative growth	12
gpaB	Gα-subunit of heterotrimeric G-protein complex of *A. fumigatus*	Asexual development	Regulation asexual development	12
gpgA	Gγ-subunit of heterotrimeric G-protein complex	Vegetative growth	Stimulation vegetative growth	11, 12, 15, 16, 23, 24
		Sexual development	Regulation of cleisthotecia formation	
		Germination	Trehalose degradation during germination	
gprA	G-protein receptor (GPCR)	Sexual development	Homothallic cleistothecia formation	15–17
gprB	G-protein receptor (GPCR)	Sexual development	Homothallic cleistothecia formation	15–16

Table 2. (Continued).

Name	Description	Developmental stage	Function	Page number
gprD	G-protein receptor (GPCR)	Vegetative growth	Repression sexual development	15
kapA	α-importin	Light response	Protein import into nucleus in the dark	18, 20
laeA	Transcription factor	Light response	Regulation of asexual development, sexual cleistothecia and Hülle cell formation	19, 20
lreA	White collar blue light receptor	Blue light response	Stimulation sexual development in the light, repression asexual development in the light	19, 20
lreB	White collar blue light receptor	Blue light response	Stimulation sexual development in the light, repression asexual development in the light	19, 20
MAT1-1	α-homeodomain transcription factor	Sexual development	Regulation of sexual reproduction	15
MAT1-2	High mobility group domain (HMG)-transcription factor	Sexual development	Regulation of sexual reproduction	15
medA	Temporal regulation conidiophore formation	Asexual development	Regulation of conidiophore development	9, 10, 16, 17, 24
		Sexual development	Regulation of cleistothecia and Hülle cell formation	
mpkB	Mitogen activated protein kinase (MAPK)	Sexual development	Signalling in cleistothecia and Hülle cell formation	14, 16, 17
nosA	$Zn(II)_2Cys_6$ transcription factor	Sexual development	Regulation of cleistothecia formation (primordium maturation)	16, 17, 19
nsdC	Zinc finger transcription factor	Vegetative growth	Repressing asexual development	14, 16, 17
		Sexual development	Regulation of cleistothecia formation	
nsdD	GATA-like transcription factor	Asexual development	Repressing asexual development	14, 16–18, 21
		Sexual development	Regulation of cleistothecia and Hülle cell formation	
olvA	Homologue of *aygA*	Spore protection	Melanin biosynthesis in *A.niger*	3, 9, 21
phnA	Phosducin like protein	Vegetative growth	Positive regulation Gβγ stimulating vegetative growth	11
pkaA	Protein kinase	Vegetative growth	Stimulation vegetative growth	11, 12, 24
		Germination	Signalling involved in germination	
pkaB	Protein kinase activity	Vegetative growth	Potential backup for pkaA	11, 12, 24
		Asexual development		
		Germination	Germination spores	
ppoA	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (psiBα)	17–19
			regulating asexual and sexual development	
ppoB	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (psiBβ) regulating asexual and sexual development	17–19
ppoC	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (psiBβ) regulating asexual and sexual development	17–19
pptA	Polyketide synthase	Spore protection	Melanin biosynthesis in *A. niger*	21
rasA	GTPase of the RAS superfamily	Asexual development	Polarised growth during germination	23, 24
		Germination		
rgsA	Regulator of G-protein signaling	Asexual development	Enhancing intrinsic activity GanB (G-α subunit)	11, 12, 23, 24
			Regulation *brlA*	
rodA	Hydrophobin	Asexual development	Formation rodlet layer during conidiophore development	3, 9, 14
rodB	Hydrophobin	Asexual development	Formation rodlet layer during Conidiophore development	14
rolA	RodA-like Hydrophobin	Asexual development	Cutinase recruitment during conidiophore formation in *A. oryzae*	14
rosA	$Zn(II)_2Cys_6$ transcription factor	Sexual development	Cleistothecia formation (VeA dependent)	16, 17, 21
schA	Ser/Thr protein kinase	Germination	Signalling leading to germination	24
sfaD	Gβ-subunit of heterotrimeric G-protein complex	Vegetative growth	Regulation vegetative growth	11, 12, 15, 16, 23, 24
		Sexual development	Regulation cleistohtecia formation	
		Germination	Trehalose degradation during germination	
sfgA	Gal4-type $Zn(II)_2Cys_6$ type transcription factor	Vegetative growth	Repression asexual development	12, 13

Table 2. (Continued).

Name	Description	Developmental stage	Function	Page number
steA	*STE*-like transcription factor	Sexual development	Repression Cleistothecia formation, and regulating Hülle cell formation	14, 16, 17, 19, 24
steC	C_2H_2 zinc finger transcription factor *MAPKKK*	Asexual development	Mitogen activated protein kinase kinase kinase (MAPKKK)	13, 14, 16
			Cleistothecia formation	
		Sexual development	Heterokaryon formation	
stuA	APSES domain transcription factor	Asexual development	Spatial regulation conidiophore formation	9, 10, 16, 17, 24
		Sexual development	Cleistothecia and Hülle cell formation	
		Germination	Germination	
tpsA	α-α-Trehalose-6-phosphate synthase	Spore protection	Trehalose biosynthesis	22, 23
tpsB	α-α-Trehalose-6-phosphate synthase	Spore protection	Trehalose biosynthesis	22
treB	Neutral trehalase B	Germination	Degradation intracellular trehalose during germination	22, 23
veA	Velvet-protein with nuclear localisation signal	Light response	Regulation sexual development (inhibition asexual development)	10, 16–21, 23
		Asexual and Sexual development		
velB	Velvet-like protein	Light response	Regulation asexual/sexual development	10, 18–20, 22
		Asexual and Sexual development		
		Germination	Regulation trehalose synthetic genes during germination	
vosA	Velvet-like transcription factor	Light response	Repression conidiophore formation in the dark	9, 10, 12, 13, 19, 22, 24
		Asexual and Sexual development		
		Germination	Regulation trehalose synthesis genes during germination	
wetA	Synthesis cell wall layers	Asexual development	Regulation conidiophore maturation and formation	9, 10, 12, 14, 24
yA	Conidial laccase	Asexual development	Production dark green pigment in *A. nidulans*	9

proteins (Levin *et al.* 2007a, Levin *et al.* 2007b, Masai *et al.* 2006, Wösten *et al.* 1991). Proteins are formed throughout the *A. niger* mycelium (Levin *et al.* 2007a, Levin *et al.* 2007b, Wösten *et al.* 1991) (Fig. 2) but they are mainly secreted at the periphery. Growth is observed in this outer zone but also in the innermost centre (Fig. 2). Spatial growth and protein production is not affected when 6 d old colonies are transferred to fresh medium for 16 h. However, after transfer protein secretion is not only observed at the periphery of the colony but also in central parts of the mycelium (Fig. 2). These data show that non-growing zones of the mycelium abundantly secrete proteins upon transfer to fresh medium (Levin *et al.* 2007a). This is a remarkable finding considering the fact that protein secretion is generally assumed to take place in growing hyphae only (Moukha *et al.* 1993, Wessels 1989, Wessels 1990, Wösten *et al.* 1991).

The finding that 7 d old macro-colonies are heterogeneous with respect to RNA accumulation, growth and protein secretion raised the question whether heterogeneity is also observed between and within micro-colonies. Indeed, micro-colonies within liquid shaken cultures are heterogeneous with respect to size and gene expression (de Bekker *et al.* 2011b). A population of small and a population of large micro-colonies can be distinguished by flow cytometry in cultures of *A. niger* that consist of pellets with a maximum diameter of 1 mm. These populations differ 90 µm in diameter. Similarly, two populations of micro-colonies were distinguished when expression of the glucoamylase gene *glaA* and the ferulic acid esterase gene *faeA* were monitored. Notably, the population of lowly expressing micro-colonies is larger than the population of small pellets. This indicates that size of micro-colonies is not the only determinant for expression of genes encoding secreted proteins (de Bekker *et al.* 2011b). It is not yet clear how heterogeneous gene expression is between zones of micro-colonies. At least, the total amount of RNA per hypha is about 50 times higher at the periphery of 1 mm wide micro-colonies when compared to the center (de Bekker *et al.* 2011b).

Heterogeneous gene expression is not only observed between micro-colonies or between zones of micro- or macro-colonies of *Aspergillus*; it is also observed between hyphae in a particular zone. It has been described that only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase (Wösten *et al.* 1991). This observation is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone (Vinck *et al.* 2005). In fact, two populations of hyphae can be distinguished at the outer zone of the colony; those highly and those lowly expressing *glaA*. The hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins (Vinck *et al.* 2011). Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and show a high 18S rRNA content. Thus, two populations of hyphae are present at the periphery of a colony; those that are lowly and those that are highly metabolically active. From the fact that the lowly active hyphae have a growth rate similar to that of the highly active hyphae it has been concluded that a "low" activity of hyphae is sufficient to support hyphal growth. However, a "high" metabolism would be needed to support secretion of large amounts of proteins (Vinck *et al.* 2011). Recently, it has been described that transcriptionally and translationally

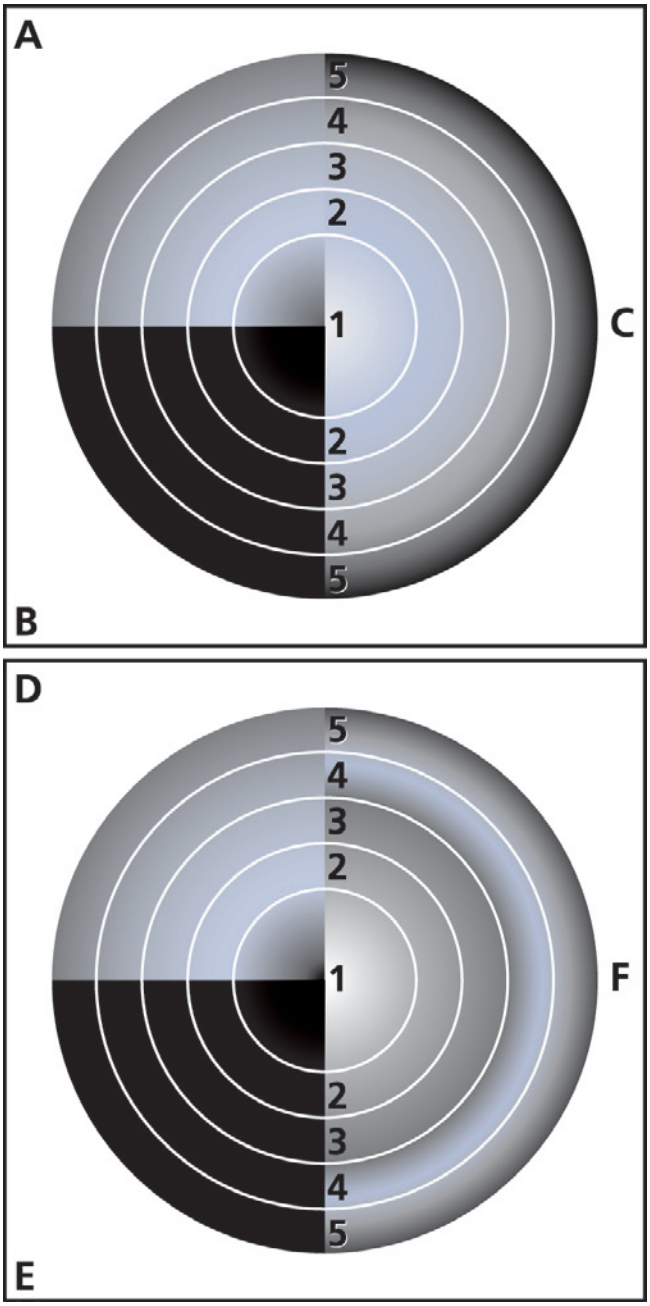

Fig. 2. Growth (A, D), protein synthesis (B, E) and protein secretion (C, F) in a 7 d old xylose grown sandwiched colony of *A. niger* before (A–C) and after transfer (D–F) to fresh medium. (Adapted from Levin *et al.* 2007).

highly active and lowly active hyphae also occur at the periphery of micro-colonies. However, the existence of distinct populations of these types of hyphae seems to be less robust when compared to macro-colonies grown on solid medium (van Veluw *et al.* 2013). Possibly, signalling between hyphae is involved in maintaining or enhancing heterogeneity. Gradients of signalling molecules cannot be formed between hyphae in liquid shaken cultures, which may explain why heterogeneity is less evident in these cultures.

Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own composition of RNA (de Bekker *et al.* 2011a). So far, we can only guess why hyphae are heterogeneous at the colony periphery. The leading hyphae explore the substrate and they may be exposed to rapid changes in the environment. A heterogeneous hyphal population may contribute to the survival under such conditions. Notably, the transcription factor FlbB, which is involved in asexual development (see below), has been shown to accumulate at 60 % of the tips of newly formed branches (Etxebeste *et al.* 2009). This is another example of heterogeneity within the *Aspergillus* mycelium.

Heterogeneity within the mycelium is surprising considering the fact that the cytoplasm of a fungal mycelium is assumed to be continuous. This is based on the fact that the septa within and between hyphae are porous allowing streaming of water, (in)organic compounds, proteins and even organelles (Jennings 1984, Jennings 1987, Bleichrodt *et al.* 2013). Heterogeneity between hyphae would require a certain immobility of molecules. This could be caused by the fact that many proteins are part of large complexes that are immobilised at membranes (Gavin *et al.* 2006). For instance, the yeast GPD homologs were found to be part of 17 protein complexes (Gavin *et al.* 2006). One of such protein complexes includes two transmembrane proteins that may well decrease the streaming rate by temporally immobilising the complex at the membrane. In agreement, the streaming rate of a fusion between GFP and GpdA was lower in *A. niger* than that of GFP itself (Bleichrodt *et al.* 2013). Closure of septa is another mechanism to maintain differences in composition between hyphae. Septa of vegetative hyphae of *A. oryzae* and *A. niger* (Bleichrodt, 2012) and the basidiomycete *Schizophyllum commune* (van Peer *et al.* 2009a, van Peer *et al.* 2009b) can be in a closed or open state. The incidence of closed septa in *A. niger* and *S. commune* depends on the environmental conditions and is reversible.

ASEXUAL DEVELOPMENT

After a period of vegetative growth, air-exposed colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae (Fig. 3). One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2–3 µm. The second type of aerial hyphae has a diameter of about 4–5 and 6–7 µm in the case of *A. nidulans* and *A. niger,* respectively. These so-called stalks can differentiate into conidiophores (Fig. 3). The conidiophore stalk of *A. nidulans* extends about 100 µm into the air and is formed from a specialised foot-cell within the substrate mycelium (Adams *et al.* 1998). When the stalk has reached its maximum height, the tip swells and forms a vesicle with a diameter of 10 µm. In biserate species like *A. nidulans* and *A. niger*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. This results in a second layer of sterigmata called phialides. The phialides give rise to chains of mainly uninucleate conidia. As a result, more than 10.000 conidia can be produced per conidiophore. *Aspergillus oryzae* can be both uniserate and biserate. In the case of uniserate species, spore producing phialides are positioned directly at the surface of the conidiophore vesicles.

The 2–3 µm wide aerial hyphae of *A. nidulans* and *A. niger* are formed about 8 h after inoculation of spores on complete medium. Although timing of this type of aerial hyphae seems to be medium-independent, the density of aerial hyphae is lower in the case of minimal medium. The first stalks of *A. nidulans* and *A. niger* are formed 10 h after spore inoculation on complete medium and growth at 37 °C and 30 °C, respectively. In both cases, conidiophores are formed 20 h post-inoculation. Formation of aerial hyphae in both aspergilli starts in the centre of the colony and moves outwards ending a few millimeters from the edge of the mycelium. This observation implies that the competence of hyphae to form aerial hyphae is acquired faster when a colony gets older (Adams *et al.* 1998). The process of aerial growth has been

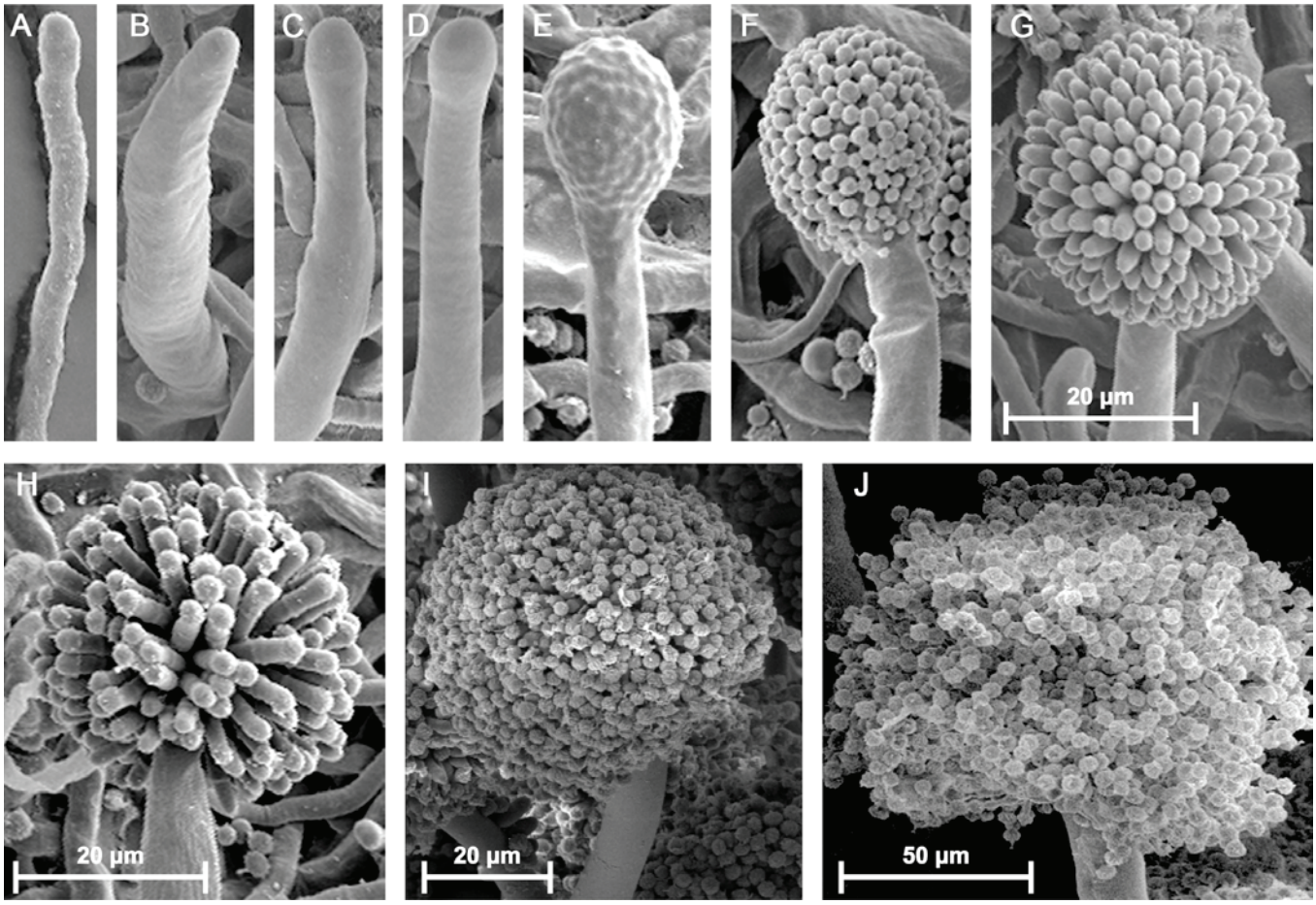

Fig. 3. Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2–3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C,D). Buds are formed on the vesicle (E) that develop into metulae (F, G). Phialides are formed on top of the metulae (H), which give rise to chains of conidia (I, J). The bar in G also holds for A–F.

proposed to involve signaling of the cell density of the vegetative mycelium (Lee & Adams 1994b, Wösten *et al.* 1999a, Wösten & Willey 2000). The signaling molecule would induce hydrophobin genes. These genes encode proteins that lower the water surface tension to enable hyphae to breach the interface to grow into the air (Wösten *et al.* 1999b, Wösten 2001). Which hydrophobin is secreted into the aqueous environment in *Aspergillus* cultures with the aim to lower the water surface tension is not yet known.

Aspergillus nidulans can also form conidia in submerged cultures (Adams *et al.* 1998). In this case, conidiation is induced when the culture gets stressed or when nutrients are limited (*e.g.* limitation of the carbon and the nitrogen source). On the other hand, formation of conidiophores in air-exposed colonies is assumed to be induced by an internal signal that activates a genetic program of sporulation (see below) (Adams *et al.* 1998). In both cases, competence to sporulate is acquired in a time-dependent way (Skromne *et al.* 1995). Like *A. nidulans, A. niger* can also form conidiophores in submerged conditions. However, these conidiophores do not form spore chains (Fig. 1F).

Aspergillus nidulans strains in which the *fluG* (*fluffy*) gene is inactivated (*i.e.* a Δ*fluG* strain) do form aerial hyphae but conidiophores are not being formed in excess of nutrients (Lee & Adams 1994b). During nutrient deprivation, however, some conidiophores are being formed on a solid medium. Similarly, submerged cultures of the Δ*fluG* strain start to sporulate in the absence of a carbon source (Lee & Adams 1996). These data indicate that FluG is involved in a developmental program of sporulation but not in the stress-related sporulation pathway. Formation of conidiophores in the Δ*fluG* strain can be rescued by growing the mutant next to a wild-type strain. Complementation is also observed when the strains are physically separated by a dialysis membrane with a size exclusion of 6–8 kDa. This indicates that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores. A similar phenomenon is observed in *Penicillium* species (Roncal & Ugalde 2003). In this case, an extracellular molecule called conidiogenone induces conidiation. Conidiogenone is a diterpene that accumulates during vegetative growth. At a certain point, a certain threshold level is exceeded and conidiation is initiated (Roncal & Ugalde 2003).

Regulation of asexual development

Formation of conidiophores has been well studied in *A. nidulans.* Experimental evidence has shown that mechanisms underlying asexual development in *A. fumigatus* and *A. oryzae* are similar but not identical in *A. nidulans* (see below). So far, formation of conidiophores and conidia has not been studied in *A. niger.* However, its genomic sequence predicts that mechanisms of asexual development are also similar, if not identical, to that in *A. nidulans* (Pel *et al.* 2007). About 1300 genes have been found to be up-regulated in whole colonies of *A. nidulans* during asexual reproduction (Timberlake 1980). Recently, RNA was isolated from the vegetative mycelium and from aerial structures (aerial hyphae, conidiophores, and spores) of 7 d old colonies of *A. niger*. Micro-array analysis showed that 34 genes are found in the top 100 of

most highly expressed genes of both the vegetative mycelium and the aerial structures (Bleichrodt *et al.* 2013). These genes include histones, ribosomal proteins, and a hydrophobin homologous to dewA.Of the 8 predicted hydrophobin genes (Pel *et al*. 2007, Jensen *et al*. 2010), 6 are within the top 100 of most highly expressed genes in the aerial structures. This top 100 also includes the pigmentation genes *fwnA, olvA* and *brnA* (for these genes see Dormancy and Germination). Seven genes encoding carbohydrate degrading enzymes are in the top 100 of highest expressed genes in the vegetative mycelium. One of these genes is the glucoamylase gene *glaA* (Bleichrodt *et al.* 2013).

Regulation by fluG, brlA, abaA, wetA, medA, stuA, and vosA

FluG is believed to be at the start of the developmental program leading to asexual sporulation in *A. nidulans*. Indeed, overexpression of *fluG* in vegetative hyphae is sufficient to cause sporulation under conditions that normally suppress conidia formation (Lee & Adams 1996). The *fluG* transcripts are present in relatively constant levels during late vegetative growth and conidiation. Notably, a 4-fold higher *fluG* expression level is found in germinating spores during their isotropic growth (3 h after inoculation) when compared to polar growing germlings (5 h after inoculation) (Breakspear & Momany 2007). This suggests that *fluG* is not only involved in conidiophore formation but also in germination.

FluG activates the *brlA* (*bristle*) gene. A *ΔbrlA* strain of *A. nidulans* forms stalks that do not stop their growth after they have reached a length of 100 µm. These stalks can reach a length 20–30 times longer than those of the wild-type, which results in the characteristic bristle phenotype (Adams *et al.* 1988). Moreover, isotropic growth is not initiated at the apex of the stalks of the *ΔbrlA* strain. As a result, conidiophore vesicles are not being formed. Conidiophore development becomes independent from *fluG* by placing *brlA* under control of an inducible promoter (Adams *et al.* 1988). Similar results have been obtained in *A. oryzae* (Ogawa *et al.* 2010, Yamada *et al.* 1999). Inactivation of *brlA* in *A. oryzae* results in the inability to form conidiophores. In contrast, fully developed conidiophores are formed in submerged culture when the *brlA* gene is expressed under the control of the *amyB* promoter. BrlA is also essential for conidiophore formation in *A. fumigatus* (Mah & Yu 2006). However, in contrast to *A. nidulans* (Adams *et al.* 1988) and *A. oryzae* (Ogawa *et al.* 2010), the *A. fumigatus* gene seems to function earlier in conidiophore development. This is based on the fact that conidiophore development is completely abolished in a Δ*brlA* strain of *A. fumigatus*. The appearance of the colonies of this strain is more similar to that of the fluffy mutants of *A. nidulans* (see below) (Mah & Yu 2006). In addition, the *A. fumigatus* gene seems to function independent from *fluG.* At least, a Δ*fluG* strain of *A. fumigatus* still sporulates in air-exposed cultures. Possibly, *A. fumigatus* has more than one *brlA* activating mechanism (Mah & Yu 2006). The *brlA* gene of *A. fumigatus* has also been shown to be involved in suppressing ribosomal protein genes during nitrogen stress (Twumasi-Boateng *et al.* 2009). This finding conforms to the general starvation response in fungi, which involves both down-regulation of ribosomal protein biogenesis and induction of sporulation (Bahn *et al.* 2007, de la Serna *et al.* 1999, Gasch *et al.* 2000, Li *et al.* 1999, Mogensen *et al.* 2006, Warner 1999). However, down-regulation of ribosomal protein encoding genes is not impaired during carbon stress in *A. fumigatus* (Twumasi-Boateng *et al.* 2009). Nevertheless, these findings suggest that *brlA* of *A. fumigatus* is not only a regulator of formation of conidiophores but also influences the vegetative mycelium by affecting its protein synthesising capacity.

Transcription of *brlA* in *A. nidulans* results in two transcripts that are called *brlAα* and *brlAβ*. Both transcripts are essential for proper conidiophore development (Prade & Timberlake 1993) and are controlled at the transcriptional (*brlAα* and *brlAβ*) and translation level (*brlAβ*) (Han & Adams 2001). Transcript *brlAβ* contains a short upstream ORF (µORF) and a downstream ORF that encodes the same polypeptide as BrlAα but with an N-terminal extension of 23 aa (Prade & Timberlake 1993). Both polypeptides contain two C_2H_2 zinc finger DNA binding motifs. The *brlAα* and *brlAβ* transcripts have different functions during asexual development. As mentioned above, inactivation of *brlA* results in indefinitely elongating stalks. In contrast, aberrant primary conidiophores develop in the Δ*brlAβ* strain that can form secondary conidiophores (*i.e.* a conidiophore that develops from another conidiophore). Asexual development proceeds further in the Δ*blrAα* strain but conidia are not produced (Fischer & Kües 2006). So far, it is not known whether transcription of *brlA* of *A. oryzae* and *A. fumigatus* also results in two transcripts.

BrlA activates a central regulatory pathway controlling temporal and spatial expression of conidiation specific genes (Boylan *et al.* 1987, Mirabito *et al.* 1989). This cascade is complex and involves, amongst others, the regulatory genes *abaA*, *wetA, stuA, medA,* and *vosA* (Fig. 4). Gene *abaA* (*abacus*) is a regulatory gene that is activated in *A. nidulans* by BrlA during sterigmata differentiation (Boylan *et al.* 1987, Breakspear & Momany 2007). A Δ*abaA* strain forms metulae that bud apically resulting in chains of cells with metula-like, rather than phialide-like, properties. In other words, phialides are not produced and therefore conidia are not formed (Boylan *et al.* 1987, Clutterbuck 1969, Sewall *et al.* 1990). The interaction of AbaA with *brlA* is complex (Fig. 4A). Gene *abaA* is activated by BrlA and, in turn, AbaA stimulates formation of *brlAα* transcripts but represses *brlAβ* accumulation (Adams *et al.* 1998, Andrianopoulos & Timberlake 1994, Han & Adams 2001, Sewall *et al.* 1990). This is caused by AbaA binding to a responsive element in the *brlAβ* locus (Han & Adams 2001). The net result of *abaA* inactivation is that *brlA* is over-activated (Aguirre 1993). The positive feedback loop of *brlA* itself is likely to be independent of AbaA because the over-expression of *brlAβ* activates expression of *brlAα* in an *abaA* mutant (Han & Adams 2001) (Fig. 4A). Taken together, both BrlA and AbaA control transcript levels of *brlAα* and *brlAβ*. AbaA regulates several other genes including *abaA* itself, *medA, wetA* (Fig. 4A), *vosA* (Fig. 4B), and the structural genes *yA* and *rodA* (for their functions see below) (Andrianopoulos & Timberlake 1994). Recently *abaA* was identified in *A. oryzae* (Ogawa *et al.* 2010) and *A. fumigatus* (Tao & Yu 2011). The role of *abaA* in *A. oryzae* is similar to that in *A. nidulans.* In the case of *A. fumigatus abaA* also delays autolysis and cell death.

During the late phase of conidiation, *wetA (wet white)* is activated by *abaA* (Fig. 4A). Normal conidiophores are formed by *wetA* mutants. However, the conidia do not form pigments, are not water repellent, and go in autolysis (Marshall & Timberlake 1991, Sewall *et al.* 1990). Gene *wetA* activates a set of genes in phialides and spores (*e.g. wA*), which are involved in making the conidial wall impermeable and mature (Marshall & Timberlake 1991). In addition, WetA seems to activate itself (Adams *et al.* 1998, Boylan *et al.* 1987, Marshall & Timberlake 1991, Ni & Yu 2007) and represses *abaA* and *brlA* (Tao & Yu 2011) (Fig. 4A). Gene *wetA* of *A. oryzae* (Ogawa *et al.* 2010) has a role similar to that in *A. nidulans.* In the case of *A. fumigatus wetA* seems to have an additional role (Tao & Yu 2011). It would also function in germ tube formation and reduced hyphal branching.

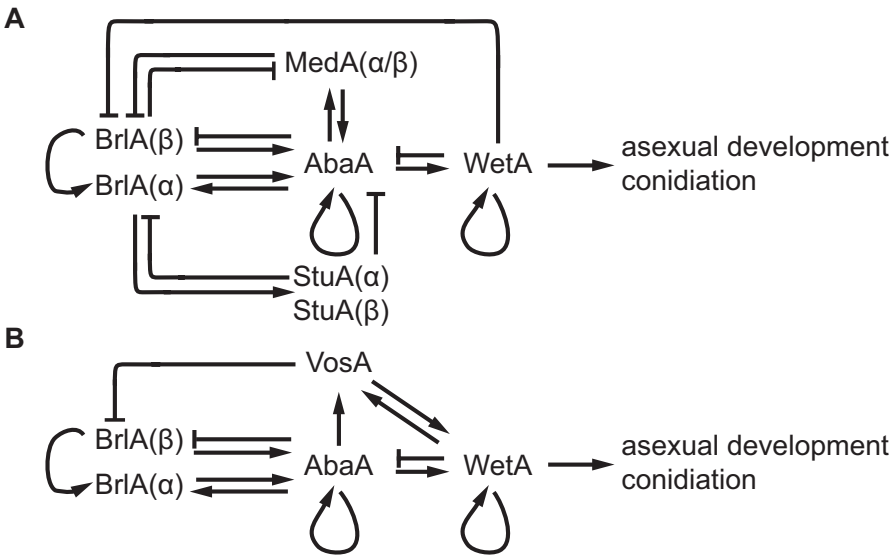

Fig. 4. The central regulatory network consisting of BrlA, AbaA and WetA initiates asexual development in *A. nidulans*. StuA and MedA (**A**) and VosA (**B**) are regulators of *brlA, abaA,* and *wetA*.

VosA (*viability of spore*) is a putative transcription factor of the velvet family (Tao & Yu 2011). This family, which is conserved in filamentous fungi, also includes VeA and VelB of *A. nidulans* (see below). Inactivation of *vosA* results in uncontrolled activation of asexual development, whereas its over-expression blocks sporulation. This may be the result of the observed inhibition of *brlA* by VosA (Tao & Yu 2011) (Fig. 4B). It should be noted that *vosA* is lowly expressed in the vegetative mycelium. Yet, these expression levels may be sufficient to control *brlA.* Gene *vosA* is particularly expressed during the formation of conidia and sexual ascospores, where it plays a role in resistance to stress conditions (see below).

The *stuA* and *medA* genes are classified as developmental modifiers. Their encoded proteins affect *brlA* and *abaA* expression (Fig. 4A). Mutations in *stuA* (*stunted*) of *A. nidulans* results in shortened aerial hyphae, shortened conidiophores and the absence of metulae and phialides. Conidiophores that are formed have reduced vesicles with abnormal numbers of nuclei. Only a few conidia can directly bud from the conidiophore vesicle. Thus, the morphology of the conidiophores is aberrant in *stuA* mutants, but neither temporal development nor conidiophore density is affected (Wu & Miller 1997). Gene *stuA* has a similar role in asexual development in *A. fumigatus* (Sheppard *et al.* 2005). StuA is a helix-loop-helix transcription factor with two transcription start sites. This leads to *stuAα* and *stuAβ* transcripts, of which the former is most important for correct development (Aguirre 1993, Miller *et al.* 1991, Miller *et al.* 1992). Expression of *stuA* depends on *brlA.* As a result, transcript levels of s*tuA* are increased 20-fold in conidiating cultures (Breakspear & Momany 2007, Busby *et al.* 1996, Miller *et al.* 1992). In turn, StuA directly or indirectly represses and spatially restricts *brlA* and *abaA* expression (Fig. 4A). With this ability *stuA* is involved in proper spatial distribution of AbaA and BrlA (Miller *et al.* 1992, Wu & Miller 1997). The StuA protein also stimulates *stuA* expression. This seems to be an indirect effect because its responsive elements are absent in the promoter (Wu & Miller 1997).

The *medA* (*medusa*) gene is conserved in filamentous fungi. Like other regulators, *medA* is transcribed at two initiation sites. While *stuA* of *A. nidulans* is required for proper spatial expression of *abaA* and *brlA, medA* is involved in proper temporal expression of these genes (Adams *et al.* 1998, Busby *et al.* 1996). Accumulation of both *brlA* transcripts is observed earlier in development in a Δ*medA* strain. Moreover, the mutant strain shows higher levels of *brlAβ,* but not *brlAα,* transcripts. As a result, the ratio of *brlA*α and *brlAβ* transcripts is lowered. Gene *medA* thus acts as a repressor of *brlA* expression. In contrast, it is an activator of *abaA* expression. This is concluded from the observation that *abaA* transcription levels are reduced or even absent in the *medA* mutant (Busby *et al.* 1996, Miller *et al.* 1992). The molecular basis of MedA function is still unclear. A Δ*medA* strain forms repeated layers of sterigmata and frequent reinitiated secondary conidiophores (Clutterbuck 1969, Sewall *et al.* 1990). This phenotype resembles that of a strain of *A. nidulans* in which the chitin synthase genes *chsA* and *chsC* genes have been inactivated (Ichinomiya *et al.* 2005). In the latter strain, *abaA* expression is reduced. This indicates that *chsA* and *chsC* regulate expression of *abaA*, most probably in an indirect way. The Δ*chsA*Δ*chsC* mutant shows a defective septum formation (Ichinomiya *et al.* 2005). Therefore, it was proposed that MedA is involved in septum formation on conidiophore structures. Taken together, conidiophore morphogenesis requires a finely tuned balance of at least BrlA, AbaA, MedA, and StuA (Busby *et al.* 1996), and possibly VosA and other velvet complex genes (Boylan *et al.* 1987, Ni & Yu 2007).

Trimeric G-protein signaling

Trimeric G-protein signaling is involved in the decision to grow vegetatively or to start asexual development. Gene *flbA* (*fluffy low brlA expression*) encodes an RGS domain protein, which negatively regulates vegetative growth signaling (Fig. 5). It does so by stimulating the intrinsic GTPase activity of the Gα subunit FadA (*fluffy autolytic dominant*) of a heterotrimeric G-protein. As a result, the Gα subunit is converted into the inactive GDP bound state (D'Souza *et al.* 2001, Yu *et al.* 1996, Yu *et al.* 1999) (Fig. 5). Overexpression of *flbA* in vegetative cells inhibits hyphal growth and stimulates conidiophore development even under conditions that normally prevent sporulation (Lee & Adams 1994a, Lee & Adams 1996). On the other hand, a mutation in *flbA* results in reduced *brlA* expression and a fluffy phenotype (hence the name *fluffy low brlA expression*). The Δ*flbA* strain does not form conidiophores. Instead, the mycelium proliferates uncontrolled and masses of undifferentiated aerial hyphae are formed. Both the submerged and aerial hyphae autolyse when colonies mature (Lee & Adams 1994a, Wieser *et al.* 1994). The autolytic phenotype of the *flbA* mutant can be partially overcome by deleting a class V endochitinase B (*chiB*). However, reduced cell viability cannot be restored in this way (Shin *et al.* 2009). Inactivation of *fadA* (Δ*fadA* or dominant-interfering *fadA* mutant) can also counteract the autolytic

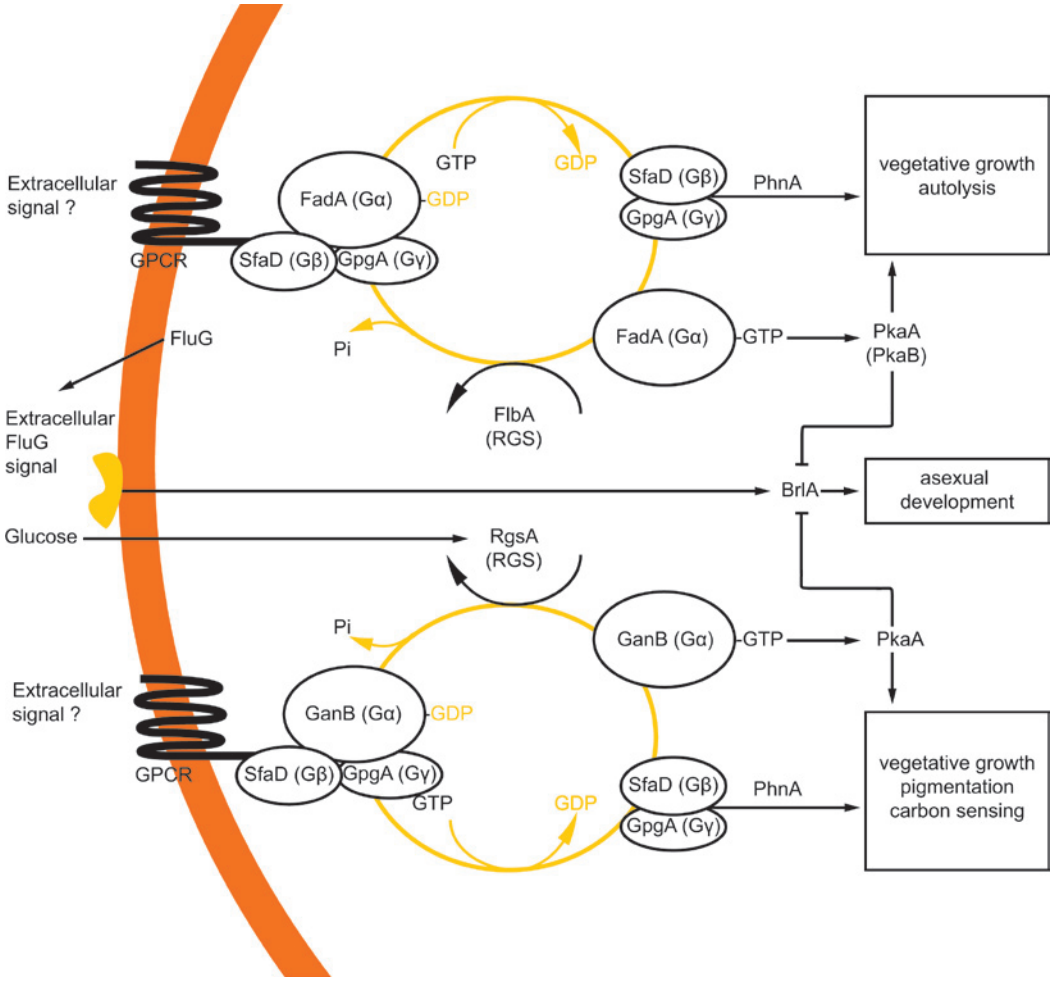

Fig. 5. Signaling cascades resulting in vegetative growth or asexual reproduction in *A. nidulans*. Signalling involves FluG (see Figure 6) and independently, two heterotrimeric G-protein complexes, both consisting of SfaD and GpgA (the Gβγ subunits) and the Gα subunits FadA and GanB, respectively. GTP-bound FadA and GanB stimulate vegetative growth via the cAMP PkaA pathway and repress asexual reproduction via *brlA*. The RGS proteins FlbA and RgsA hydrolyze the GTP bound to FadA and GanB, respectively, thereby repressing vegetative growth and promoting asexual development. The SfaD-GpgA dimer also stimulates vegetative growth. This is regulated by PhnA. (Adapted from Yu 2006).

phenotype of the *flbA* mutant. This is in agreement with the function of FlbA to convert FadA into the inactive GDP bound state (Fig. 5). A constitutively active *fadA* mutant, *fadA*^{G42R}, results in autolytic mutants similar to the *flbA* mutant (Hicks *et al.* 1997, Yu *et al.* 1996). The constitutively active *fadA*^{G42R} mutant phenotype cannot be suppressed by overexpression of *flbA* (Yu *et al.* 1999).

In its inactive GDP-bound form, FadA of *A. nidulans* forms a heterotrimeric G-protein with the β- and γ-subunits encoded by *sfaD* and *gpgA*, respectively (Rosén *et al.* 1999, Seo *et al.* 2005, Yu *et al.* 1996, Yu *et al.* 1999). When FadA becomes GTP bound, this α-subunit dissociates from SfaD and GpgA (Fig. 5). Deletion of *sfaD* (Rosén *et al.* 1999) or *gpgA* (Seo *et al.* 2005) suppress the phenotype of the *flbA* mutant. Moreover, in a wild-type background reduced vegetative growth is observed in these deletion strains. Inactivation of *sfaD* (Rosén *et al.* 1999) but not *gpgA* (Seo *et al.* 2005) also causes hyper-sporulation. The Δ*sfaD*Δ*gpgA* double mutant shows a phenotype identical to those of the Δ*sfaD* mutant (Seo *et al.* 2005). This shows that Δ*sfaD* is epistatic to Δ*gpgA* and that SfaD can induce inappropriate conidiation even in the absence of GpgA. Notably, constitutive activation of *fadA* in the absence of *sfaD* is sufficient to cause proliferation of undifferentiated hyphae (Seo *et al.* 2005, Wieser *et al.* 1997). Taken together, FadA and SfaD-GpgA have overlapping functions in stimulating vegetative growth (Rosén *et al.* 1999, Seo *et al.* 2005). The phosducin like protein A (PhnA) seems to be involved in positively regulating Gβγ signaling in *A. nidulans*. Deletion of *phnA* results in a phenotype similar to that of a Δ*sfaD* strain (Seo & Yu 2006). This would agree with the role of phosducin like proteins to act as chaperones for Gβγ assembly (Yu 2006). Finally, deletions in *sfaD*, *fadA* or *gpgA* do not suppress conidiation defects in a *fluG* mutant. Therefore, the FadA/SfaD/GpgA vegetative growth-signaling cascade seems to be distinct from that of the FluG pathway (Seo *et al.* 2005).

GTP-bound-FadA promotes vegetative growth and inhibits asexual and sexual development by activating a cAMP-PKA signaling cascade (Shimizu & Keller 2001) (Fig. 5). The cAMP dependent protein kinase A catalytic subunit (PKA) encoded by *pkaA* has a major role in the stimulation of vegetative growth and the repression of conidiation (Lafon *et al.* 2005, Lafon *et al.* 2006, Seo *et al.* 2005, Yu & Keller 2005). Inactivation of *pkaA* results in hyper-sporulation and reduced radial growth (Shimizu & Keller 2001). On the other hand, overexpression of *pkaA* leads to decreased sporulation accompanied by a fluffy-like appearance. Deletion of the other *pka* gene in the genome of *A. nidulans, pkaB*, causes no apparent phenotype (Ni *et al.* 2005). However, over-expression of *pkaB* reduces conidiation and increases vegetative growth on solid medium. Moreover, it complements the reduced radial growth of the Δ*pkaA* strain. Apparently, PkaB functions as a backup for PkaA (Ni *et al.* 2005, Seo *et al.* 2003).

The FlbA/FadA/SfaD/GpgA pathway seems to be conserved in *A. nidulans, A. oryzae* and *A. fumigatus* (Mah & Yu 2006, Ogawa *et*

al. 2010, Yu 2006). Like in *A. nidulans,* the SfaD-GpgA complex is involved in stimulating vegetative growth in *A. fumigatus* (Shin *et al.* 2009). However, there are some differences in the case of the other components. Deletion of *flbA* in *A. nidulans* (Wieser *et al.* 1994), *A. oryzae* (Ogawa *et al.* 2010) or *A. fumigatus* (Mah & Yu 2006) results in low *brlA* expression, and reduced conidiation. In contrast to *A. nidulans* and *A. oryzae,* the autolysis phenotype is missing in *A. fumigatus*. Moreover, in both *A. oryzae* and *A. fumigatus* hyphal proliferation is reduced in the Δ*flbA* strain, blocking formation of aerial hyphae in the case of *A. oryzae* (Ogawa *et al.* 2010). Like in *A. nidulans,* FadA of *A. oryzae* (Ogawa *et al.* 2010) and *A. fumigatus* (Mah & Yu 2006) repress conidiation. Remarkably, in *A. oryzae* it also represses vegetative growth (Ogawa *et al.* 2010), while in *A. fumigatus* vegetative growth is stimulated (Liebmann *et al.* 2004, Shimizu & Keller 2001).

Apart from FadA, two other Gα subunits are present in *A. nidulans* (GanA and GanB) and *A. fumigatus* (GpaA and GpaB), and three in *A. oryzae* (GanA, GanB, GaoC) (Chang *et al.* 2004, Lafon *et al.* 2006, Liebmann *et al.* 2003, Rosén *et al.* 1999, Seo *et al.* 2005, Yu *et al.* 1996). In contrast to *ganB,* the functions of *ganA* and *gaoC* have not been established yet (Chang *et al.* 2004, Han *et al.* 2004a, Lafon *et al.* 2006). Like FadA, GanB in its inactive form interacts with SfaD and GpgA (Seo *et al.* 2005) (Fig. 5), which in fact are the only β and γ subunits of trimeric G-proteins in *A. nidulans, A. fumigates*, and *A. oryzae* (Lafon *et al.* 2006). The Δ*ganB* strain shows hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts. Constitutive activation of GanB results in reduced hyphal growth and a severe defect in asexual sporulation (Chang *et al.* 2004). Like FadA, GanB therefore seems to be involved in repression of *brlA* and inhibition of asexual sporulation (Chang *et al.* 2004).

RgsA (*regulator of G protein signaling*) is a repressor of GanB signaling. Colonies of the Δ*rgsA* strain are reduced in size, form more aerial hyphae, and accumulate dark brown pigments (Han *et al.* 2004a). Expression of a constitutively active RgsA results in hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts (Han *et al.* 2004a). The presence of glucose is claimed to result in the increase of *rgsA* mRNA levels, and this would result in down-regulation of GanB mediated signaling. In cases of stress or unfavorable carbon sources, *rgsA* levels decrease and as a consequence GanB-GTP signaling is activated (Han *et al.* 2004a). In *A. fumigatus* the outcome of the signaling pathway involving the GanB orthologue GpaB is different. Growth and conidium formation of a Δ*gpaB* strain is slightly decreased (Liebmann *et al.* 2004). This and other data show that GpaB signaling in *A. fumigatus* promotes asexual sporulation via PKA. However, the PkaC1 cascade in *A. fumigatus* is complex, since it also promotes vegetative growth, when activated by GpaA (Liebmann *et al.* 2003).

Upstream activators of brlA

FluG activates sporulation by a derepression pathway that involves the SfgA protein (Fig. 6). Gene *sfgA* (*suppressor of fluG*) is predicted to encode a transcription factor with a Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA binding motif (Seo *et al.* 2006). Mutations in *sfgA* bypass the need for FluG during asexual development. The Δ*sfgA* strain shows hyperactive sporulation in liquid submerged cultures. Overexpression of *sfgA* results in delayed and reduced levels of *brlA* mRNA, and in colonies with reduced conidiation. Apparently, the primary role of FluG is to remove the repressive effects of SfgA (Seo *et al.* 2006). The low FluG levels in young colonies would result in *sfgA*-mediated repression of conidiation. Once the FluG factor has accumulated above a certain threshold, it inhibits the repression of conidiation by SfgA (Seo *et al.* 2006). SfgA has also been proposed to negatively regulate FlbA (Fig. 6). By this, the repression of SfgA by FluG will result in both activation of conidiation and inhibition of FadA-mediated stimulation of growth.

Apart from *flbA*, four other regulatory genes, *flbB*, *flbC, flbD,* and *flbE,* have been identified that act upstream of *brlA* (Wieser *et al.* 1994). All *flb* mutant strains show low *brlA* expression and a fluffy phenotype (Wieser *et al.* 1994). The mutants grow indeterminately and produce masses of aerial hyphae. The *flb* mutants can restore conidiation in *fluG* loss-of-function mutants (Wieser *et al*. 1994). These results indicate that the *flb* genes are involved in responding to the diffusible signaling molecule, produced by FluG, which is necessary for conidiation (Wieser *et al*. 1994). SfgA acts downstream of *fluG* but upstream of *flbA-D*. As mentioned above, FluG is assumed to repress *sfgA* thus releasing the repression of the *flb* genes. Notably, *flb* genes are involved in regulation of *fluG* expression (Ruger-Herreros *et al.* 2011). A repressing function of FlbA on *fluG* expression is indicated by a 7-fold higher *fluG* expression in a Δ*flbA* strain. In contrast, *fluG* expression is stimulated by FlbB and FlbC. For instance, a Δ*flbC* shows no *fluG* mRNA accumulation (Ruger-Herreros *et al.* 2011).

The *flbC* gene encodes a transcriptional regulator containing two C_2H_2 zinc finger DNA binding domains (Kwon *et al.* 2010a). The Δ*flbC* strain shows delayed and reduced conidiation, while overexpression causes restricted hyphal growth and delayed conidiation. In wild type colonies, FlbC is localised in nuclei of vegetative hyphae and in conidiophores (*i.e.* not in conidia). Here, FlbC activates *brlA, abaA,* and *vosA* but not *wetA.* Overexpression of *flbC* not only inhibits vegetative growth in a wild-type strain but also in a Δ*abaA* or Δ*brlA* background. Thus, FlbC plays a direct role in repressing vegetative growth, independent of *brlA* or *abaA* (Fig. 6). The deletion of *flbC* also results in highly enhanced sexual fruiting body formation (see below) (Kwon *et al.* 2010a). Taken together, FlbC has a repressive role in sexual development, but positively regulates germination and asexual development (Kwon *et al.* 2010a). FlbC acts in a pathway parallel to that of *flbB, flbD*, and *flbE* (Garzia *et al.* 2010, Wieser & Adams 1995). Absence of FlbC does not affect expression of *flbB* or *flbE* and vice versa. Moreover, double mutants cause additive effects, resulting in a prolonged delay in conidiation (Kwon *et al.* 2010a). It has been proposed that FlbC coordinates activation of *brlA* together with a FlbB/FlbD transcriptional complex (Etxebeste *et al.* 2010a, Garzia *et al.* 2010) (Fig. 6). Promoter binding regions of FlbC and FlbB/FlbD may overlap (Garzia *et al.* 2010, Han & Adams 2001).

The *flbB* gene encodes a fungal specific bZIP-type transcription factor (Etxebeste *et al.* 2008). A Δ*flbB* strain shows defective branching patterns, delayed conidiation with a fluffy appearance and, under stress conditions, a high autolysis rate. Overexpression of *flbB* results in reduced conidiophore vesicle formation, and a reduced number of metulae (Etxebeste *et al.* 2008, 2009). FlbB is located within the cytoplasm at the hyphal apex during early vegetative growth. In contrast, it preferentially accumulates at the most apical nucleus during later stages of growth (Etxebeste *et al.* 2008). The repressor SfgA may be a key intermediate in the process of nuclear localisation of FlbB. This is indicated by the fact that FlbB is found in all nuclei of Δ*sfgA* hyphae, rather than only at the hyphal tip as observed in the wild-type strain. Gene *flbE* encodes a protein without any known conserved domain (Garzia *et al.* 2009). Expression of *brlA* and *vosA* is delayed in the Δ*flbE* strain (Kwon *et al.* 2010a). This is accompanied by absence of conidiophore formation, a fluffy appearance of the colonies, accelerated vegetative growth, and accelerated autolysis and

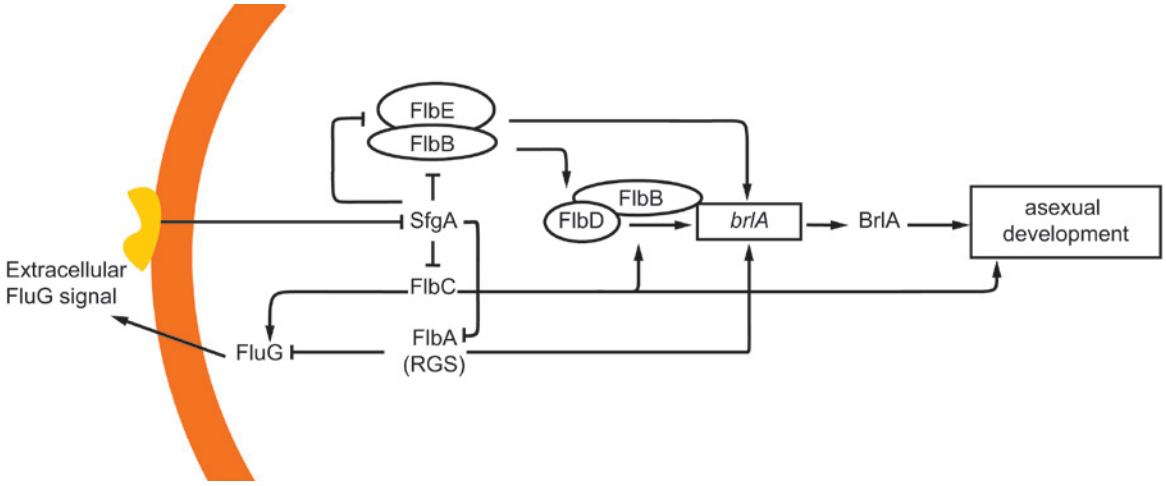

Fig. 6. Model of upstream regulation of *brlA*. FluG is involved in the formation of an extracellular factor that activates an unknown receptor. At a certain concentration of FluG, the general suppressor SfgA is inhibited removing the repression of the *flb* genes. FlbB and FlbE form a complex that activates *brlA* leading to asexual development. FlbC activates *brlA* together with the FlbB/FlbD transcription complex. FlbC also activates *fluG* and regulatory genes that act downstream of BrlA. FlbA activates *brlA* by inactivating FadA and probably plays a role in repressing *fluG*. (Adapted from Etxebeste *et al.* 2010).

cell death. FlbE is localised at hyphal tips. In fact, it co-localises with FlbB. Localisation of these proteins at the hyphal tip depends on the presence of F-actin. This is concluded from the fact that disintegration of the actin filaments causes mis-localisation of FlbB and FlbE. Localisation of FlbB and FlbE was also lost in a Δ*flbE and* a Δ*flbB* strain, respectively. These results indicate that these proteins depend on each other for proper localisation at the hyphal apex. It has also been shown that FlbB stability is affected by the absence of a functional form of FlbE (Garzia *et al.* 2009). FlbE may thus protect FlbB from proteolytic degradation. It may do so by physical interaction with FlbB. At least, such an interaction was shown *in vivo* (Garzia *et al.* 2009). Taken together, FlbE and FlbB function in close association with each other and are functionally interdependent (Garzia *et al.* 2009).

The FlbB/FlbE complex is a requisite for *flbD* expression in the wild-type (Garzia *et al.* 2010) (Fig. 6). FlbD is a c-Myb transcription factor. Deletion of its encoding gene results in delayed conidiation and a fluffy phenotype (Wieser *et al.* 1994, Wieser & Adams 1995). Overexpression causes sporulation in liquid submerged cultures. This is due to inappropriate activation of *brlA* (Wieser & Adams 1995). As mentioned above, the FlbB/FlbE complex is found at hyphal apices (Garzia *et al.* 2009). In contrast, FlbD is found in all nuclei of vegetative hyphae of *A. nidulans*. Thus, other transcription factors seem also to be involved in the regulation of *flbD* expression (Etxebeste *et al.* 2010a, Garzia *et al.* 2009, 2010). Not only depends *flbD* expression on the presence of FlbB, FlbD also interacts with this protein (Etxebeste *et al.* 2010a, Garzia *et al.* 2009, 2010). The underlying mechanism is so far unknown but might involve a translationally modified form of FlbB (Garzia *et al.* 2010). Both FlbD and the FlbB/FlbE complex seem to activate *brlA* expression (Garzia *et al.* 2010) (Fig. 6). Overexpression of *flbD* restores the conidiation defect in the Δ*flbE* strain (Kwon *et al.* 2010a), suggesting an additive effect of both pathways. Interestingly *flbB* and *flbD* transcripts disappear shortly after *brlA* activation (Etxebeste *et al.* 2008, Wieser & Adams 1995). However, the mechanism underlying this effect is independent of *brlA* levels (Garzia *et al.* 2010).

The *flb* genes are conserved in *A. fumigatus, A. oryzae* and *A. nidulans* (Kwon *et al.* 2010a, Ogawa *et al.* 2010). The phenotypes of the Δ*flb* strains of *A. oryzae* are similar to those of *A. nidulans*. These results indicate that the functions of these regulatory genes are conserved between *A. oryzae* and *A. nidulans* (Ogawa *et al.* 2010). The *A. fumigatus flbE* could complement the Δ*flbE* phenotype in *A. nidulans* (Kwon *et al.* 2010a), suggesting a similar role for these orthologues. Indeed, FlbE in *A. fumigatus* is necessary for proper control of conidiation and *brlA* and *vosA* expression. However, deletion of *flbE* does not cause an elevated vegetative proliferation or accelerated autolysis or cell death in *A. fumigatus* (Kwon *et al.* 2010a). Inactivation of *flbB* in *A. fumigatus* results in delayed and reduced sporulation, and precocious cell death. Moreover, expression of *brlA* and *abaA* is affected. In contrast to *A. nidulans,* the FlbB protein is in *A. fumigatus* encoded by two transcripts, *flbBα* and *flbBβ*. The longest transcript, *flbBβ,* is constitutively expressed, while the *flbBα* transcript is found during progression of conidiation (Xiao *et al.* 2010). Both transcripts are needed for proper asexual development. The *flbB* gene of *A. nidulans,* encoding one transcript, only partially complements the Δ*flbB* strain of *A. fumigatus.* FlbC and FlbD functions in *A. fumigatus* are probably similar to *A. nidulans,* but characterisation is ongoing at the moment (Xiao *et al.* 2010).

MAPK pathways and downstream targets

A mitogen activated protein kinase (MAPK) module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK is phosphorylated in response to external stimuli. Active (*i.e.* phosphorylated) MAPKKK phosphorylates MAPKK, which in turn phosphorylates MAPK. Phosphorylated MAPK enters the nucleus and activates transcription factors by phosphorylation (Dickman & Yarden 1999). A MAPK pathway has been shown to repress asexual development and to stimulate sexual development in *A. nidulans* (see below). This pathway includes MAPKKK SteC (Wei *et al.* 2003). Growth of the *ΔsteC* strain on minimal medium results in reduced growth and hyphae are more curled and more branched than the wild-type. Conidiophore development is initiated as in wild-type, but the length distribution of the stalks is affected. Metulae and phialide morphology is normal in the majority of the conidiophores. In about 2 % of the conidiophores, however, only a few metulae arise from the vesicle and these metulae do not differentiate normally. In addition, secondary conidiophores grow out of the vesicle. Finally, the mutant produces 1–2 % of very large conidia in addition to wild-type-like spores. A translational fusion of SteC and GFP localises in metulae, phialides and young

conidia but not in conidiophore stalks. The targets of SteC during asexual development have not yet been identified. A functional study of MAPKK genes in *A. nidulans* is lacking and inactivation of the MAPK gene, *mpkB*, does not affect sporulation (Paoletti *et al.* 2007). Similarly, the putative target of *mpkB, steA,* is not involved in asexual development (Vallim *et al.* 2000). In contrast, a potential other target of *mpkB, nsdD* (*never in sexual development*), does affect conidiation (Han *et al.* 2001). A Δ*nsdD* strain starts to conidiate earlier. On the other hand, over-expression of *nsdD* suppresses formation of conidiophores. Similarly, deletion of the transcription factor gene *nsdC* results in derepression of conidiation. In this case, the effect depends on the carbon source. Derepression is observed on carbon sources favoring sexual development. This indicates that NsdC acts as a repressor of asexual development under those conditions (Kim *et al.* 2009).

The role of hydrophobins in asexual development

Regulators activate target genes that fulfill a structural or enzymatic role in the formation of asexual structures. Genes have been identified that are upregulated in conidiophores and / or conidia (Bleichrodt *et al.* 2013, van Leeuwen *et al.* 2013a, b). Hydrophobin genes are examples of such target genes. Hydrophobins mediate the escape of hyphae into the air and make aerial structures such as conidiophores and conidia hydrophobic (Wösten 2001). This hydrophobicity ensures that reproductive structures do not fall back in the substrate under humid conditions and serves dispersal of conidia by wind or vectors. Hydrophobins may also affect the cell wall architecture (van Wetter *et al.* 2000) and mediate attachment to hydrophobic substrates (Wösten 2001). In the case of *A. fumigatus* it has also been shown that hydrophobins prevent immune recognition by the host (Aimanianda *et al.* 2009, Aimanianda & Latgé 2010, Bruns *et al.* 2010, Dagenais *et al.* 2010, Paris *et al.* 2003). Moreover, the hydrophobin RolA of *A. oryzae* recruits cutinase by adsorbing to the substrate of the enzyme. As a result, the substrate is efficiently degraded (Takahashi *et al.* 2005).

The *A. nidulans, A. fumigatus, A. oryzae,* and *A. niger* genomes contain 6, 4-5, 2, and 8 hydrophobin genes, respectively (Jensen *et al.* 2010). One or more of the hydrophobins in each species enable hyphae to grow into the air by lowering the surface tension of the aqueous environment (see above). The aerial structures are then coated with hydrophobins to make them hydrophobic. So far, it has not been established which hydrophobins line aerial hyphae and the conidiophore stalk and vesicle of aspergilli. However, hydrophobins have been identified that coat sterigmata and conidia. The hydrophobin gene *rodA* (*rodlet)* of *A. nidulans* is expressed during the final stages of conidiophore formation. It is not expressed by vegetative hyphae and conidia (Boylan *et al.* 1987, Stringer *et al.* 1991). Expression of *rodA* is mediated by BrlA but not by AbaA or WetA. This is based on the finding that a Δ*brlA* strain does not express *rodA* but expression of the hydrophobin gene is not affected in the Δ*abaA* and Δ*wetA* strains. A Δ*rodA* strain forms wettable conidiophores and conidia. This is accompanied by the absence of the rodlet layer (Stringer *et al.* 1991). As a consequence, Δ*rodA* conidia adhere to each other in water. This affects their dispersal by air flow. The rodlet layer is also absent at the surface of metulae and phialides of the Δ*rodA* strain (Stringer *et al.* 1991). Experimental data imply that the RodA protein is produced by the sterigmata and diffuses to the outer surface of these structures as well as to that of the conidia to form the rodlet layer. *Aspergillus fumigatus* contains an ortholog of *rodA*. Inactivation of this gene results in a phenotype similar to that in *A. nidulans* (Paris *et al.* 2003, Thau *et al.* 1994). Moreover, *rodA* of *A. fumigatus* is involved in attachment of spores to particular substrates. The adhesion of the Δ*rodA* conidia is reduced in the case of collagen and bovine serum albumin but is not affected in the case of pneumocytes, fibrinogen, and laminin.

The *dewA* (*detergent wettable*) hydrophobin gene is expressed in sporulating cultures but not in cultures that grow vegetatively (Boylan *et al.* 1987, Stringer & Timberlake 1995). Unlike RNA of *rodA*, transcripts of *dewA* are present in conidia (Breakspear & Momany 2007). In agreement, immuno-detection showed that DewA hydrophobin is specifically present in cell walls of conidia, especially in mature spores. Expression of *dewA* is not only abolished in the *ΔbrlA* strain but also in the Δ*abaA* and Δ*wetA* strains. Forced expression of *brlA* or *abaA* has only resulted in *dewA* expression in strains with an intact copy of *wetA*. Thus, *dewA* is regulated by *brlA* and *abaA* via *wetA* (Stringer & Timberlake 1995). Conidia of Δ*dewA* are still covered with the rodlet layer. Thus, DewA is not essential for the rodlet layer on spores. Yet, when present, it may be part of it. The conidia of the Δ*dewA* strain do not wet in water. However, they wet more easily compared to wild-type spores when detergent is added to the water. A role of DewA in surface hydrophobicity is also indicated from the fact that wettability of the Δ*dewA*Δ*rodA* strain is much more pronounced when compared to the Δ*dewA* and the Δ*rodA* strains. *Aspergillus fumigatus* also contains a hydrophobin that is present at the surface of conidia but whose presence is not essential for the rodlet layer of conidia (Paris *et al.* 2003). This hydrophobin, RodB, is different in sequence when compared to DewA. As mentioned above, the surface of Δ*rodA* conidia of *A. fumigatus* lacks the rodlet layer. Instead, the surface is granular. In contrast, the surface of Δ*rodA*Δ*rodB* conidia is amorphous. Taken together, RodB of *A. fumigatus* may be part of the rodlet layer of conidia when RodA is present. In the absence of RodA, RodB forms a granular structure and does not form rodlets.

SEXUAL DEVELOPMENT

About one-third of the described species of *Aspergillus* have a known sexual stage (Geiser 2009). However, analysis of genome sequences suggests that most, if not all, *Aspergillus* species should be able to reproduce sexually (Dyer & Paoletti 2005). Indeed, *A. fumigatus*, *A. flavus, A. parasiticus,* and *A. nomius* have been shown to have a sexual stage (Horn *et al.* 2009a, b, 2011, O'Gorman *et al.* 2009). In the case of *A. fumigatus*, this was 145 years after this fungus was described for the first time. Clearly, the conditions under which sexual reproduction takes place differs between aspergilli; some of which being more restrictive (*e.g. A. fumigatus*) than others (*A. nidulans*).

Aspergillus species with a sexual state can be either homothallic or heterothallic. Homothallic species undergo sexual reproduction without the need to cross with a compatible partner. *Aspergillus nidulans* (Paoletti *et al.* 2007) and *Neosartorya fischeri* (Rydholm *et al.* 2007) are known to be homothallic. It should be noted that *A. nidulans* can also be heterothallic and can thus cross with a partner. In contrast, *A. flavus* (Horn *et al.* 2009a), *A. parasiticus* (Horn *et al.* 2009b), *A. fumigatus* (O'Gorman *et al.* 2009), and *A. nomius* (Horn *et al.* 2011) have been shown to be exclusively heterothallic. The genomic sequences of *A. niger* (Pel *et al.* 2007) and *A. oryzae* (Rokas & Galagan 2008) predict that these fungi are either heterothallic or truly asexual (Pál *et al.* 2008). The sequenced

A. niger strains only contain the *MAT1-1* mating type locus (see below) but otherwise seem to emcompass a complete set of genes enabling sexual development.

Sexual development in *A. nidulans* is a highly complex process, ultimately resulting in fruiting bodies of 125–200 µm in diameter that are called cleisthotecia. Sexual development is influenced by both environmental and intrinsic signals, and occurs only when all prerequisites are met. The process of sexual development starts after 40–50 h of cultivation at 37 °C in the center of the colony and mature cleistothecia can be found after approximately 96 h (Seo *et al*. 2004). The production of Hülle cells (see below) represents the first sign of sexual development. Subsequently, hyphae fuse to form a dikaryon. As mentioned, in the case of *A. nidulans* these hyphae may originate from the same colony (self-fertilisation) or from another individual (out-crossing). The hyphae that fuse are morphologically similar. Thus, male antheridia and female ascogonia cannot be distinguished (Benjamin 1955). A population of dikaryotic cells originates from a single cell fusion event, and gives rise to a single cleistothecium (Champe *et al.* 1994). The Hülle cells surround the dikaryotic hyphae and form an increasingly packed "nest". They differentiate into thick-walled globose cells (Champe & Simon 1992, Pöggeler *et al.* 2006) that are believed to provide protection and nutrition (Zonneveld 1977). An intertwined network of ascogenous dikaryotic hyphae develops within the cleistothecial shell. Nuclear fusion takes place inside the ultimate branches of the dikaryotic hyphae, which represent the young asci. This is immediately followed by meiosis and a post-meiotic mitosis, thus resulting in eight nuclei. These nuclei are then separated from each other by membranes, giving rise to eight red-pigmented spores in each of the 10.000 asci within one fruiting body (Pöggeler *et al.* 2006). A second post-meiotic mitosis makes that mature ascospores are binucleate (Braus *et al.* 2002).

Regulation of sexual development

Mating type loci

Strains of heterothallic ascomycetes contain either a *MAT1-1* or a *MAT1-2* mating type locus. These loci are not related in sequence, and are therefore called ideomorphs. The *MAT1-1* locus contains a gene encoding an α-domain transcription factor, while the *MAT1-2* encodes a high mobility group-domain (HMG) transcription factor (Turgeon & Yoder 2000). The homothallic species *A. nidulans* contains both the *MAT1-1* and *MAT1-2* mating type loci, which reside on different chromosomes (Paoletti *et al.* 2007). Inactivation of *MAT1-1* or *MAT1-2* does not affect vegetative growth or asexual sporulation. Moreover, Hülle cells and cleistothecia are formed under conditions inducing the sexual cycle. However, the cleistothecia are lower in number and smaller than those of the wild-type strain (Paoletti *et al.* 2007). In addition, no ascospores are formed within the cleistothecia. This and the fact that fruiting body development can be induced by placing both transcriptional activator genes under an inducible promoter shows that *MAT1-1* and *MAT1-2* are the master switches of sexual reproduction.

In filamentous ascomycetes, the *MAT* loci determine cell type identity and may confer nuclear recognition and proliferation (Coppin *et al.* 1997, Shiu & Glass 2000). In addition, *MAT* loci regulate expression of a pheromone-signaling system. This system, which is generally involved in the detection of a mating partner, has been best studied in *Saccharomyces cerevisiae* (Banuett 1998). In *S. cerevisae* the a- and α-cells each produce a peptide pheromone and a receptor for the pheromone of the other partner. Binding of the pheromone to the receptor that is produced by the compatible partner triggers G protein-mediated signaling via a mitogen activated protein kinase (MAPK) cascade. As a result, a homeodomain transcription factor gene is activated that induces cell cycle arrest, and activates the mating process. Components of a pheromone-signaling pathway similar to that of *S. cerevisae* have been detected in *A. nidulans* and other sequenced *Aspergillus* species, including pheromone and pheromone receptor genes (Pál *et al.* 2008). Notably, expression of the pheromone and pheromone receptor genes is not affected when the *MAT* genes are inactivated in *A. nidulans*. Apparently, these genes are not under control of the mating type loci (Paoletti *et al.* 2007). They may be induced by environmental conditions that stimulate sexual reproduction. In contrast, *MAT1-1* of *A. fumigates* does stimulate the pheromone gene *ppgA,* while it is repressed by *MAT1-2* (Szewczyk & Krappmann 2010).

Pheromones, pheromone receptors, and intracellular signalling

In *A. nidulans* only one putative pheromone gene has been identified, which has homology to the gene encoding the α-pheromone of *S. cerevisae* (Dyer *et al.* 2003). Until now, it has not been confirmed that the encoded product binds to a pheromone receptor(s). Alternatively, psi factor (see below) may bind to the receptor protein(s) (Seo *et al.* 2004). Receptor proteins have been identified in *A. nidulans* on basis of homology with the pheromone receptor genes of *S. cerevisae* (Seo *et al.* 2004). Inactivation of these genes*, gprA* and *gprB* (*G protein receptor*), does not affect growth rate, Hülle cell formation, and asexual sporulation. However, Δ*gprA* and Δ*gprB* strains produce less cleistothecia that are smaller than wild-type fruiting bodies under homothallic conditions. The Δ*gprA* and Δ*gprB* cleistothecia produce only 5 % of the ascospores when compared to the wild- type but viability of these spores is not affected. The Δ*gprA*Δ*gprB* strain does not form cleistothecia at all under homothallic conditions but Hülle cells are still formed. Notably, sexual development is not impaired under heterothallic conditions; *i.e.* when strains are crossed in which either or both *gprA* and *gprB* have been inactivated. Heterokaryon formation in this case can be selected by using parental strains that have different auxotrophic markers. Taken together, pheromone receptors genes, and therefore the pheromone signaling pathway, is only required for homothallic sexual development (Seo *et al.* 2004). Genes *gprA* and *gprB* represent two out of sixteen genes encoding seven-transmembrane-spanning G protein coupled receptor proteins (GPCR) (Lafon *et al.* 2006). Gene *gprD* is another GPCR gene. Inactivation of *gprD* results in a strain with impaired vegetative growth and exhibiting extremely enhanced sexual development (Han *et al.* 2004a, b). Deletion of *gprA* and *gprB* rescue the growth defects of the Δ*gprD* strain and fruiting bodies are no longer formed. Thus, GprD functions downstream of GprA/B (Han *et al.* 2004a, b).

In general, binding of pheromones sensitises the pheromone receptors. As a result, the receptors physically interact with a heterotrimeric G protein. This induces the exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein, resulting in its dissociation from the βγ-subunits (Fig. 7). Genes *sfaD* and *gpgA* are the only β and γ subunit encoding genes in the genome of *A. nidulans*. Inactivation of *sfaD* or *gpgA* abolishes and severely affects cleistothecia formation under homothallic and heterothallic conditions, respectively. Moreover, under both conditions more Hülle cells are formed (Rosén *et al.* 1999, Seo *et al.* 2005). The genome of *A. nidulans* contains three α-subunit encoding genes; *ganA, ganB*, and *fadA* (see above). It has not fully been established

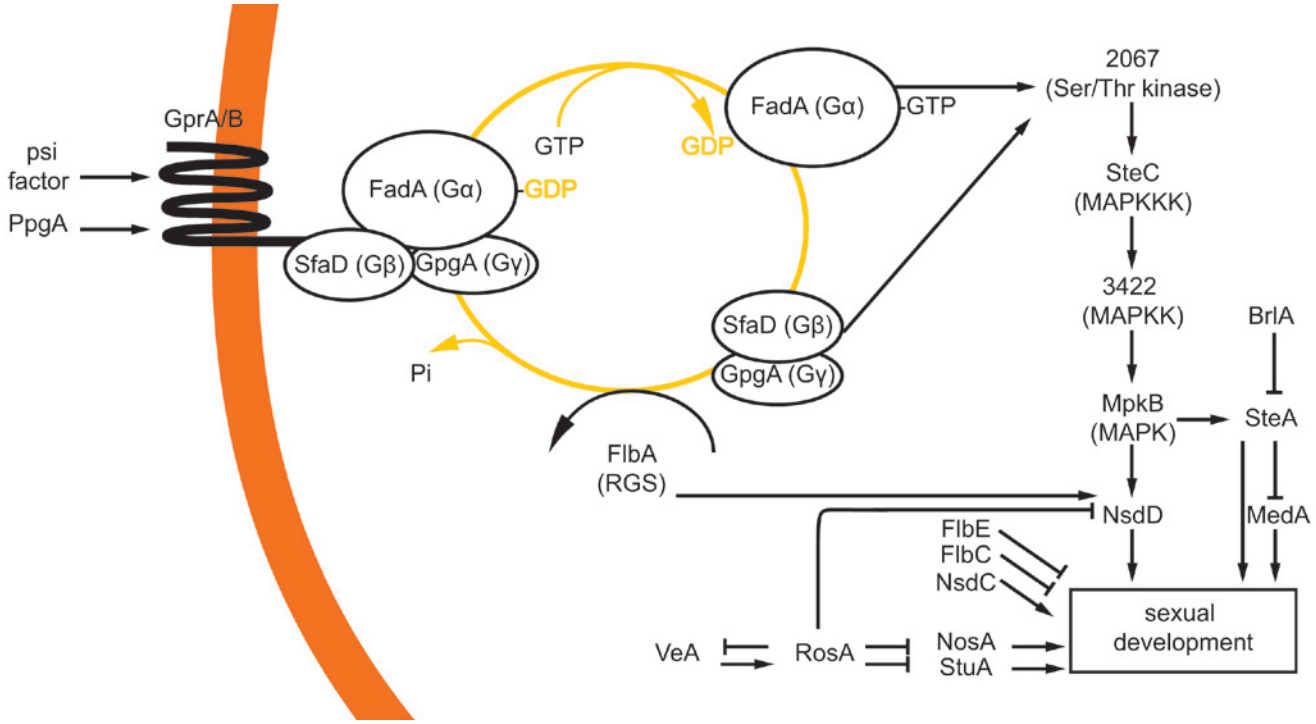

Fig. 7. GprA/B mediated signalling resulting in sexual development of *A. nidulans*. Signalling involves the heterotrimeric G-protein consisting of SfaD, GpgA and FadA. FadA bound to GTP activates a MAP kinase cascade (hypothetical protein AN2067.2, SteC, hypothetical protein AN3422.2 and MpkB). This in turn, activates the regulators NsdD and SteA that induce sexual development. In addition, SteA inhibits MedA that is also involved in activating sexual development. StuA, NsdC, and NosA also activate the sexual development program, while FlbC and FlbE are repressors of this pathway. RosA is a transcriptional inhibitor of *veA, nsdD, nosA* and *stuA*. (Adapted from Seo *et al.* 2004, Yu 2006).

which of the α-subunits is the cognate Gα protein of GprA and GprB. A role of GanA and GanB in sexual development, if any, has not yet been reported. On the other hand, the role of *fadA* in sexual development has been studied. Cleistothecia formation is not affected in a Δ*fadA* strain under heterothallic conditions but *fadA* is essential for homothallic sexual development. Under homothallic conditions, cleistothecia formation is abolished in the Δ*fadA* strain but Hülle cell formation is not affected (Rosén *et al.* 1999, Seo *et al.* 2005). The RGS protein FlbA that controls FadA-mediated signaling (see above) also plays a role in fruiting body development (Han *et al.* 2001). Inactivation of *flbA* completely abolishes cleistothecia and Hülle cell formation under homothallic conditions. The developmental modifier genes *medA* and *stuA*, which play a crucial role in asexual development, also function in sexual differentiation (Fig. 7). The *ΔmedA* strain only forms unorganised masses of Hülle cells and does not differentiate cleistothecia or ascogenic tissue (Busby *et al.* 1996). Sexual development is delayed in the Δ*stuA* strain and it forms only 1 % of the cleistothecia that are formed by the wild-type. Moreover, the cleistothecial shell of the Δ*stuA* strain is much more fragile when compared to the wild-type (Wu & Miller 1997). Thus, *medA* and *stuA* are activators of sexual development.

Activation of the heterotrimeric G-protein by pheromone binding results in activation of a mitogen activated protein kinase pathway (MAPK). As mentioned above, the MAPK module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. The MAPKKK SteC is involved in formation of cleistothecia (Wei *et al.* 2003). The Δ*steC* strain grows slower, forms more branched hyphae, and has an altered conidiophore morphology. Moreover, heterokaryon formation is inhibited. This is probably due to the inability of hyphae to fuse, possibly by the fact that hyphal recognition is blocked (Wei *et al.* 2003). The Δ*steC* strain also has a block in cleistothecium development. Only a few small nest-like structures and single Hülle cells are being produced by this strain. The fact that deletion of both *gprA* and *gprB* results in a phenotype similar to that of the Δ*steC* strain strongly indicates that a MAPK cascade functions in GprA/B mediated signaling (Fig. 7). This would be similar to that found in the yeast mating response. In fact, the *A. nidulans* genome is predicted to contain all the major components of such a yeast MAPK cascade (Seo *et al.* 2004, Yu 2006) (Fig. 7). However, so far the role of all these genes has not yet been confirmed. For instance, a functional study of MAPKK genes in *A. nidulans* is lacking. However, it has been shown that inactivation of the MAPK gene, *mpkB*, abolishes sexual reproduction. Both Hülle cells and cleistothecia are not formed (Paoletti *et al.* 2007). This indicates that MpkB is part of the GprA/B mediated signaling pathway that results in formation of cleistothecia and ascospores.

Transcriptional activators

A target of MpkB seems to be SteA (Vallim *et al.* 2000). This homeodomain-C_2H_2-zinc finger transcription factor is homologous to Ste12, which in *S. cerevisae* is activated by the MAPK signaling pathway involved in mating. A Δ*steA* strain does neither form cleistothecia nor primordial structures but Hülle cell formation is not affected. Expression of *steA* does not depend on *medA* and *stuA*. In contrast, *medA* but not *stuA* is repressed by *steA*. This suggests that *steA* and *stuA* are part of independent pathways regulating sexual development and that *medA* acts downstream of *steA* (Vallim *et al.* 2000) (Fig. 7). Another target of MpkB could be *nsdD* (*never in sexual development*). This gene encodes a putative GATA-like transcription factor with a type IVb zinc finger DNA-binding domain (Han *et al.* 2001). Inactivation of *nsdD* abolishes formation of cleistothecia. In contrast, over-expression of the gene results in increased formation of fruiting bodies on solid medium and Hülle cells are formed even in submerged culture. As a result of over-expression the phenotype of the *veA1* mutation (see below) is overruled. This indicates that *nsdD* acts downstream of this gene or functions in an overlapping

pathway (Han *et al.* 2001). The *nsdD* gene of *A. fumigatus* has a similar function as its counterpart in *A. nidulans.* The Δ*nsdD* mutant of *A. fumigatus* accumulates dark pigments and sexual development is abolished (Szewczyk & Krappmann 2010). A third gene encoding a transcriptional regulator involved in sexual development is *nsdC* (*never in sexual development*) (Kim *et al.* 2009). This gene encodes a putative transcription factor carrying a novel type of zinc-finger DNA-binding domain consisting of two C_2H_2's and a C_2HC motif. Inactivation of *nsdC* completely abolishes fruiting body formation. On the other hand, overexpression of the gene promotes cleistothecia formation, even under repressing stress conditions (Kim *et al.* 2009). The phenotype of the Δ*nsdC* strain is not affected by over-expression of *steA* or *nsdD.* These results suggest that NsdC, NsdD, and SteA are non-redundant proteins and thus are all essential for fruiting body development.

Gene *nosA* (*number of sexual spores*) (Vienken & Fischer 2006) and *rosA* (*repressor of sexual development*) (Vienken *et al.* 2005) encode putative $Zn(II)_2Cys_6$ transcription factors that share 43 % amino acid identity. Transcript levels of these genes are usually very low but the genes are up-regulated during carbon starvation and during asexual development. RosA is an inhibitor and NosA an activator of sexual development. Asexual and sexual development is not affected under standard laboratory conditions when *rosA* is inactivated in a strain with *a veA1* mutation (see below). However, the number of cleistothecia increases when compared to the wild-type strain when the Δ*rosA* strain contains a fully functional *veA* gene (Vienken *et al.* 2005). These data indicate that RosA function depends on VeA. The difference in the number of cleistothecia between the wild-type strain and the Δ*rosA* strain is even higher under low-glucose or high-osmolarity conditions. When grown submerged, Hülle cells are produced in the Δ*rosA* strain, while sexual development in the wild-type is completely absent under this condition (Vienken *et al.* 2005). Asexual development is not affected in a Δ*nosA* strain with a *veA1* mutation. However, this strain was unable to undergo sexual development (Vienken & Fischer 2006). Asexual development in the light is also not affected when the Δ*nosA* strain contains a fully functional *veA* gene. On the other hand, sexual development in the dark is blocked in the primordial stage in this mutant strain. Differentiated cells such as ascus mother cells and Hülle cells are hardly formed and only a few very small cleistothecia (about 30 mm in diameter instead of 300 mm) are produced. These fruiting bodies do produce viable ascospores. Expression of *nosA* was increased in a Δ*rosA* strain (Vienken & Fischer 2006). Similarly *nsdD, veA,* and *stuA* are up-regulated in this mutant strain (Vienken *et al.* 2005). Taken together, RosA represses sexual development, whereas NosA is required for primordium maturation. The balance between these two $Zn(II)_2Cys_6$ transcription factors determines whether vegetative hyphae undergo sexual development.

THE BALANCE OF SEXUAL AND ASEXUAL DEVELOPMENT

There is a balance between sexual and asexual development. *Aspergillus nidulans* forms conidia in the light, whereas ascospores are preferentially produced in the dark (Adams *et al.* 1998, Bayram *et al.* 2010, Mooney *et al.* 1990, Purschwitz *et al.* 2006). Several light sensors and regulatory proteins are involved in light-regulated development. Moreover, the balance between sexual and asexual reproduction is the result of psi factor and cross-talk between regulatory pathways that are involved in these developmental pathways. The light-regulated pathway, the regulation by psi factor, and the cross-talk between regulatory pathways of asexual and sexual reproduction are part of a complex regulatory network.

The role of SteA, BrlA, FlbC, and FlbE

SteA is assumed to be the transcription factor that is activated by MpkB (Fig. 7) thereby promoting sexual development. Expression of *steA* is increased in undifferentiated hyphae of a *ΔbrlA* strain (Vallim *et al.* 2000). Repression of *steA* in uninduced hyphae has been related to the BrlAβ protein. The regulatory interaction between *brlA*, *medA*, and *steA* is a clear example of cross-talk between the developmental programs of asexual and sexual sporulation. FlbC and FlbE also play a role in the cross-talk between the asexual and sexual developmental pathway. FlbC and FlbE are upstream activators of *brlA* involved in asexual development (Fig. 6) but at the same time repress the sexual pathway (Fig. 7) (Kwon *et al.* 2010a, b). Both a Δ*flbC* and a Δ*flbE* strain show elevated cleitothecium formation and abundant formation of Hülle cells (Kwon *et al.* 2010a, b).

The role of psi factor

Aspergillus nidulans produces oleic- and linoleic-acid-derived oxylipins that are known as psi factor (precocious sexual inducer). These hormone-like molecules modulate the timing and balance between sexual and asexual spore development (Calvo *et al.* 2002). Psi factor is mainly a mixture of secreted hydroxylated linoleic (18 : 2) and oleic (18 : 1) molecules termed psiα and psiβ, respectively (Champe *et al.* 1987, Champe & el-Zayat 1989). The positioning of the hydroxy groups distinguishes the psi compounds as psiA (5',8'-dihydroxy-), psiB (8'-hydroxy-), and psiC (designating a lactone ring at the 5' position of psiA) (Mazur *et al.* 1990, 1991). Purified psiAα enhances asexual sporulation. On the other hand, psiBα and psiCα stimulate sexual reproduction and inhibit asexual spore development (Champe *et al.* 1987, Champe & el-Zayat 1989). It has therefore been proposed that the ratio of psiAα to psiBα and psiCα determines whether asexual or sexual sporulation dominates (Champe *et al.* 1987, Champe & el-Zayat 1989). Gene *ppoA* (Tsitsigiannis *et al.* 2004a), *ppoB* (Tsitsigiannis *et al.* 2005), and *ppoC* (Tsitsigiannis *et al.* 2004b), encode putative fatty acid oxygenases. The former gene is required for the production of psiBα, while the latter two genes are involved in the synthesis of psiBβ (Fig. 8). Deletion of *ppoA* reduces psiBα levels and, as a consequence, increases the ratio of asexual to sexual spore numbers 4-fold. On the other hand, over-expression of *ppoA* results in elevated levels of psiBα and decreases the ratio of asexual to sexual spore numbers 6-fold. An increased ratio of sexual to asexual spore numbers is also observed after deletion of *ppoC* (Tsitsigiannis *et al.* 2004a), whereas an opposite phenotype is observed after inactivation of *ppoB* (Tsitsigiannis *et al.* 2005). This is unexpected since the *ΔppoB* and *ΔppoC* strains produce similar psiB profiles (Tsitsigiannis *et al.* 2004a, 2005). Several explanations for this phenomenon have been proposed, one of which is the possibility that the products of the *ppo* genes have more than one substrate. This would generate oxylipins, of unknown nature, that would also affect differentiation (Garscha *et al.* 2007). Another explanation may be related to the finding that the products of the fatty acid oxygenases affect the expression of *ppo* genes and thereby impact the composition of psi factor. Gene *ppoC* is upregulated in a Δ*ppoB* strain, whereas *ppoA* is repressed

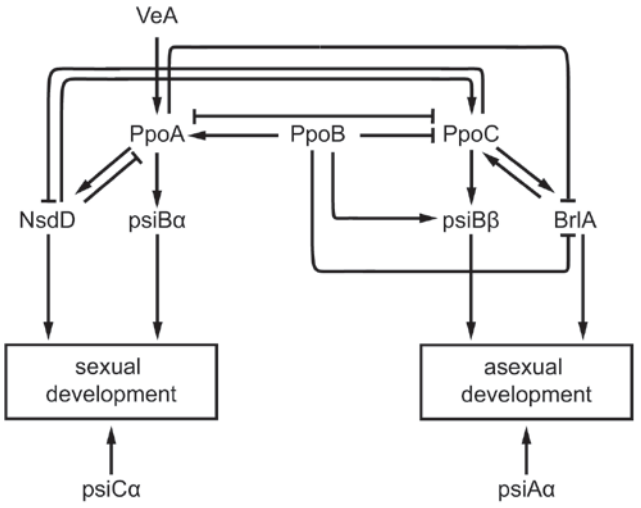

Fig. 8. Oxylipins, known as psi factor, regulate timing and balance between sexual and asexual development. The hormone like structures psiBα and psiCα stimulate sexual development, whereas psiAα and psiBβ stimulate asexual development. Psi factor results from *ppoA, ppoB,* and *ppoC* activity. Expression of these genes is regulated by the products resulting from the Ppo proteins and by BrlA and NsdD. In turn, the products resulting from the Ppo genes regulate expression of *brlA* and *nsdD.*

(Tsitsigiannis *et al.* 2005) (Fig. 8). On the other hand, *ppoA* mRNA levels are higher in a Δ*ppoC* strain, whereas *ppoC* mRNA levels are lower in a strain over-expressing *ppoA* (Tsitsigiannis *et al.* 2004a).

Deletion of *ppoB* significantly increases *brlA* expression but has a minor effect on expression of the regulatory genes of sexual reproduction *nsdD* and *veA* (Tsitsigiannis *et al.* 2005) (Fig. 8). This would explain why the net formation of conidia is upregulated in a Δ*ppoB* strain when compared to ascospores. Changes in sporulation ratios in the Δ*ppoA* and Δ*ppoC* strains also agree with the relative expression of *brlA* and *nsdD* (Tsitsigiannis *et al.* 2004a, b). Expression of *brlA* is down-regulated in a Δ*ppoA*Δ*ppoB*Δ*ppoC* strain, whereas higher levels of *nsdD* and *veA* transcripts are found. This correlates with the increased number of ascospores when compared with the conidia. Notably, sexual development precedes asexual development in the Δ*ppoA*Δ*ppoB*Δ*ppoC* strain, whereas the opposite is observed in the wild-type. Taken together, *ppo* genes determine timing and ratio of sexual and asexual reproduction (Tsitsigiannis *et al.* 2005). The transcripts of *ppoA* and *ppoC* are developmentally regulated in differentiated tissues (Tsitsigiannis *et al.* 2004a, b). Moreover, PpoA has been localised in metulae of conidiophores and Hülle cells (Tsitsigiannis *et al.* 2004b). These observations suggest that Ppo enzymes and/or their substrates are spatially and temporally regulated in reproductive tissues. This would result in a temporal and spatial distribution profile of the oxylipins, which in turn orchestrates the sexual and asexual sporulation schedule (Tsitsigiannis *et al.* 2005). This schedule is associated with two feedback loops (Fig. 8). Genes *ppoC* and *ppoA* regulate the expression of *nsdD* and *brlA*. On the other hand, both *brlA* and *nsdD* activate *ppoC,* whereas *nsdD* represses *ppoA* (Tsitsigiannis *et al.* 2004a, b). In the other loop Ppo oxylipin products would antagonistically signal generation of Ppo substrates (Tsitsigiannis *et al.* 2004a, 2005).

Aspergillus niger (Wadman *et al*. 2009) and *A. fumigatus* (Garscha *et al.* 2007) produce the same oxylipins as *A. nidulans.* The *A. niger* genome contains the *ppo* genes *ppoA*, *ppoC*, and *ppoD*. A *ppoB* homologue is not present (Wadman *et al.* 2009). *Aspergillus niger* strains in which the *ppoA* or *ppoD* gene are inactivated are not affected in oxylipin production and sporulation. In contrast, a multi-copy *ppoC* strain has reduced conidia formation. This shows that oxylipins also play a role in *A. niger* development but it could well be that the function of the individual psi factor components is different.

The role of light

Regulatory genes involved in light response

The *veA1* (*velvet*) mutant of *A. nidulans* was isolated and characterised for the first time in 1965 (Käfer 1965). Sexual development in such strains (among which many lab strains) is generally retarded and reduced, while asexual development is more pronounced (Yager 1992). Deletion of *veA* has a more dramatic effect. A Δ*veA* strain does not produce cleistothecia not even in the dark (Kim *et al.* 2002). On the other hand, over-expression of *veA* results in reduced asexual development and increased production of cleistothecia, even under conditions where the wild-type hardly forms these sexual structures (*i.e.* in liquid medium or on solid medium containing a high concentration of potassium chloride). Taken together, VeA is a positive regulator of sexual development, while simultaneously suppressing asexual development (Kim *et al.* 2002). In contrast, the *veA* gene of *A. fumigatus* (Krappmann *et al.* 2005) and *A. parasiticus* (Calvo *et al.* 2004) have been shown to stimulate asexual reproduction. However, it should be noted that the positive effect on conidia formation is especially clear under adverse environmental conditions. The influence of these conditions on the phenotype of Δ*veA* of *A. nidulans* has not yet been studied.

The *veA* gene of *A. nidulans* is expressed throughout culturing both in liquid and solid medium but its expression increases during sexual development. The *veA* gene encodes a velvet protein that contains a nuclear localisation signal (NLS) (Kim *et al.* 2002, Stinnett *et al.* 2007). VeA is predominantly present in the cytoplasm in the light (white or blue light), but migrates to the nucleus in the dark or when exposed to red light. Migration of VeA to the nucleus depends on the interaction between its NLS and the importin α, KapA (Stinnett *et al.* 2007) (Fig. 9). The velvet protein encoded by the *veA1* allele is predicted to encode a truncated VeA that lacks the first 36 N-terminal amino acids (Kim *et al.* 2002). As a consequence, it misses a non-functional NLS. Indeed, interaction of KapA with the mutant protein is reduced. The VeA1 protein locates mainly in the cytoplasm both in the dark and in the light. This explains why development is affected in the *veA1* strain (Stinnett *et al.* 2007).

Apart from KapA, VeA interacts with the regulator of secondary metabolism LaeA (Bok & Keller 2004) and the VeA-like protein VelB (Bayram *et al.* 2008b, Bok & Keller 2004) (Fig. 9). VeA interacts with LaeA in the nucleus, whereas the complex of VeA and VelB can be found both in the cytoplasm and in the nucleus (Bok & Keller 2004) (Fig. 9). The interaction with VeA explains why VelB can migrate into the nucleus despite the absence of an apparent NLS (Bayram *et al.* 2008b). Like the Δ*veA* strain, the Δ*velB* strain does not show light-dependent development. The Δ*velB* strain is unable to form cleistothecia in the light and in the dark. The effect on asexual sporulation, however, is not as strong as in the Δ*veA* strain. Over-expression of *veA* in the Δ*velB* strain and vice versa does not complement the developmental defects. These data show that the VeA/VelB complex mediates light regulated sexual and asexual development. The binding partner LaeA also has a role in asexual and sexual development. In a *veA1* background inactivation of *laeA* has no effect on asexual reproduction (Bayram *et al.* 2008b, Sarikaya Bayram *et al.* 2010). However, conidiophore formation is reduced both in the light and in the dark when *laeA* is

inactivated in a strain with a functional *veA* gene (Sarikaya Bayram *et al.* 2010). The Δ*laeA* strain in the wild-type background produces five times less conidia in the light when compared to the wild-type. The absolute number of conidia of the Δ*laeA* strain is similar in the light and in the dark. An opposite effect is observed for cleistothecia production. Fruiting body formation is markedly increased in the Δ*laeA* strain when grown in the light. As a consequence, the number of cleistothecia in the mutant strain is similar in the light and the dark. These results are explained by experiments that suggest that the Δ*laeA* strain is entirely blind, which results in a dark-phenotype (low number of conidiophores and high number of cleistothecia) independent of illumination. A Δ*laeA*Δ*veA* strain shows a Δ*veA* phenotype (*i.e.* absence of cleistothecia formation both in light and dark). Thus, *veA* is epistatic to Δ*laeA* (Sarikaya Bayram *et al.* 2010). Taken together, LaeA is a negative regulator of sexual development when *A. nidulans* is grown in the light. LaeA also has a role in the formation of Hülle cells in the dark. Dark grown colonies of the Δ*laeA* strain form less of these cells that feed the cleistothecia. Consequently, the diameter of the cleistothecia as well as the number of ascospores is reduced 5-fold when compared to the wild-type (Sarikaya Bayram *et al.* 2010).

LaeA plays an important role in regulating the levels of the VeA-like proteins VelB and VosA in a light-depended way. When a wild-type colony is exposed to light, the amount of VosA and VelB (see below) is decreased. As a consequence, the amount of VosA/VelB complex is reduced, which releases the repression on asexual development (Fig. 9). In parallel, the reduction of VelB in the light affects sexual development. In the *ΔlaeA* strain, high amounts of VosA and VelB are found in the light, and as a consequence asexual developmental is inhibited, whereas sexual development is promoted (Sarikaya Bayram *et al.* 2010). In addition, LaeA controls levels of VeA and inhibits a post-translational modification of this protein (Sarikaya Bayram *et al.* 2010). The function of the post-translational modification is not yet clear.

LaeA regulates directly or indirectly downstream regulatory genes involved in sexual and asexual development (Sarikaya Bayram *et al.* 2010). Expression of regulatory genes of sexual development, *steA* and *nosA* (Vallim *et al.* 2000, Vienken & Fischer 2006), is transiently reduced during vegetative growth of Δ*laeA* strains. Of interest, the *ΔnosA* strain has a similar phenotype as Δ*laeA* strains with respect to fruiting body formation (Vienken & Fischer 2006). Hülle cell formation and, as a consequence, the size of cleistothecia is strongly reduced. This indicates that Hülle cell formation is regulated by *laeA* via *nosA*. In agreement, over-expression of *nosA* in a Δ*laeA* strain rescues, albeit moderately, the small cleistothecia phenotype (Sarikaya Bayram *et al.* 2010). A Δ*laeA* strain also shows reduced levels of transcripts of *abaA* but not of *brlA.* The effect on *abaA* expression may well explain the reduced ability of Δ*laeA* strains to form conidia. Similar to LaeA, VeA affects expression of downstream genes involved in asexual development. The higher conidiation profile in a Δ*veA* strain is accompanied by a change in expression of *brlA* (Kato *et al.* 2003). In both the Δ*veA* strain and wild-type colonies the *brlAβ* transcript is present. However, in the Δ*veA* strain the *brlAα* transcript is dominant (Kato *et al.* 2003). The relation of reduced *veA* levels and an increase in the ratio between the *brlAα* and *brlAβ* transcripts is also observed when expression of these genes is analysed after a light exposure of 30 and 60 minutes (Ruger-Herreros *et al.* 2011). Taken together, these data indicate that *veA* affects the *brlAα/brlAβ* transcript ratio (Kato *et al.* 2003). Other developmental genes that are linked to *veA* are the psi factor oxilipin genes that control the balance between asexual and sexual reproduction (see above). Expression of the oxylipin gene *ppoA* is completely abolished in a Δ*veA* strain (Fig. 8). Both the Δ*veA* strain (Kim *et al.* 2002) and the Δ*ppoA* strain (Tsitsigiannis *et al.* 2004b) show an increase in the ratio of conidia and ascospore formation. Notably, expression of *veA* is increased in the Δ*ppoA*Δ*ppoB*Δ*ppoC* strain, which suggests the existence of a regulatory loop between *ppo* genes and *veA* (Tsitsigiannis *et al*. 2005).

Photoreceptors involved in light response

Light has to be sensed in order to enable regulatory proteins to activate or inhibit developmental pathways. Large numbers of conidia are produced in white light. Asexual sporulation also occurs in red (680 nm) or blue (450 nm) light but this process is reduced when compared to white light. When red and blue light are combined, formation of conidia is similar to that in white light (Purschwitz *et al.* 2008). Both blue and red light alone effectively inhibit sexual development to an extent similar to white light. Three light sensors have been identified in *A. nidulans*. FphA is a phytochrome that represents a red-light receptor. Blue light is sensed by the white-collar complex consisting of LreA and LreB, while the photolyase/cryptochrome CryA senses blue light as well as UVA (Bayram *et al.* 2010). Development is regulated via interplay of these receptors.

FphA (*fungal phytochrome*) represents the phytochrome of *A. nidulans*. It contains an N-terminal photosensory module (GAF and PHY domain) that harbors the chromophore. The C-terminal part of FphA contains a histidine kinase related domain, which is expected to be involved in the regulatory output. In addition, it contains a predicted ATPase domain and two NLS sequences. FphA is responsible for the effect of red light on development of *A. nidulans* (Blumenstein *et al.* 2005). Conidiation is slightly reduced when *fphA* is inactivated (Purschwitz *et al.* 2008). Moreover, whereas a wild-type strain produces conidia in red light, the Δ*fphA* strain still reproduces sexually. Yet, the number of cleistothecia is only 10 % of that found in the dark (Blumenstein *et al.* 2005). Under the latter condition, formation of cleistothecia is similar in the wild-type and the Δ*fphA* strain (Purschwitz *et al.* 2008). These data show that FphA is an activator of asexual development and a repressor of sexual reproduction. FphA is located in the cytoplasm and in the nucleus (Blumenstein *et al.* 2005, Purschwitz *et al.* 2008). FphA that resides in the nucleus interacts with the LreB subunit of the white-collar complex that signals blue light (see below) (Purschwitz *et al.* 2008) (Fig. 9). Nuclear-located FphA also has a physical interaction with VeA (Purschwitz *et al.* 2008). The light control of VeA might be activated during development via a direct interaction with FphA. VeA is highly phosphorylated, and FphA has a light driven histidine kinase activity (Brandt *et al.* 2008). It might therefore be that FphA phosphorylates VeA. However, up to now no downstream phosphor transfer initiated by FphA has been observed (Bayram *et al.* 2010, Purschwitz *et al.* 2009). Moreover, it is not clear whether photoreceptor-linked VeA also interacts with VelB and/or LaeA.

LreA and LreB (*light response)* form the white-collar blue light receptor, and are orthologues of the best characterised white collar proteins WC-1 and WC-2 of *N. crassa* (Purschwitz *et al.* 2008). The LreA protein contains a light-, oxygen-, or voltage-sensitive (LOV) domain that harbors the flavin adenine dinucleotide co-factor. It also contains two protein-protein interaction domains called PER-ARNT-SIM (PAS) domains. In addition, LreA is characterised by a NLS and a GATA-type zinc-finger DNA binding domain. LreB is a smaller protein than LreA. It contains a NLS sequence, only one PAS domain, and the GATA-type zinc-finger DNA binding domain. The light sensing domain is thus lacking in

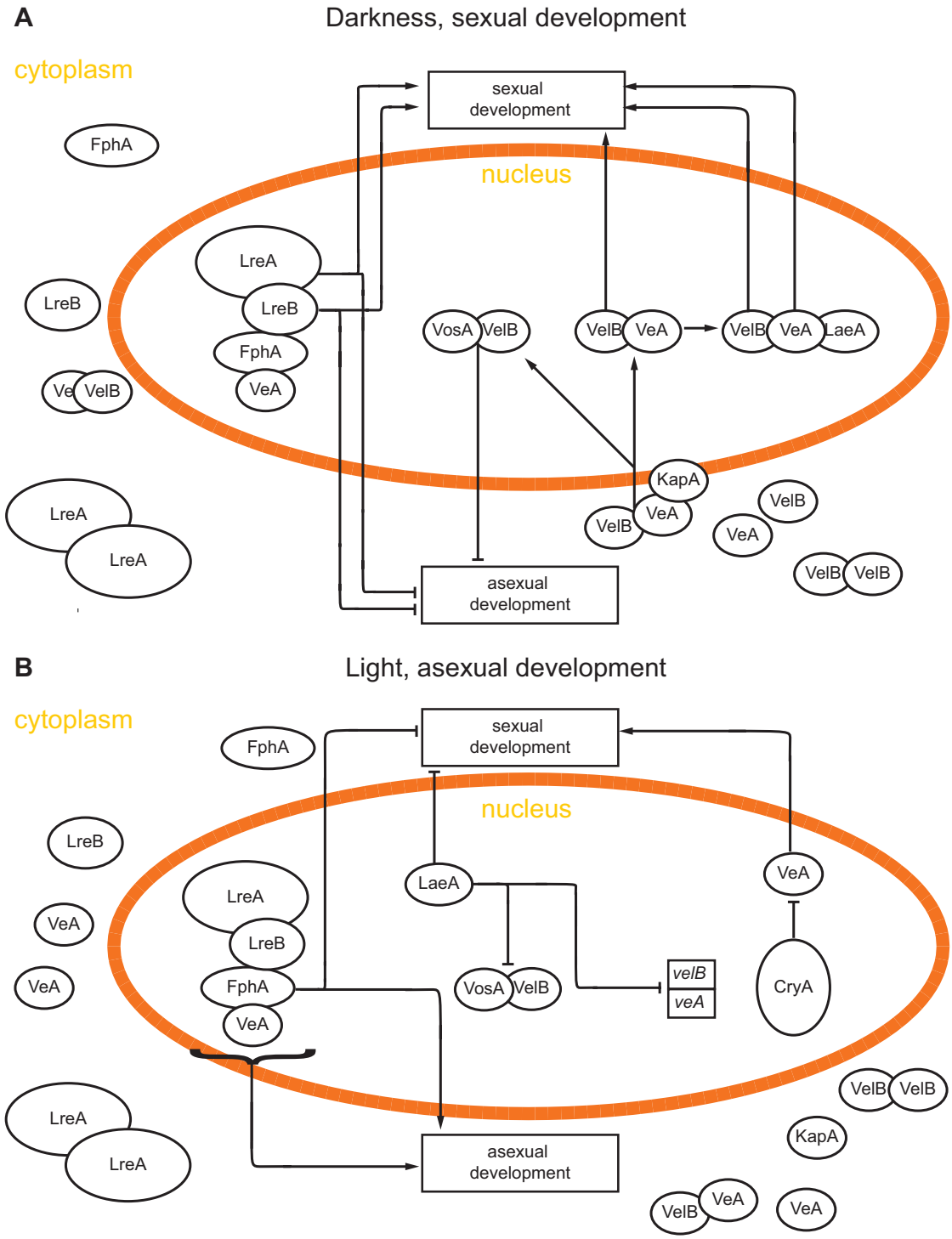

Fig. 9. Light-regulated development in *A. nidulans.* (**A**) In the dark VelB enters the nucleus together with VeA and α-importin KapA. In the nucleus, VeA and VelB act as a dimeric complex or as a trimeric complex together with LaeA to positively regulate sexual development. VelB also forms a complex with VosA that negatively regulates asexual development. (**B**) In the light, activity of LaeA results in reduced levels of VelB and VosA. As a consequence, the inhibition of asexual development by the VelB-VosA complex is released. Moreover, the reduction of VelB levels abolishes stimulation of sexual development. Light is detected by the red light receptor FphA and the blue light receptor proteins LreA and LreB. These light receptors form a complex in the nucleus together with VeA. The cryptochrome/photolyase CryA also plays a role in light regulated development. Like FphA, it is a repressor of sexual development. (Adapted from Sarikaya Bayram *et al.* 2010).

LreB. Asexual development is somewhat increased in the Δ*lreA* and Δ*lreB* strains, irrespective of the presence of light. This finding suggests that LreA and LreB repress conidia formation (Purschwitz *et al.* 2008). Notably, the number of conidia in the Δ*lreA*Δ*fphA* strain, the Δ*lreB*Δ*fphA* strain, and the Δ*lreA*Δ*lreB*Δ*fphA* strain is strongly decreased when compared to the wild-type. Yet, both in the dark and in the light a basal level of conidiation is observed in these strains. Cleistothecia production in the dark is reduced by 70 % and 30 %, respectively, in the Δ*lreA* and Δ*lreB* strains when compared to the wild-type. Strains Δ*lreA*Δ*fphA*, Δ*lreB*Δ*fphA*, and Δ*lreA*Δ*lreB*Δ*fphA* behave like the Δ*lreB* strain. When exposed to white light, cleistothecium formation is nearly absent in the Δ*lreA* and Δ*lreB* strains. This effect is suppressed by inactivation of *fphA* in these strains. Taken together, LreA and LreB act as activators of the sexual cycle and their activity is repressed by light through the action of FphA (Purschwitz *et al.* 2008). LreB also has a function in activation of the asexual cycle by induction of the *brlA* gene (Ruger-Herreros *et al.* 2011). It may be that LreB directly controls *brlA*

expression via its DNA binding domain (Fig. 9). Another possibility is that the photoreceptor complex activates other transcriptional regulators that in turn bind to the *brlA* promoter.

CryA of *A. nidulans* is a combined cryptochrome/photolyase that resides in the nucleus. It repairs UV induced DNA damage. It also regulates fruiting body formation under both UVA and blue light conditions (350–370 and 450 nm) but does not have a known DNA binding domain (Bayram *et al.* 2008a). The *cryA* (*cryptochrome*) gene has a basal expression during vegetative growth and early asexual and sexual development. Expression increases during late asexual and sexual sporulation, and therefore suggests a role of CryA in late developmental processes. The Δ*cryA* strain shows no phenotype on solid medium under standard laboratory conditions but in submerged cultures Hülle cells, but not cleistothecia, are formed (Bayram *et al.* 2008a). These data suggest that CryA is a negative regulator of sexual development. The phenotype of the Δ*cryA* strain resembles that of the Δ*rosA* strain (Vienken *et al.* 2005). Hülle cells are also formed in submerged cultures when *veA* or *nsdD* are over-expressed (Han *et al.* 2001, Kim *et al.* 2002). Expression levels of *veA*, *nsdD*, and *rosA* are increased in the Δ*cryA* strain. This suggests that the cryptochrome regulates these transcription factors (Bayram *et al.* 2008a) (Fig. 9). These and other expression data indicate that CryA represses *veA* expression, whereas VeA stimulates *nsdD* expression. NsdD subsequently activates expression of its own negative regulator *rosA* (Bayram *et al.* 2008a). The *rosA*/*nsdD* feedback loop results in the presence of NsdD during development of Hülle cells and not during later stages of sexual development. In this way, NsdD can fulfill its function in the early stages of sexual reproduction.

DORMANCY AND GERMINATION

Dormancy

Spores should not germinate on the conidiophore or in the ascocarp but rather when they have been dispersed. Moreover, spores should only germinate when environmental conditions are favorable for fungal growth. This requires mechanisms to keep spores dormant. During dormancy, spores may be exposed to various stress conditions such as UV-radiation, drought, and relatively high temperatures. Conidia of *Aspergillus* are moderately resistant to these stress situations due to a number of resistance mechanisms.

Volatiles that prevent germination of spores on reproductive structures

The volatile 1-octen-3-ol is produced by fungi as a degradation product of linoleic acid. It inhibits germination of conidia of *Penicillium paneum* and *A. nidulans* (Chitarra *et al.* 2004, Herrero-Garcia *et al.* 2011). The critical concentration of 1-octen-3-ol needed to inhibit germination is obtained when spores are present at a high density. This situation is met on a conidiophore and in this situation the volatile acts as a self-inhibitor. Upon spore dispersal, the concentration of 1-octen-3-ol drops. As a result, outgrowth of the conidia is no longer self-inhibited but only depends on the environmental conditions.

Resistance of conidia against UV

Conidia of the genus *Aspergillus* survive UV due to the presence of melanin or melanin-like pigments in their cell wall. The melanin-(like) pigment is also a virulence factor (Tsai *et al.* 1999). Melanin contained in the cell wall of *Aspergillus* conidia is synthesised via the 1,8-dihydroxynaphtalene (1,8-DHN) pathway (Fig. 10), which is conserved in the Aspergilli (Baker 2008, Tsai *et al.* 1999). The DHN pathway results in brown or black melanin. Absence of enzymatic steps or modification of melanin precursors results in pigments with different colours.

A cluster of six genes has been identified that is involved in the production of the bluish-green pigment in the conidia of *A. fumigatus* (Tsai *et al.* 1999). Inactivation of one of each genes in the cluster results in spores with different colours. Gene *alb1* (*albino*) encodes the polyketide synthase of the DHN pathway (Fig. 10). This polyketide synthase produces the heptaketide naphtopyrone YWA1 (Watanabe *et al.* 2000), which is the precursor for the green spore pigment of *A. nidulans* (Watanabe *et al.* 1999). In the case of *A. fumigatus, ayg1* (*Aspergillus yellowish green*) converts YWA1 into 1,3,6,8 tetrahydroxynaphtalene (1,3,6,8-THN), which is further modified in the DHN pathway (Fujii *et al.* 2004). Gene *arp2* (*Aspergillus reddish-pink*) encodes the hydroxynaphthalene (HN) reductase that forms both scytalone and vermelone (Tsai *et al.* 1999), whereas *arp1* encodes the dehydratase that converts scytalone in 1,3,8-THN, which is the precursor for Arp2 (Tsai *et al.* 1999).The combination of *alb1, ayg1, arp1*, and *arp2* are predicted to produce a brown-black melanin. The two other genes in the cluster are assumed to be required for the production of the bluish-green pigment of wild-type *A. fumigatus* spores. Gene *abr1* (*Aspergillus brown*) is a putative multicopper oxidase that converts vermelone to 1,8-DHN (Pihet *et al.* 2009). Subsequently, 1,8-DHN is polymerised by the laccase encoded by *abr2* (Sugareva *et al*. 2005).

In contrast to other Aspergilli such as *A. nidulans* and *A. fumigatus*, there is little evidence that supports the involvement of the DHN pathway in *A. niger* (Jørgensen *et al.* 2011). The *A. niger* genome lacks clear orthologs for *arp1* and *arp2.* Moreover, spore pigmentation of *A. niger* is insensitive to the HN reductase (Arp2) inhibitor tricyclazole A. The *fwnA* (*fawn*), *pptA* (*phosphopantetheinyl transferase*), *olvA* (*olive*), and *brnA* (*brown*) genes have been shown to be involved in the production of the characteristic black spore pigment of *A. niger*. Gene *fwnA* encodes a polyketide synthase. Inactivation of this gene results in fawn-coloured conidia. Conidia of the *ΔpptA* strain are white due to the absence of phosphopantetheinyl transferase activity. This activity is required for activation of polyketide synthases (PKSs) and non-ribosomal peptide synthases. The proteins encoded by *olvA* and *brnA* are homologous to the *A. fumigatus* AygA and Abr1 proteins, respectively.

Resistance to drought and high temperature

Dormant conidia of *A. nidulans* survive up to 6 weeks at room temperature in liquid (Fillinger *et al.* 2001) but even much longer when kept in a dried state. In a dried state, the mRNA pool of the conidia of *A. fumigatus* hardly changes even after a year of storage (Lamarre *et al*. 2008). Conidia of *Aspergillus* also survive relatively high temperatures. For example, conidia of *A. niger* survive 1 h at 50 °C (Ruijter *et al.* 2003). Compatible solutes are assumed to protect conidia against drought and heat stress. These molecules include the disaccharide trehalose and the polyols mannitol, glycerol, erithreitol, and arabinitol. The compatible solutes do not affect functioning of proteins and membranes when they accumulate to high concentrations inside the cell. Conidia of *A. nidulans*, and *A. oryzae* contain 0.7–1.4 pg trehalose and 0.5–0.8 pg mannitol (d'Enfert & Fontaine 1997, Sakamoto *et al.* 2009). Together they

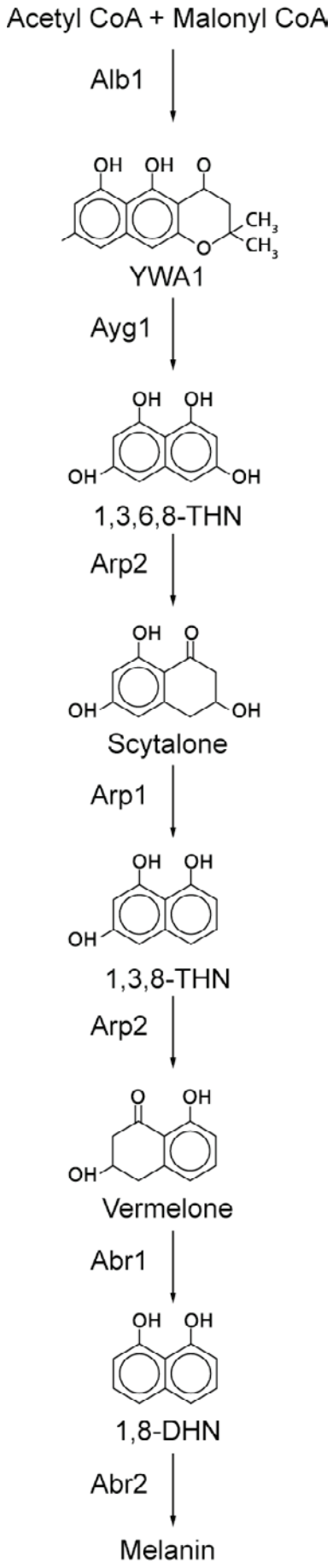

Fig. 10. Synthesis of melanin by means of the DHN pathway. Proteins of *A. fumigatus* responsible for each of the steps are indicated. Note that absence of particular enzymes and/or modification of melanin precursors will result in melanin-like pigments with colours other than brown/black. (Adapted from Fujii *et al.* 2004, Tsai *et al.* 1999, Pihet *et al.* 2009).

account for 4–6 % of the wet weight of these spores. Mannitol is the most abundant compatible solute in the case of *A. niger.* Mannitol and trehalose represent 10.9 % and 3.6 % of the dry weight of conidia, respectively (Ruijter *et al.* 2003, Witteveen & Visser 1995).

Conidia of an *A. niger* strain in which the mannitol 1-phosphate dehydrogenase gene *mpdA* is inactivated have increased trehalose (11.5 % dry weight) and reduced mannitol levels (4,0 % dry weight). Despite the fact that the total amount of compatible solutes remains unaltered, mutant conidia show 90 % viability loss after 1 h incubation at 50 °C when compared to the wild-type. The Δ*mpdA* conidia are also more sensitive to other stress conditions including freeze-thawing, drying, and hypochlorite treatment. Absence of trehalose also affects heat resistance of *A. niger* conidia (Wolschek & Kubicek 1997). *Aspergillus niger* contains two genes, *tpsA* and *tpsB,* that encode trehalose-6-phosphate synthase. This enzyme catalyses the first step in trehalose biosynthesis. Trehalose content is reduced by more than 50 % in the Δ*tspA* strain, which is accompanied by increased sensitivity to heat stress. About 3 times more conidia of the wild-type survive incubation at 55 ˚C when compared to the Δ*tspA* strain. Trehalose also has a role in protection of conidia of *A. nidulans.* The Δ*tpsA* strain of *A. nidulans* is incapable of producing trehalose (Fillinger *et al.* 2001). Consequently, conidia of the mutant strain are unable to germinate at 44 °C in a glucose medium, whereas wild-type spores do grow out to form a mycelium. Prolonged incubation at 44 °C also abolishes the ability of the Δ*tpsA* to germinate at lower temperatures. A role for trehalose to protect conidia against heat stress is also indicated by the fact that inactivation of the neutral trehalase gene *treB* promotes heat resistance of germinating conidia. This is explained by the fact that these conidia can not degrade trehalose and are therefore longer protected against temperature stress (d'Enfert *et al.* 1999).

VosA and VelB regulate the trehalose biosynthetic genes in conidia and ascospores. Levels of *vosA* transcripts and VosA protein are high in both spore types. Inactivation of *vosA* results in the lack of trehalose in spores, which is accompanied by a rapid loss of spore viability, and a dramatic reduction in tolerance of conidia to heat and oxidative stress (Tao & Yu 2011). Similarly, spores of a *ΔvelB* strain show reduced viability and stress tolerance (Sarikaya Bayram *et al.* 2010). In both *ΔvosA* and *ΔvelB* strains reduced expression of genes involved in the biosynthesis of trehalose is observed (Sarikaya Bayram *et al.* 2010). It has been proposed that a heterodimer of VelB and VosA activates expression of genes involved in trehalose biosynthesis and by this plays a role in viability and stress tolerance of spores (Sarikaya Bayram *et al.* 2010).

Proteins and metabolites other than polyols and sugars may also protect dormant conidia. High numbers of transcripts are observed in conidia of *A. niger* of a gene encoding a LEA-like protein (*late embryogenesis abundant*) and of two small heat shock proteins (van Leeuwen *et al.* 2013a). Conidia of *A. oryzae* accumulate the amino acid glutamic acid (Sakamoto *et al.* 2009) up to approximately one percent of the wet weight.

Germination

Three stages can be distinguished during germination of conidia. In the first phase of germination, dormancy is broken by environmental cues such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars (Osherov & May 2001). Spores grow isotropically in the second phase of germination. This process that is also known as swelling is observed between 2 and 6 h after inoculation of *A.*

niger at 25 °C (van Leeuwen *et al.* 2013a, b). During this stage, the diameter of the spore increases two fold or more due to water uptake. This is accompanied by a decrease in the microviscosity of the cytoplasm (Dijksterhuis *et al*. 2007). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Momany 2002). In the third phase of germination, a germ tube is formed by polarised growth. To this end, the morphogenetic machinery is redirected to the site of polarisation (d'Enfert 1997, Harris 2006, Momany 2002, Harris & Momany 2004). Polarised growth of *A. niger* can be observed 6 h after inoculation at 25 °C (van Leeuwen *et al.* 2013a, b). At a later stage, the growth speed of the germ tube increases.

Transcripts of about one third of the genes can be detected by micro-arrays in dormant conidia of *A. niger* (van Leeuwen *et al.* 2012a, b). Transcripts representing the functional gene classes protein synthesis and protein fate are enriched in the RNA pool. A strong drop in the amount of RNA is observed in the first two hours of germination (van Leeuwen *et al.* 2013a, b). Notably, transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation are over-represented in the up-regulated genes at 2 h. Moreover, the categories transcription (including rRNA synthesis and rRNA processing), energy (respiration), cell cycle & DNA processing are overrepresented in the up-regulated genes at this time point. Up-regulation of genes involved in protein synthesis has also been shown in germinating conidia of *A. fumigatus* (Lamarre *et al.* 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis do not affect this process (Osherov & May 2000). The total number of genes that are expressed in germinating conidia of *A. niger* gradually increase between 2 and 8 h after inoculation at 25 °C (van Leeuwen *et al.* 2013a). After 4 h of germination, the functional categories metabolism and cell cycle and DNA processing are over-represented in the up-regulated genes. The latter suggests that the conidium prepares itself for mitosis, which occurs a few hours later. No functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h (van Leeuwen *et al.* 2013a).

Upon activation of conidia of *A. nidulans* and *A. niger,* the compatible solute trehalose is converted to glucose (d'Enfert *et al.* 1999, van Leeuwen *et al.* 2013b). Similarly, mannitol levels quickly drop during the first 2-3 h of germination. Intracellular trehalose is degraded by the action of the neutral trehalase TreB (d'Enfert *et al.* 1999). The Δ*treB* strain of *A. nidulans* still contains 1.2 pg of trehalose after 3 h of germination and also shows a reduction in the degradation of mannitol when compared to the wild-type. Germ tube formation is not affected in the Δ*treB* strain of *A. nidulans* in the presence of an external C-source. However, it is delayed in the case the concentration of the external C-source is very low. Apparently, degradation of trehalose is needed to generate energy during germination. Interestingly, germinating Δ*treB*-spores resist a heat shock of 50 °C for 30 min, whereas more than 80 % of the wild-type spores have died after this treatment. This suggests that trehalose has a protective effect. However, experiments with a Δ*tpsA* strain that is not able to synthesise trehalose show that the situation is more complex. Three-hours-old germlings of this mutant strain show accumulation defects of trehalose after subjection to heat stress or oxidative stress. The isotropically growing wild-type spores accumulate 0.8 pg trehalose as a response to the stress, but the Δ*tpsA* strain was not able to do so. Remarkably, there was no effect on the sensitivity of these germlings for a second heat shock at 50 °C (Fillinger *et al.* 2001).

Regulation of germination of conidia

cAMP and RasA signalling

Initiation and completion of germination requires the sensing of external signals. To this end, conidia of *A. nidulans* use signaling via cAMP/protein kinase A (Fig. 11), and independently, signaling via RasA. In general, signalling via cAMP/protein kinase A (PKA) is initiated by an external signal through a heterotrimeric G-protein. Sensing of the external signal leads to the activation of the Gα-subunit of the heterotrimeric G-protein, which activates adenylate cyclase. This enzyme produces cAMP that can bind to the regulatory subunit of PKA. As a result the regulatory subunit dissociates from the catalytic subunit (PKAc). Active PKAc phosphorylates proteins and in this way controls their activity. As described, the heterotrimeric G protein GanB-SfaD-GpgA represses conidia formation in *A. nidulans* (Fig. 5), but this heterotrimeric G-protein is also a carbon source sensor involved in early cAMP-dependent germination in *A. nidulans* (Lafon *et al.* 2005). Conidia of a Δ*ganb* strain show a reduced rate of swelling and germ tube formation (Chang *et al.* 2004). Wild-type conidia start to swell 2 h after inoculation. Of these conidia, 8 % and > 90 % had formed germ tubes 4 and 6 h post inoculation, respectively. Germ tubes are hardly observed after 4 h in the case of the Δ*ganB* strain, and less than 50 % of these spores have formed a germ tube after 10 h of inoculation. In contrast, expression of a constitutive active version of GanB ($GanB^{Q208L}$) promotes germ tube formation. In this case, germination of conidia even takes place in the absence of carbon source (Chang *et al.* 2004). Gene *ganB* is also involved in the germination process of ascospores. Ascospores of the Δ*ganB* strain germinate very poorly, whereas expression of $GanB^{Q208L}$ results in precocious ascospore germination, even in the absence of carbon source. Taken together, GanB plays a positive role during germination, probably through carbon source sensing. The RgsA protein of *A. nidulans* negatively regulates GanB signaling (Han *et al.* 2004b) (Fig. 11). Like a strain expressing $GanB^{Q208L}$, conidia of the Δ*rgsA* strain germinate even in the absence of a carbon source. This effect is not observed in a Δ*ganB*Δ*rgsA* or a Δ*sfaD*Δ*rgsA* strain (Han *et al.* 2004b, Lafon *et al.* 2005).

The Δ*ganB* strain of *A. nidulans* shows a > 3-fold reduction in trehalose degradation during spore germination (Lafon *et al.* 2005). Breakdown of this disaccharide is a direct outcome of activation of the cAMP/PKA pathway during early germination (d'Enfert *et al.* 1999). A role of GanB in cAMP/PKA signaling has been proven by measuring cAMP levels in germinating spores. Addition of glucose to dormant wild-type spores results in a rapid and transient increase in cAMP levels. This increase is not observed in the Δ*ganB* strain (Lafon *et al.* 2005). Thus, GanB regulates cAMP synthesis in response to glucose at the start of germination. As described above GanB forms a heterotrimer G-protein together with SfaD and GpgA (Fig. 11). Spore germination and trehalose degradation is also affected in conidia of the Δ*sfaD* and Δ*gpgA* strains, although not as strong as observed in the Δ*ganb* strain (Lafon *et al.* 2005). A role of RgsA in GanB signaling is further supported by the finding that trehalose degradation in the Δ*rgsA* strain is increased. This effect is abolished by inactivation of *ganB* in this strain but also by inactivation of *sfaD* (Lafon *et al.* 2005). These data indicate that glucose-stimulated activation of the cAMP/PKA pathway by GanB requires a functional G-protein formed by GanB, SfaD, and GpgA (Fig. 11).

CyaA represents the adenylate cyclase of the cAMP/PKA pathway that is regulated by GanB (Fig. 11). In contrast to the wild-type, mycelium of the Δ*cyaA* strain is completely devoid of cAMP

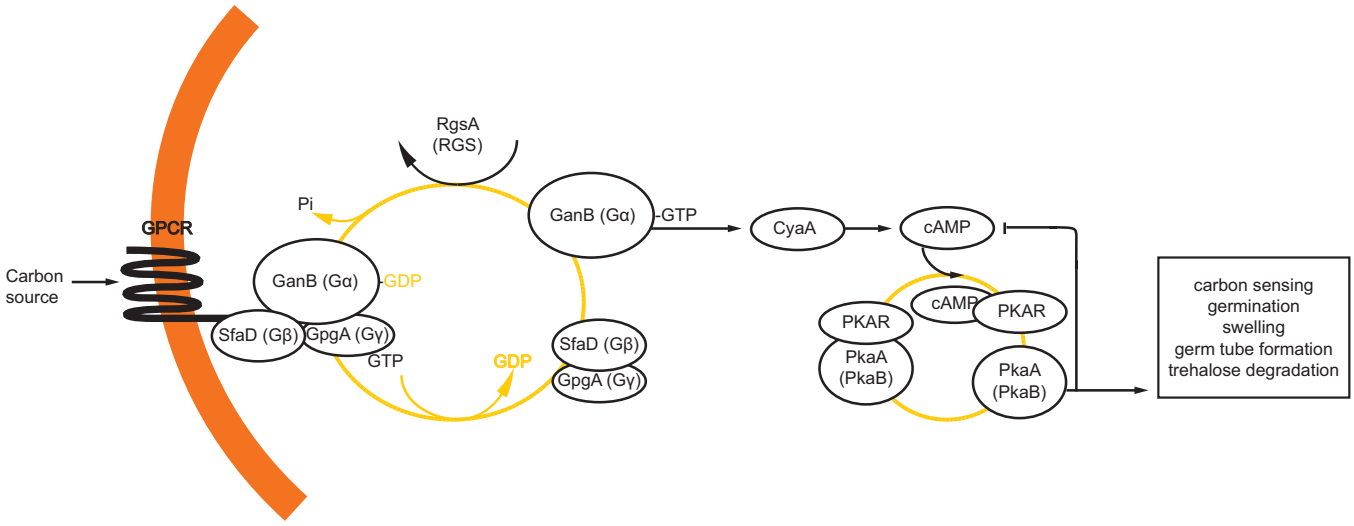

Fig. 11. The cAMP/protein kinase A signaling pathway involved in germination of spores in *A. nidulans*. The presence of a carbon source is sensed by a GPCR that activates the Gα subunit GanB. GanB-GTP activates adenylate cyclase CyaA that produces cyclic adenosine-monophosphate (cAMP). cAMP binds to the regulatory subunit of PKA (PKAR), thus releasing the catalytic subunit PkaA. Active PkaA phosphorylates downstream targets resulting in swelling, germ tube formation and trehalose degradation. PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source.

(Fillinger *et al*. 2002). This indicates that *cyaA* encodes the unique adenylate cyclase during mycelial growth. Conidia of the Δ*cyaA* strain do not degrade trehalose during the onset of germination. Moreover, germ tube outgrowth is affected. As mentioned above, cAMP produced by adenylate cyclase activates the catalytic subunit of PKA (PKAc) by releasing the regulatory subunit. In the case of *A. nidulans, pkaA* encodes the primary PKAc. Trehalose breakdown is reduced in the Δ*pkaA* conidia and germ tube outgrowth is affected, but not as strong as in the Δ*cyaA* conidia (Fillinger *et al*. 2002). Germination of conidia is also affected in *A. fumigatus* (Liebmann *et al*. 2004) and *A. niger* (Saudohar *et al.* 2002) when their closest homologue of *pkaA* is inactivated. *Aspergillus nidulans* contains a secondary *pka* gene, *pkaB*, which encodes a catalytic subunit of PKA. The Δ*pkaB* strain does not have an apparent phenotype (Ni *et al.* 2005). However, the Δ*pkaA*Δ*pkaB* strain is lethal, indicating that PkaB is involved in hyphal growth and/or spore germination. Approximately 10-fold up-regulation of *pkaB* mRNA levels rescues the defects in germination of conidia of the Δ*pkaA* strain in the presence of glucose (note that the level of up-regulation is important for the phenotypic outcome). In contrast, up-regulation of *pkaB* completely abolishes spore germination in the absence of an external carbon source. Taken together, these data indicate that PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source (Ni *et al.* 2005). Other Ser/Thr protein kinases also contribute to spore germination in *A. nidulans.* Inactivation of the Ser/Thr protein kinase gene, *schA*, in the Δ*pkaA* background results in a phenotype similar to that of the Δ*cyaA* conidia. This indicates that PkaA and SchA are activated by cAMP produced by CyaA.

The Ras signaling pathway operates independently from the cAMP/PKA signaling pathway during germination of conidia of *A. nidulans* (Fillinger *et al*. 2002). Conidia of *A. nidulans* strains expressing a dominant active form of RasA (RasA^{G17V}) do not proceed to polarised growth. Instead, swelling continues resulting in giant swollen spores (Som & Kolaparthi 1994). This suggests that high RasA activity prevents the switch from isotropic to polarised growth. There are indications that the RasA activity is regulated by a GTPase-activating protein GapA. By stimulating hydrolysis of the GTP bound to RasA it loses its activity (Harispe *et al.* 2008). Both wild-type and Δ*gapA* conidia germinate in the presence of glucose. In contrast, whereas Δ*gapA* conidia also swell in the absence of a carbon source, the wild-type does not. A similar phenotype has been reported for *A. nidulans* expressing RasA^{G17V}. This suggests that RasA plays a role in carbon source sensing during conidiation (Osherov & May 2000), and that this is regulated by GapA. RasA has been suggested to function via activation of a mitogen-activated protein kinase pathway (Fillinger *et al.* 2002). This pathway may well include the mitogen-activated protein kinase MpkA since conidia of the Δ*mpkA* strain have a defect in germination (Bussink & Osmani 1999).

Regulation by stuA and flbC

Genes *fluG, brlA, abaA, wetA, medA, stuA,* and *vosA* play a central role in conidiophore and conidia formation in aspergilli (Fig. 4). The developmental modifier StuA is a transcriptional regulator involved in proper spatial distribution of AbaA and BrlA (Miller *et al.* 1992, Wu & Miller 1997). Conidiophores of the Δ*stuA* strain of *A. fumigatus* are extremely malformed and the number of conidia that are formed is strongly reduced (Sheppard *et al.* 2005). Moreover, these conidiophores are twice the size of wild-type spores. Interestingly, they germinate faster but the underlying mechanism is not known yet. Several transcriptional activators act upstream of BrlA in the regulation of asexual development (Fig. 6). One of these regulators is FlbC, which also has a role in germination (Kwon *et al.* 2010a). Polarised growth has taken place for up to 40 % and 100 % of wild-type conidia 4 h and 6 h post-inoculation, respectively. In contrast, spores of the Δ*flbC* strain only show swelling after 4 h, whereas up to 40 % of the spores have formed germ tubes 6 h after inoculation. These findings show that FlbC has a role in germination.

CONCLUSIONS

The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. The success of aspergilli is explained by the fact that they are not very selective with respect to their abiotic growth conditions, that they can degrade a wide variety of organic molecules, and by the fact that they produce high numbers of asexual and sexual spores that are dispersed over short and

long distances. We have now a strong framework of understanding of molecular mechanisms underlying growth and development of *Aspergillus* but the picture is far from complete. Signalling cascades and transcription factors that are involved in germination, in formation of a vegetative mycelium, and in asexual and sexual development are only partly known. There is clear evidence that these regulatory processes are similar but not identical between *Aspergillus* species. The consequences of these differences for the success of the *Aspergillus* species in nature are not known. Moreover, the question why sexual reproduction in a large group of aspergilli is more restrictive than in other representatives of this genus should be answered, as well as the question what the consequences are for the fitness of the different species.

Expression profiles of conidiophores, conidia, ascocarps, ascospores, germlings, and the vegetative mycelium are clearly distinct. Heterogeneity in gene expression is also found between micro-colonies of a liquid culture of *Aspergillus*. Heterogeneity is even observed between and within zones of the vegetative mycelium. The role of heterogeneity between the leading hyphae that explore the substrate is not known but it is tempting to speculate that the existence of hyphal types increases the fitness of the colony. Heterogeneity between hyphae also has implications how RNA, protein, and metabolite profiles from whole cultures should be interpreted. An average gene expression or activity of the hyphae will be obtained when the whole culture, or for instance, whole ascocarps or conidiophores are used for analysis. This average may by far not reflect the composition or activity of particular cellular or hyphal types within the mycelium or tissue that is being analysed. As a consequence, regulatory mechanisms may not be identified or mis-interpreted. Therefore, single cells or particular cell types should be analysed to improve our understanding of growth and development of *Aspergillus* and other fungi.

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