Molecular Microbiology (2009) 71(5), 1278–1295
doi:10.1111/j.1365-2958.2009.06606.x
First published online 4 February 2009

The protein kinase ImeB is required for light-mediated inhibition of sexual development and for mycotoxin production in *Aspergillus nidulans*

Özgür Bayram,[1†] Fatih Sari,[1†] Gerhard H. Braus[1*] and Stefan Irniger[2]**
[1] *Institute of Microbiology and Genetics, Georg August University, Grisebachstr. 8, D-37077 Göttingen, Germany.*
[2] *Institute of Microbiology, Leibniz University Hannover, Schneiderberg 50, D-30167 Hannover, Germany.*

Summary

Spore formation is a common process in the developmental cycle of fungi. In the yeast *Saccharomyces cerevisiae,* Ime2 is a key protein kinase for the meiotic cell cycle, which precedes ascospore formation. Here, we analysed the *IME2*-related *imeB* gene of the filamentous ascomycete *Aspergillus nidulans. imeB* deletion strains are retarded in growth and overproduce fertile sexual fruiting bodies in the presence of light, which normally represses sexual development. *imeB* mutants also display abnormal differentiation of sexual Hülle cells in submerged cultures. Increased sexual development of *imeB* mutants is dependent on VeA, a component of the heterotrimeric *velvet* complex. A combined deletion of *imeB* with the phytochrome *fphA*, a red light receptor, results in a complete loss of light response, suggesting that ImeB and FphA cooperate in light-mediated inhibition of sexual development. Furthermore, we found that *imeB* mutants fail to produce the mycotoxin sterigmatocystin, an aflatoxin precursor, and show that ImeB is needed for expression of the sterigmatocystin gene cluster. ImeB contains a TXY motif conserved in mitogen-activated protein kinases. This sequence element is essential for ImeB function. We conclude that ImeB is a mitogen-activated protein kinase-related protein kinase required for the co-ordinated control of light-dependent development with mycotoxin production.

Accepted 9 January, 2009. For correspondence. *E-mail gbraus@gwdg.de; Tel. (+49) 551 393 771; Fax (+49) 551 393 330; **E-mail: sirnige@gwdg.de; Tel. (+49) 511 7625 945; Fax (+49) 511 7625 287. †These authors contributed equally to this work.

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd

Introduction

The reproduction cycle of most fungi involves a developmental period, in which specialized cells, termed spores, are produced. Sporulation, the morphological process leading to the formation of spores, is widespread and can take place either in the sexual or asexual life cycle of fungi. Spores not only allow fungi to survive in an unfavourable environment, but also serve for the dispersal of the fungus and the colonization of new habitats. Sporulation occurs in a highly different manner among fungal species. This diversity is also apparent in the phylum of the ascomycota, for example, when comparing yeast with filamentous fungi. In the unicellular yeast *Saccharomyces cerevisiae*, spore formation is a relatively simple process. A diploid cell produces an ascus containing four haploid spores as a result of a meiotic division. In filamentous euascomycetes, the production of different types of spores is often associated with a complex developmental programme involving several specialized cell types to form multicellular fruiting bodies (Braus *et al.*, 2002; Pöggeler *et al.*, 2006).

Regulators of sporulation have been primarily identified and analysed in few model organisms, such as *S. cerevisiae.* Protein kinases are known as important regulators of the cell division cycle and these enzymes also have fundamental roles in yeast during the meiotic cell cycle (Honigberg, 2004). A key factor is the meiosis-specific protein kinase Ime2, which is known to act in concert with the cyclin-dependent kinase Cdk1 to trigger meiosis. *IME2* gene expression is induced early in meiosis by the Ime1 transcription factor (Mitchell *et al.*, 1990) and is then required for the onset of meiotic S-phase by inducing degradation of the Cdk1 inhibitor Sic1 (Dirick *et al.*, 1998). In later periods, Ime2 induces transcription and activation of *NDT80*, a transcription factor controlling the expression of middle meiotic genes (Hepworth *et al.*, 1998), and this kinase has further essential roles for meiotic nuclear divisions (Benjamin *et al.*, 2003).

Although Ime2 is related to cyclin-dependent kinases, it does not require the association with cyclin subunits. Ime2 is an unstable protein kinase and it was shown that its inactivation is needed for the formation of normal asci (Sari

et al., 2008). Ime2 is also regulated by post-translational modifications. It is phosphorylated in early meiosis and hyperphosphorylated during middle/late meiosis (Benjamin *et al.*, 2003; Schindler and Winter, 2006).

Ime2-related proteins have been identified in various organisms. These include fungal proteins termed Pit1 and Mde3 in fission yeast (Abe and Shimoda, 2000) and Crk1 in the basidiomycete *Ustilago maydis* (Garrido and Perez-Martin, 2003). Studies with *U. maydis* showed that Crk1 is needed for sexual development and pathogenesis (Garrido and Perez-Martin, 2003; Garrido *et al.*, 2004). The most closely related proteins from mammals are male germ cell-associated kinase (MAK) and intestinal cell kinase (ICK). MAK expression is tissue-specific and occurs predominantly in testis during meiosis (Matsushime *et al.*, 1990; Jinno *et al.*, 1993). This kinase may have a role in spermatogenesis, but it is not essential for fertility, as shown by the analysis of *mak*$^{-/-}$ knockout mice (Shinkai *et al.*, 2002). All these kinases contain a TXY dual phosphorylation motif, a characteristic of mitogen-activated protein kinases (MAPK) (Payne *et al.*, 1991) and it was proposed that these kinases represent a novel subclass of the MAPK family (Garrido *et al.*, 2004).

Putative homologues of *IME2* have also been identified in genome sequencing projects of different *Aspergillus* species, including *A. nidulans* and *A. fumigatus* (Galagan *et al.*, 2005). However, a role of these proteins in filamentous ascomycete species has not yet been described. *A. nidulans* is an attractive model organism for studying fungal developmental processes, mainly because of its ability to reproduce both asexually and sexually. Asexual development is characterized by the appearance of conidiophores, the carrier structures of the asexual spores called conidiospores (Adams *et al.*, 1998; Fischer, 2002; Pöggeler *et al.*, 2006). *A. nidulans* is a self-fertile fungus and therefore has the ability to propagate sexually even in the absence of a mating partner (Coppin *et al.*, 1997). In this sexual developmental process, hyphae aggregate to so-called 'nests' and subsequently differentiate to form multinucleate Hülle cells (Braus *et al.*, 2002). Such 'nests' serve as a primary structure for the formation of primordia, which are nursed by Hülle cells and differentiate to form a closed fruiting body. In these structures, termed cleistothecia, meiosis and formation of sexual spores, known as ascospores, take place.

Earlier studies identified a variety of environmental and endogenous factors influencing *A. nidulans* development, including nutritional conditions, light, oxygen pressure, carbon dioxide and pheromones (Champe *et al.*, 1987; Champe and el-Zayat, 1989; Coppin *et al.*, 1997; Eckert *et al.*, 1999; Busch and Braus, 2007). How these signals are integrated and converted into an appropriate response is largely unknown. In *A. nidulans*, pivotal environmental conditions governing the balance between asexual and sexual development are light and the availability of oxygen (Mooney and Yager, 1990). Red light and an air interphase stimulate conidia formation and repress the development of sexual fruiting bodies. In contrast, the sexual cycle is induced when a mycelium is grown in the dark without air circulation.

Regulation of development by light requires the far-red and red light photoreceptor FphA (Blumenstein *et al.*, 2005). This phytochrome is required for inhibition of sexual development under red light conditions. *fphA* deletion strains abnormally produce sexual fruiting bodies when grown in the light. Photoreceptors CryA, LreA and LreB mediate blue- and UV light repression of fungal development (Bayram *et al.*, 2008a; Purschwitz *et al.*, 2008). Another key factor in the light response is the VeA protein. VeA acts as an activator of sexual fruiting body formation and as an inhibitor of conidiation (Mooney *et al.*, 1990; Kim *et al.*, 2002). Strains deleted for the *veA* gene fail to produce sexual fruiting bodies, whereas the overexpression of *veA* abnormally stimulates the formation of sexual structures. The function of VeA is largely unclear. Its intracellular localization is light-dependent, cytoplasmic in the light and nuclear in the dark (Stinnett *et al.*, 2007). In the nucleus, VeA can interact with FphA and other light sensor molecules (Purschwitz *et al.*, 2008). We have recently shown that VeA forms a trimeric protein complex with the VelB protein and LaeA, a global regulator of secondary metabolism, in darkness (Bayram *et al.*, 2008b). This complex co-ordinates the light signal with fungal development and secondary metabolism. *A. nidulans* produces various secondary metabolites, including the aflatoxin precursor sterigmatocystin (ST) and penicillin (Keller *et al.*, 2005). Genes responsible for ST biosynthesis are arranged in a cluster (Brown, 1996), which is regulated epigenetically by the methyltransferase LaeA (Bok and Keller, 2004; Keller *et al.*, 2005) and transcriptionally by the zinc finger transcription factor AflR (Fernandes *et al.*, 1998).

In this study, we provide evidence that the Ime2-related protein kinase ImeB of *A. nidulans* is involved in light-mediated regulation of development and in mycotoxin production. Deletion of the *imeB* gene results in an efficient sexual development on solid media in the light, and in the production of Hülle cells in liquid medium. Deletion strains fail to express the ST gene cluster and produce no detectable level of mycotoxin. We also show that the TXY motif conserved in MAP kinases is indispensable for ImeB function.

Results

Identification of an Ime2-related protein kinase from A. nidulans

Previous studies have demonstrated that the

© 2009 The Authors

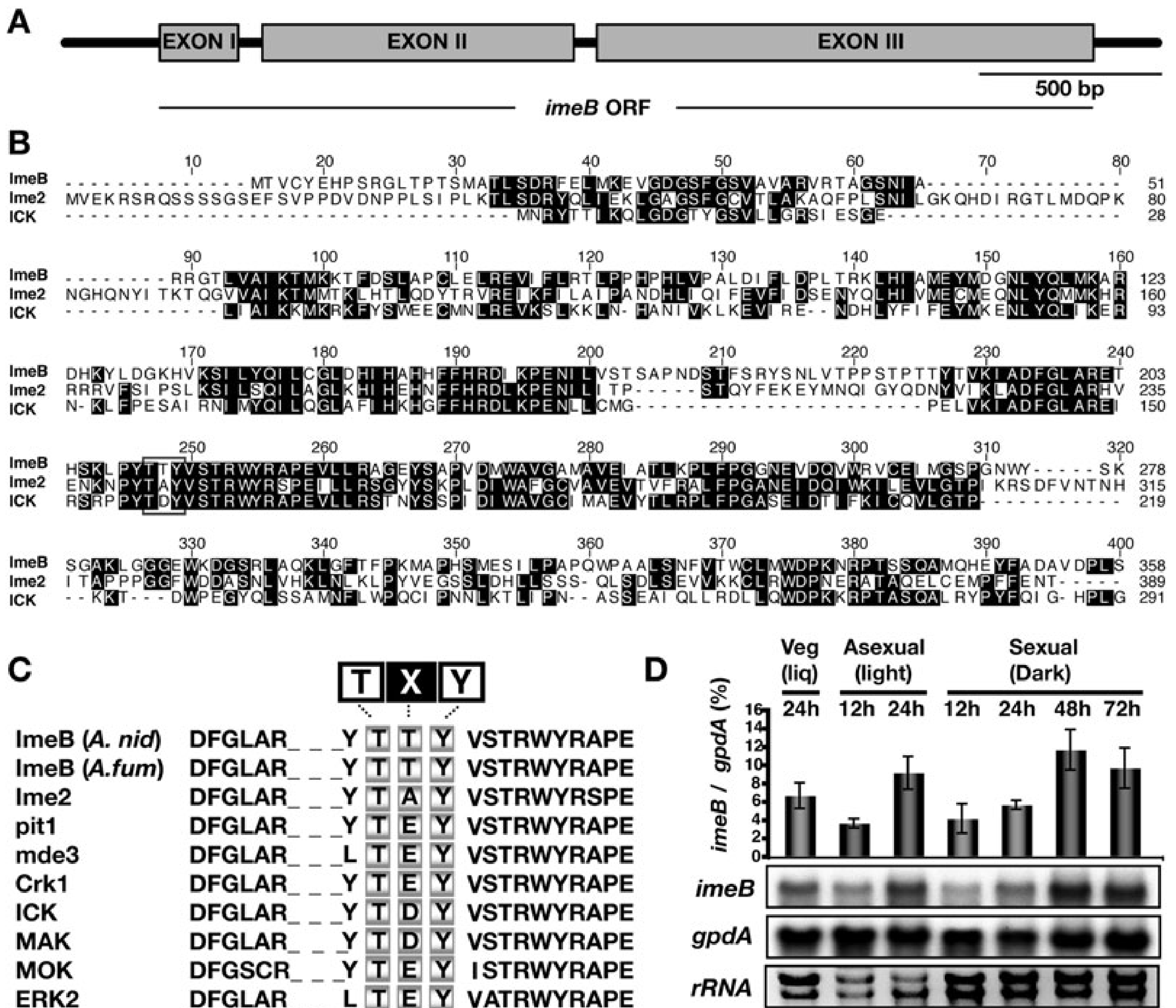

Fig. 1. Alignment of N-terminal parts of ImeB-related proteins and expression of *imeB* in *Aspergillus nidulans*.
A. Illustration of the *ImeB* locus consisting of three exons and two introns.
B. Alignment of N-terminal regions of *A. nidulans* ImeB, Ime2 from *S. cerevisiae* and mammalian ICK. Amino acids identical in at least two sequences were shaded. The alignment was generated with DNAstar using Megalign (Clustal W).
C. Conserved MAPK motifs from different organisms. Sequence alignment of the catalytic domain of ImeB (*A. nidulans*), a hypothetical ImeB homologue from *A. fumigatus* (Nierman *et al*., 2005), Ime2 from *S. cerevisiae* (Yoshida *et al*., 1990), Mde3 and Pit1 from *Schizosaccharomyces pombe* (Abe and Shimoda, 2000), Crk1 from *Ustilago maydis* (Garrido and Perez-Martin, 2003), mammalian ICK (Togawa *et al*., 2000), MAK (Matsushime *et al*., 1990) and MOK (Miyata *et al*., 1999), and ERK2 from *Rattus norvegicus* (Boulton *et al*., 1991). The TXY motifs of the kinases are highlighted.
D. Expression pattern of *imeB* at different developmental stages. A wild-type strain (FGSCA4) was pre-grown in liquid medium for 18 h, subsequently transformed onto solid medium to induce either the asexual or sexual development. At indicated time points mycelia were harvested and a Northern analysis was performed. A probe specific to the coding region of the *imeB* gene was labelled with ^{32}P prior to hybridization. rRNA stained with ethidium bromide was used as loading control and a glycolytic gene, *gpdA*, served as an internal control. Relative expression levels were quantified by using AIDA Bio-Package (Raytest, Germany). Asexual and sexual development was verified by microscopic examination of conidiation or cleistothecia formation respectively.

meiosis-specific protein kinase Ime2 has a pivotal function in the sporulation programme of the yeast *S. cerevisiae* (Honigberg, 2004). Genes related to *IME2* are present in various organisms from yeast to mammals, but it is unknown whether Ime2-related proteins may have a role in the often complex developmental cycle of euascomycetes. Inspection of the *A. nidulans* genome revealed a gene (AN6243.3) named *imeB*, encoding a protein with high similarity to Ime2. *In silico* analysis of the *imeB* locus revealed three exons interrupted by two introns (Fig. 1A),

© 2009 The Authors

the presence of which were also confirmed by sequencing the cDNA of the gene amplified from a cDNA library (data not shown).

Alignment of the deduced *A. nidulans* ImeB protein, consisting of 782 amino acids, with Ime2 showed that the N-terminal parts of the proteins are highly conserved, with 40% similarity between the two proteins (Fig. 1B). In contrast, C-terminal parts of the proteins do not have significant homology (not shown). In yeast, the catalytic domain of the kinase is located in the N-terminal region, whereas the C-terminal region is known to have regulatory functions (Honigberg, 2004). In the N-terminal parts, ImeB and Ime2 also showed high similarity to mammalian proteins, such as ICK. An *imeB* homologue is also present in the genome of the pathogenic fungus *A. fumigatus* (Nierman *et al*., 2005). Comparison of ImeB with other related proteins from different organisms show a highly conserved region encompassing the TXY motif (Fig. 1C). TXY motifs are highly conserved in the activation loop of MAP kinases and are known to be phospho-acceptor sites for MAP kinase kinases (Payne *et al*., 1991). Thus, ImeB belongs to a family of protein kinases conserved throughout the eukaryotic kingdom.

imeB *mRNA is induced during asexual and sexual development*

We analysed *imeB* expression in vegetative mycelium and at different stages of the life cycle of *A. nidulans*. For this purpose, a wild-type strain (FGSC A4) was cultivated either in liquid medium or on agar plates. In liquid cultures, *A. nidulans* produces vegetative mycelium. On solid medium, this fungus can be induced to undergo asexual development by incubation in the light and by aeration. In contrast, the sexual life cycle is promoted when the fungus is grown on agar plates in the dark without aeration. Total RNA was isolated from liquid cultures and from different time points after transfer of the mycelium to solid medium. Northern hybridization with an *imeB* DNA probe showed that *imeB* is expressed at low levels during vegetative growth in liquid culture (Fig. 1D). Its expression gradually increased after transfer of mycelium to solid medium, under conditions promoting asexual conidiation (12 and 24 h) as well as under conditions inducing sexual development (12–72 h). mRNA levels of the control gene *gpdA* remained constant and were used as loading control. Thus, *imeB* expression is increased during both the asexual and sexual developmental programmes.

ImeB is a predominantly nuclear protein

To analyse the subcellular localization of the ImeB protein, we constructed a fusion protein of ImeB fused at its N-terminus with e-GFP. An *imeB* cDNA was cloned into the expression plasmid pCMB17apx, allowing the expression of the *e-gfp*::*imeB* gene fusion from the alcohol dehydrogenase (*alcA*) promoter, which can be induced by different non-fermentable carbon sources (Lubertozzi and Keasling, 2006). This plasmid was transformed to strain AGB152, together with a plasmid containing an *mrfp*::*h2A* fusion, encoding a red fluorescent protein fused to histone H2A allowing to visualize nuclei (Bayram *et al*., 2008b). For induction of the *e-gfp*::*imeB* construct, media were supplemented with glycerol and threonine and then the strain was grown overnight on a microscope slide. Fluorescence microscopy showed that e-GFP::ImeB colocalized with the red fluorescence signal indicating that the fusion protein is mainly localized to the nucleus in germinating spores (Fig. 2A, upper panel) and adult hyphae (Fig. 2A, lower panel). Localization of the e-GFP::ImeB fusion protein was similar in hyphae grown either in the light or in the dark (data not shown). In addition to this overexpression construct, we also replaced the endogenous *imeB* locus with *imeB*::*sgfp*, expressed from the native *imeB* promoter (Fig. 2B). This fully functional ImeB::sGFP fusion is also targeted to the nucleus. Therefore, ImeB protein is a predominantly nuclear protein, which is not affected in its localization by illumination.

imeB *deletion strains have a reduced growth rate and produce aberrantly increased numbers of cleistothecia in the light*

To address the cellular function of ImeB, a deletion cassette was constructed by using a 2.1 and 2.5 kb fragment of the 5′ and 3′ flanking regions of *imeB* gene respectively, and the pyrithiamine-resistance gene, *ptrA*, as selective marker (Fig. S1). After transformation of the deletion cassette into the wild-type strain FGSC A4 and selection on pyrithiamine-containing plates, an *imeB* deletion strain was identified by Southern analysis. Probing with an *imeB* probe confirmed the replacement of *imeB* by *ptrA* (Fig. S1). A verified deletion strain was backcrossed with a wild-type strain to demonstrate that the phenotype of the *imeB* mutant is coupled to the replacement of the *imeB* gene with *ptrA*.

Initial analysis of the *imeB* deletion strain showed a reduced growth rate compared with wild-type strains, as manifested by reduced diameters of colonies on agar plates (Fig. 3A and C). The slower growth phenotype of *imeB* strains on solid medium could also be observed when *imeB* deletion strains are grown in liquid, and furthermore, a delay in germination of both condiospores and ascospores was also apparent (data not shown).

We further observed that *imeB* mutants display unusual sexual development in the presence of light, which normally represses fruiting body formation (Fig. 3A and B).

© 2009 The Authors

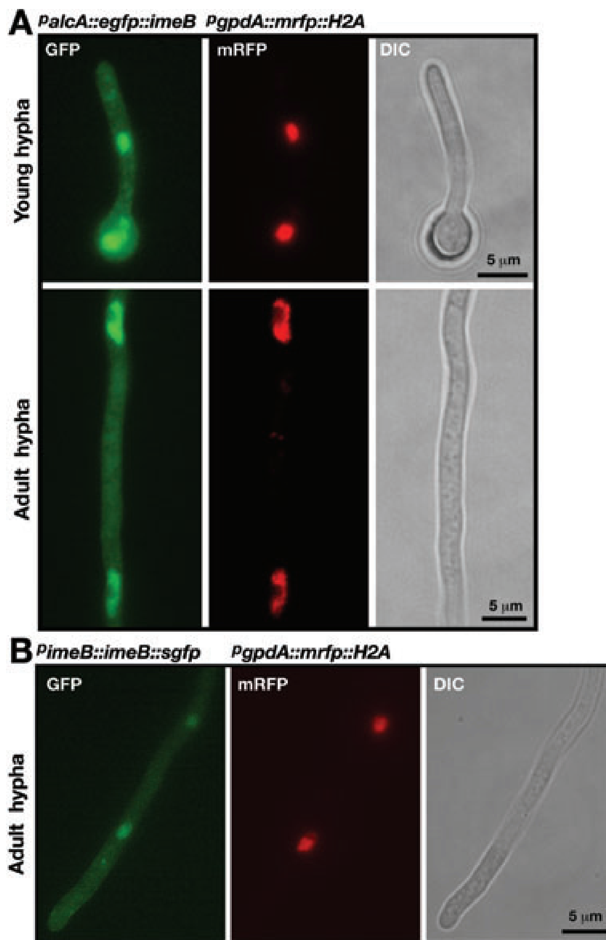

Fig. 2. Nuclear localization of e-GFP::ImeB and ImeB::sGFP fusion proteins.
A. An *A. nidulans* strain containing the fully functional *e-gfp*::*imeB* (AGB325; *imeB* N-terminally tagged) construct under control of the inducible *alcA* promoter was grown for 7 h (young hypha) or 24 h (adult hypha) in liquid media containing 100 mM threonine and 2% glycerol for induction of the constructs. An *mrfp*::*H2A* (histone H2A) construct was used for visualization of the nuclei.
B. A strain with a fully functional *imeB*::*sgfp* construct (AGB446, *imeB* C-terminally tagged) expressed from the native *imeB* promoter and *mrfp*::*H2A* was grown for 24 h. An adult hypha is shown.

imeB deletion strains evidently fail to respond to light and, as a consequence, produce abnormally many cleistothecia (Fig. 3B). However, when incubated in the dark without aeration, wild-type and mutant produced approximately the same number of cleistothecia, indicating that increased sexual fruiting body formation is specific to plates grown in light. Cleistothecia produced by the *imeB* deletion strain were fully fertile (data not shown). Crossing experiments showed that *imeB* deletion strain has no deficiency in mating (not shown) and heterokaryon formation (Fig. S2).

The slow growth phenotype and the abnormal cleistothecia production of the *imeB* mutant could be complemented by ectopic integration of a 5 kb genomic fragment of the *imeB* locus, which includes approximately 1.5 kb of both 5′ and 3′ flanking regions, into the *imeB* deletion strain (Fig. 3A and B). Our results suggest that ImeB is required for normal growth and for inhibition of sexual development when the fungus grows under conditions of illumination and aeration.

Deletion of imeB *results in abnormal formation of Hülle cells in submerged cultures*

Inspection of the *imeB* deletion strain also revealed a distinct phenotype in liquid medium. In contrast to wild-type cells, we observed the formation of Hülle cells in submerged cultures (Fig. 4A and B). Hülle cells, which are specific for sexual development and presumably nurse the maturating fruiting bodies, are normally not produced in submerged cultures. An unusual formation of these cells has previously been reported for strains overexpressing the developmental regulators *veA* (Kim *et al.*, 2002) or *nsdD* (Han *et al.*, 2001), and in *rosA* (Vienken *et al.*, 2005) or *cryA* deletion strains (Bayram *et al.*, 2008a).

Hülle cell formation of the *imeB* mutant was most pronounced after long incubation (48–72 h) in a submerged culture. However, prolonged incubation in liquid medium did not result in further development of cleistothecia. The phenotype of abnormal Hülle cell formation could be reversed with a 5 kb *imeB* genomic fragment ectopically integrated into the genome (Fig. 4A and B).

We next examined whether the Hülle cells produced in liquid cultures of *imeB* mutants are physiologically active, by testing whether they are capable of expressing Hülle cell-specific genes such as *mutA,* which encodes an α–1,3 glucanase. A *mutA*-driven *gfp* expression can be visualized in physiologically active Hülle cells (Wei *et al.*, 2001). Expression of *^p^mutA*::*sgfp* in the *imeB* deletion strain produced a green fluorescence signal specifically in a large fraction of Hülle cells, which indicates that these cells are in a physiologically active state (Fig. 4C). This abnormal Hülle cell formation in liquid medium underlines the model that ImeB acts as repressive regulator of sexual development.

Abnormal sexual development of imeB *mutants depends on VeA*

The *veA* gene is a key factor for sexual development (Kim *et al.*, 2002) and is a major constituent of the trimeric *velvet* complex required for co-ordination of the light-dependent development and secondary metabolism (Bayram *et al.*, 2008b). To gain insights into the interdependence of ImeB and VeA, we wanted to test whether sexual development of *imeB* mutants is dependent on

© 2009 The Authors

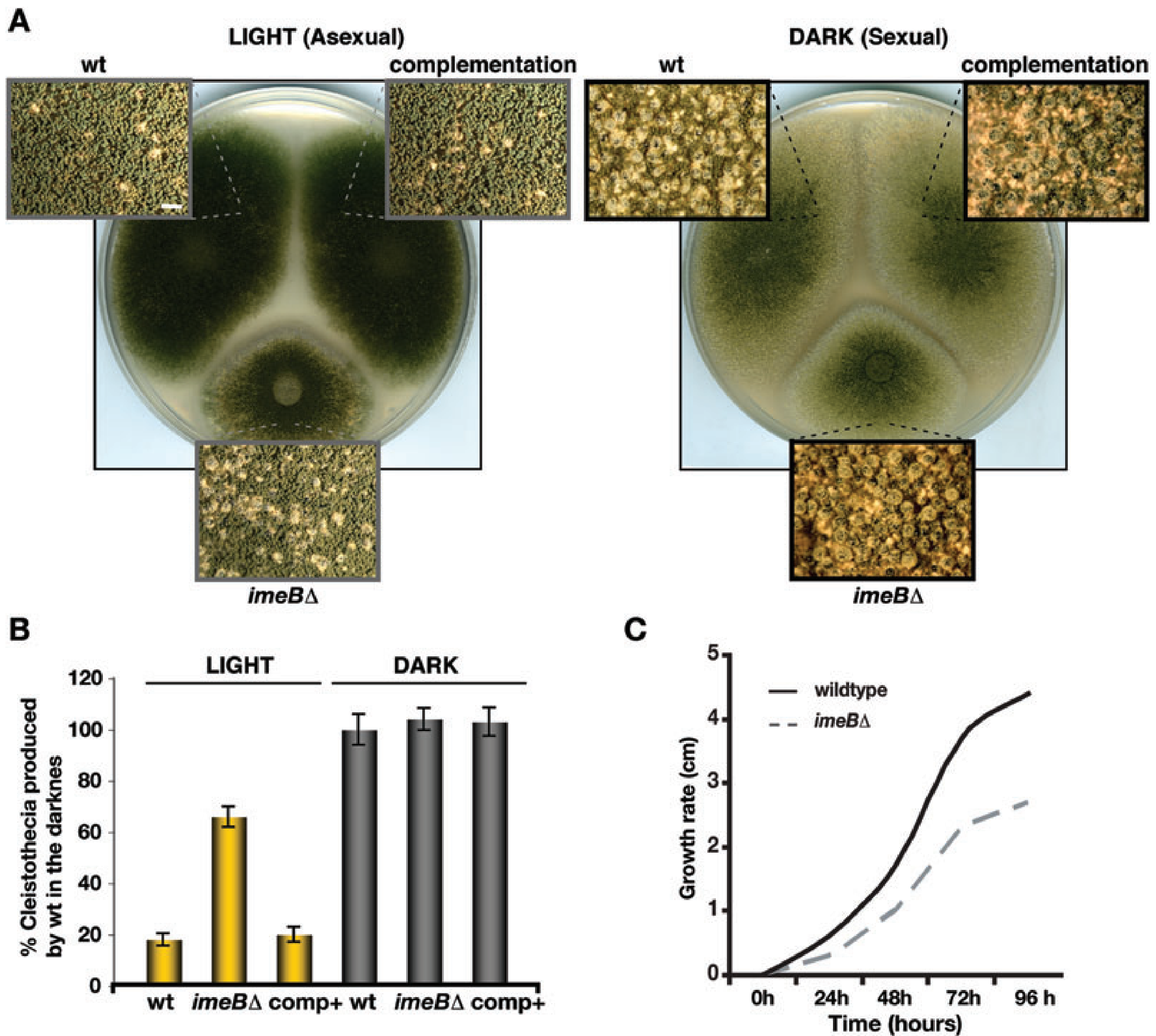

Fig. 3. Reduced growth and increased cleistothecia production of *imeB* deletion mutants under conditions of illumination and aeration.
A. A wild-type (FGSC A4), the *imeB*Δ (AGB322) and the complementation strain (ABG321) were point-inoculated (2×10^3 spores) on solid minimal media. Strains were grown at 37°C for 5 days in conditions promoting either asexual or sexual development. Enlargement of the colonies (boxed) shows conidiophores and cleistothecia. White horizontal bar represents 200 μm.
B. Quantification of the cleistothecia production from (A). Values are given in per cent to the number of cleistothecia produced by wild-type in dark (set as 100%; approximately 600 cleistothecia per 10 mm^2). Five independent 10 mm^2 squares were counted under a stereomicroscope. Vertical bars indicate standard deviations.
C. Comparison of growth of a wild-type (FGSCA4) and the *imeB*Δ strain on solid minimal media. Strains were point-inoculated (2×10^3 spores) and colony size was measured (in cm).

VeA function. Therefore, we aimed to delete the *imeB* gene in a *veA* deletion strain, but we did not receive a strain containing both deletions. We then used a laboratory strains that has a mutant allele of *veA*, designated as *veA1*. The *veA1* allele encodes a *velvet* protein, truncated of the first 37 amino acids in its N-terminus (Kim *et al*., 2002), and therefore, only a small number of cleistothecia are produced in the dark (Mooney *et al*., 1990).

We analysed the light-dependent development of *veA1 imeB* double mutants on agar plates (Fig. 5A). A strong increase of the number of sexual fruiting bodies in *imeB* mutants in the light was only seen in the wild-type (*veA*+) background, but not in the *veA1 imeB* strain (Fig. 5B). The findings that the *imeB* deletion caused an only modestly increased number of cleistothecia in a strain with reduced VeA function implies that VeA is needed for the abnormal sexual development of *imeB* strains.

© 2009 The Authors

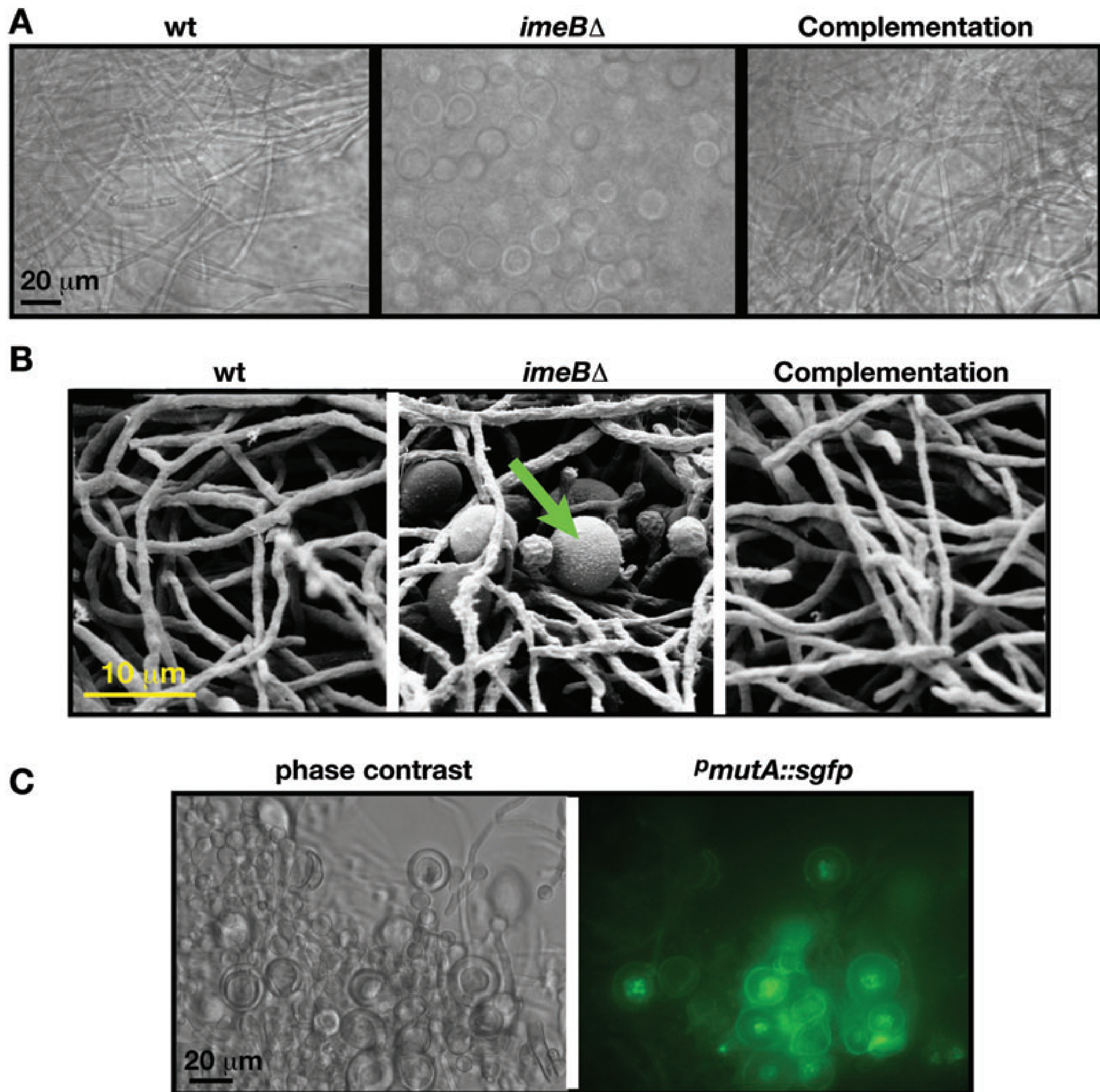

Fig. 4. Hülle cell formation of *imeB* deletion mutants in liquid culture.
A. Phenotype of the *imeB* deletion strain in submerged culture. A wild-type (FGSCA4), a *imeB*Δ mutant (AGB322) and a complementation strain (AGB321) were grown vegetatively at 37°C. Phase contrast pictures photographed after 48 h of vegetative growth.
B. Scanning electron microscopy of a wild-type, the *imeB*Δ mutant and the complementation strain, grown in liquid minimal media for 48 h. The arrow indicates a typical Hülle cell.
C. Phase contrast and fluorescence microscopy of an *imeB*Δ strain bearing the *PmutA*::*sgfp* construct (AGB326). For analysis of physiologically active Hülle cells, the strains were incubated in liquid minimal media for 48 h.

Cooperative repression of sexual development in the light by ImeB and the red light receptor phytochrome FphA

We next analysed the connections of ImeB with the red light receptor FphA (Fig. 5C). We therefore constructed an *fphA imeB* strain and compared this double mutant with single mutants with respect to light-mediated sexual development. Light-dependent cleistothecia formation was examined under white light (90 µWm^2), red light (30 µWm^2) and dark conditions. In white light, the *imeB* strain produced about 70% of cleistothecia compared with dark

© 2009 The Authors

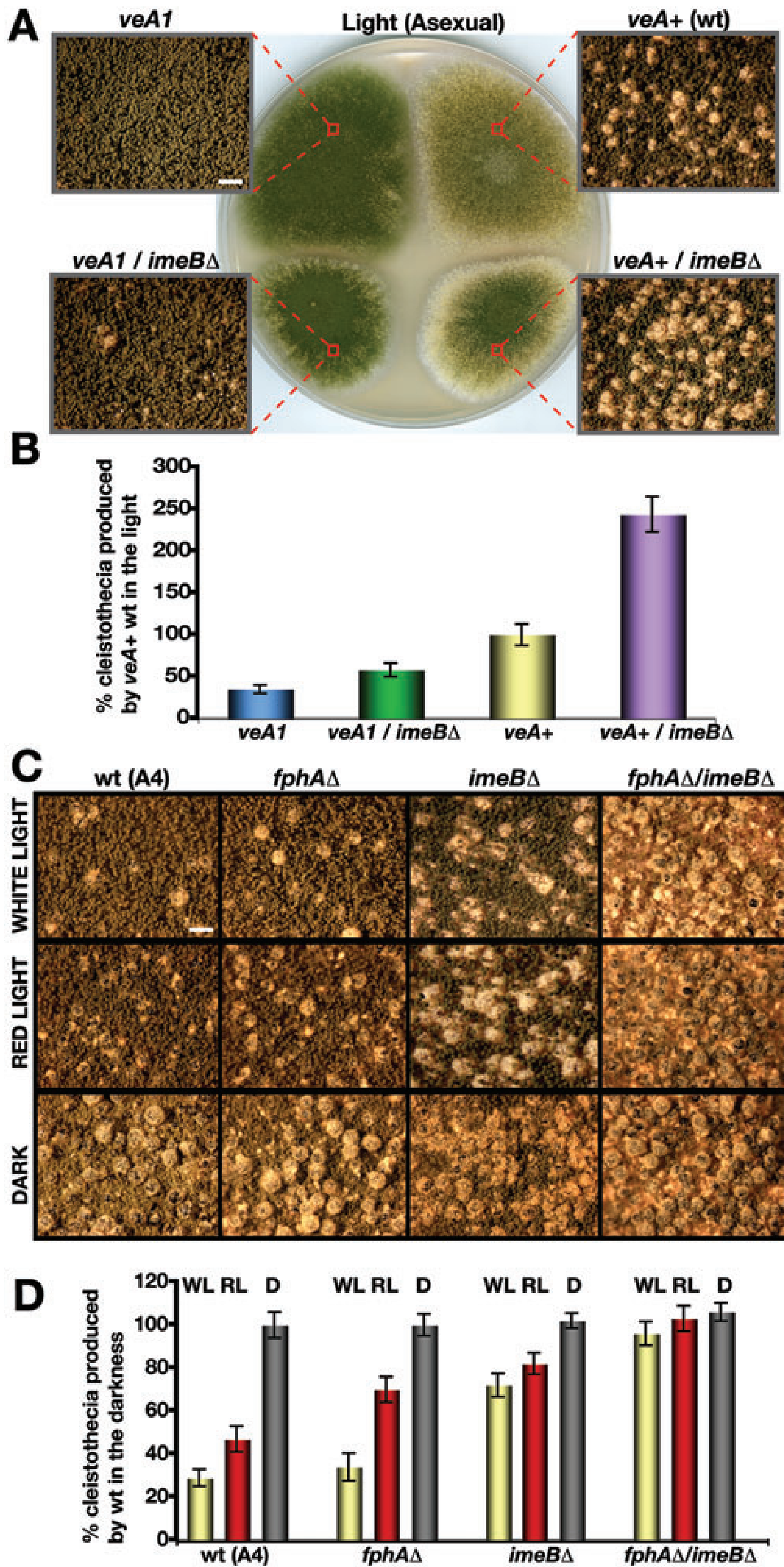

Fig. 5. Phenotype of an *imeB* deletion in the *veA1* and in the *fphA* strain backround.
A. Comparison of the cleistothecia production of *imeB*Δ strain in *veA*+ (wt) and *veA1* (N-terminal truncated *veA*) backgrounds on the plates under illumination conditions. Enlargement of the colonies (boxed) shows conidiophores and cleistothecia. White horizontal bar represents 200 μm.
B. Quantification of the cleistothecia production from (A). Values are given in per cent to the number of cleistothecia produced by wild-type (set as 100%). Five independent 10 mm^2 squares were counted under a stereomicroscope. Vertical bars indicate standard deviations.
C. A wild-type (FGSC A4), the *imeB*Δ (AGB322), *fphA*Δ (SAB2) and *fphA*Δ/*imeB*Δ (AGB449) double knockout strains were grown at 37°C for 5 days under white light, red light and darkness conditions.
D. Quantification of the cleistothecia production from (C) as described in (B).

© 2009 The Authors

(Fig. 5C and D), the *fphA* strain about 30%. When illuminated with red light, this number was slightly increased in *imeB* mutants, but more pronounced in *fphA* strain, as previously shown (Blumenstein *et al*., 2005). Remarkably, the *fphA imeB* double deletion strain was almost blind to any kind of light including the blue light spectrum (not shown). Under illumination conditions, this strain produces a similar number of cleistothecia as in darkness. These data demonstrate that *fphA imeB* strains fail to respond to light. This indicates that ImeB and FphA are apparently involved in different light response pathways, and that the absence of both of pathways has an additive effect and results in complete loss of light response.

ImeB is required for the expression of the strerigmatocystin gene cluster

Aspergillus nidulans produces several secondary metabolites including the aflatoxin precursor product ST (Keller *et al*., 2005). Light-dependent development and secondary metabolism processes are co-regulated by the trimeric VelB-VeA-LaeA complex (Bayram *et al*., 2008b). As *imeB* deletion strains are impaired in regulation of development, we tested whether *imeB* strains are also affected in the production of the mycotoxin ST. To this end, wild-type and *imeB* strains were analysed for their ST levels (Fig. 6A). Thin-layer chromatographic (TLC) analysis of the *imeB* mutants revealed that *imeB* strain did not produce any detectable ST neither in the light nor dark, while wild-type produced substantial level of ST (Fig. 6A). We next analysed whether the ST gene cluster and the *laeA* gene, encoding a putative methyltransferase needed for expression of the ST cluster, are expressed in *imeB* deletion strains. mRNA levels of *laeA* were similar in wild-type and *imeB* deletion strains (Fig. 6B). In contrast, expression level of *aflR*, encoding a transcription factor required for expression of ST biosynthesis genes (Fernandes *et al*., 1998), was reduced to 50% expression levels. We used the *stcU* gene of the ST cluster as an indicator for the expression of the ST gene cluster and found that *stcU* RNA was almost undetectable in the *imeB* deletion strain. Loss of *stcU* mRNA and reduction in *aflR* transcripts were reversed by introducing the *imeB* locus back to the mutant strain (comp+). These results demonstrate that ImeB is indispensable for expression of the ST gene cluster and for production of mycotoxin.

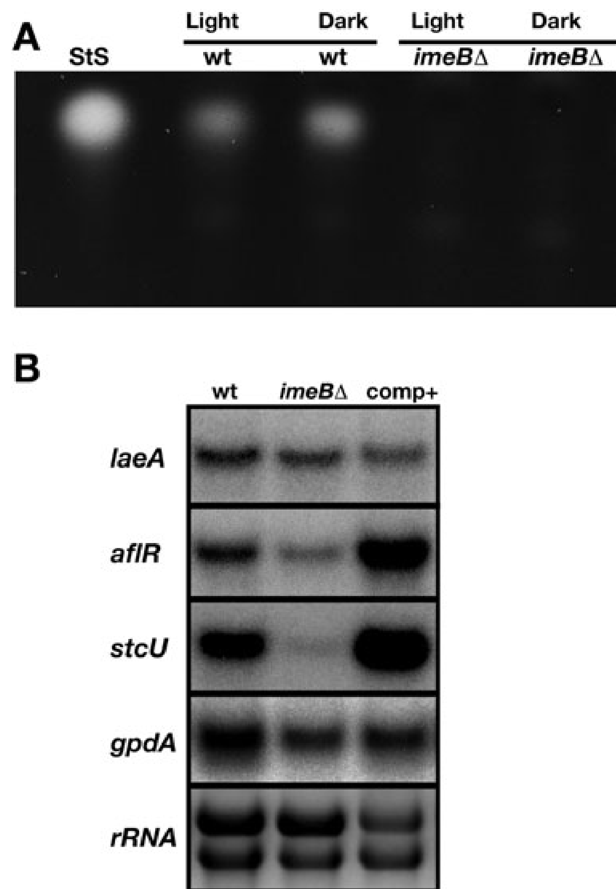

Fig. 6. Loss of ST production and drastic reduction of ST biosynthetic gene expression in *imeB* mutants.
A. TLC analysis of chloroform extracts of the wild-type (FGSCA4) and *imeB*Δ (AGB322) grown on plates both in light and dark at 37°C for 5 days. StS, ST standard.
B. Expression of the regulators of the ST gene cluster from 24 h asexually growing plates. The global regulator *laeA*, a transcription factor *aflR* and a structural gene *stcU* levels were monitored in wt, *imeB*Δ (AGB322) and complementation (AGB321) strains. *gpdA* gene expression and ethidium bromide stained rRNA served as loading control.

mRNA levels of sexual development regulators are increased in the imeB *deletion strain*

The impact of ImeB on expression of the ST gene cluster prompted us to test whether ImeB may also affect the expression of other genes, particularly regulators of sexual development. A wild-type strain, the *imeB* deletion strain and the complementation strain (comp+) were grown vegetatively in submerged cultures for 48 h, as well as on plates in the presence of light (24 h) and on plates in the absence of light (48 h). RNAs were isolated and hybridized with DNA probes from the *veA*, *velB*, *stuA* and *nsdD* genes (Fig. 7). *veA* (Kim *et al*., 2002) and *velB* (Bayram *et al*., 2008b) are components of the *velvet* complex, whereas *stuA and nsdD* encode transcription factors required for early sexual development (Miller *et al*., 1992; Dutton *et al*., 1997; Han *et al*., 2001). Signal intensities were quantified relative to the constitutively expressed *gpdA* mRNA. In liquid medium, transcript levels of *veA*, *velB* and *stuA* were increased by 30–100% in *imeB* mutants compared with the wild-type and the complementation strain (Fig. 7A). *nsdD* mRNA was not significantly affected. On plates in the light, the relative

© 2009 The Authors

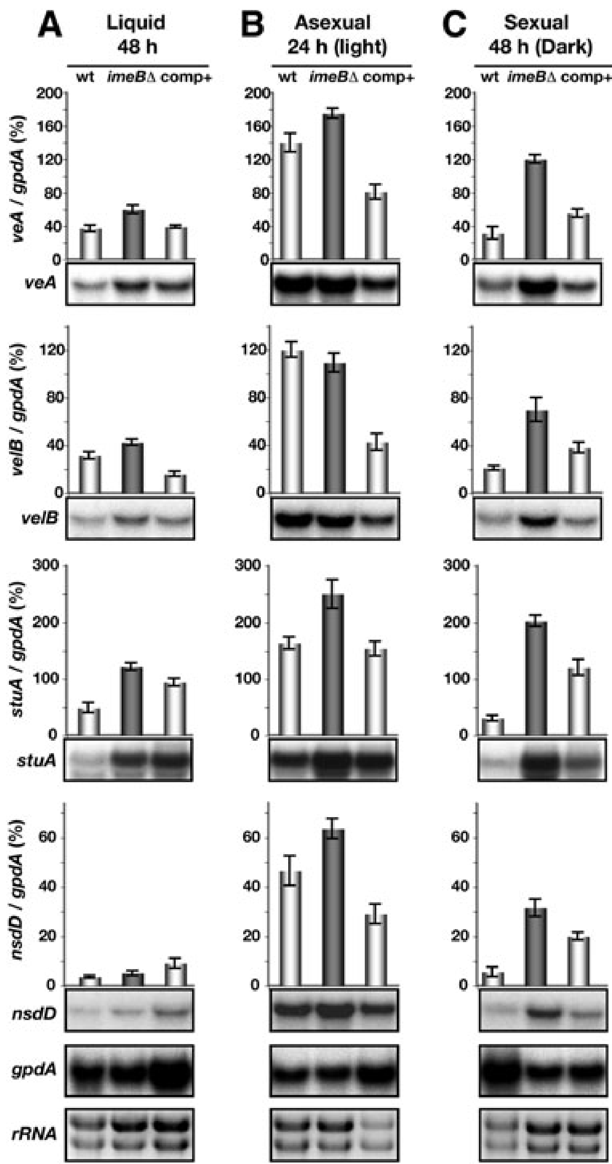

Fig. 7. Increased expression of several developmental regulators in *imeB* deletion mutants. A wild-type (FGSCA4), the *imeB*Δ (AGB322) and the complementation strain (ABG321) were grown in submerged cultures vegetatively for 48 h (A), on plates asexually (in the light) for 24 h (B) and on plates sexually (in the dark) for 48 h (C). Total RNA was isolated and expression levels of genes encoding components of the *velvet* complex (*veA*, *velB*) and of genes encoding the developmental regulatory proteins *stuA* and *nsdD* were quantified by using internal *gpdA* mRNA levels as reference. Ethidium bromide-stained rRNA was used as an additional loading control. Relative expression levels were quantified by using AIDA Bio-Package (Raytest Germany).

amounts of each of the mRNAs were rather variable, but appear to be moderately elevated in *imeB* mutants (Fig. 7B). In contrast, a two- to threefold increase of transcript levels was detectable on plates cultivated in the dark (Fig. 7C), indicating that the most pronounced effect of ImeB on transcript levels occurs during sexual development. We also hybridized the same membrane with a probe for the *rosA* gene, encoding a repressor of sexual development (Vienken *et al*., 2005) and found that mRNA levels were similar in wild-type and *imeB* mutant strains (data not shown).

We conclude that ImeB mediates at least partial down-regulation of the expression of several important positive regulators of sexual development.

Overexpression of imeB *results in efficient sexual differentiation independently of light and repression of asexual development*

We next examined the phenotypic changes when ImeB is present at increased levels in *A. nidulans*. The inducible *niiA* promoter has been applied previously to analyse regulators of sexual development (Han *et al*., 2001; Kim *et al*., 2002; Todd *et al*., 2006). For this purpose, we over-expressed the *imeB* gene by constructing a plasmid, in which the endogeneous promoter was replaced by the *niiA* promoter (Muro-Pastor *et al*., 1999). The *niiA* promoter is induced by nitrate and repressed by ammonium. The *^{p}niiA*::*imeB* construct was introduced ectopically into the *A. nidulans* wild-type strain AGB152 as a single copy.

A wild-type strain, the *^{p}niiA*::*imeB* strain and a control strain carrying only *^{p}niiA* were inoculated to medium containing either nitrate or ammonium. Growth on nitrate plates caused the formation of enormous numbers of sexual structures in the *^{p}niiA*::*imeB* strain, irrespective of the presence or absence of light (Fig. 8A). Almost no conidiophores were produced in this overexpression strain (see also Fig. S3). Quantifications of *imeB* mRNA levels showed that expression from the nitrate promoter leads to a 50-fold increase in mRNA compared with internal *imeB*, whereas the levels of the control *gpdA* mRNA remained constant (Fig. 8B). When grown on plates containing ammonium, level of *imeB* RNA was still 13-fold higher than endogenous level. This *imeB* overexpression did not have distinct effects on development (Fig. 8A).

The findings that a strong overexpression of *imeB* leads to efficient cleisthothecia formation irrespective of light conditions indicate that ImeB has also a function in promoting sexual fruiting bodies.

The sequence motif TTY is essential for the function of ImeB

ImeB contains the sequence motif TTY corresponding to the characteristic TXY motif embedded in the activation loop of MAP kinases. A dual phosphorylation on tyrosine and threonine is a prerequisite for kinase activation (Payne *et al*., 1991). To address the question whether the TTY sequence element is crucial for activity of ImeB, we replaced separately single amino acids of the TTY motif

© 2009 The Authors

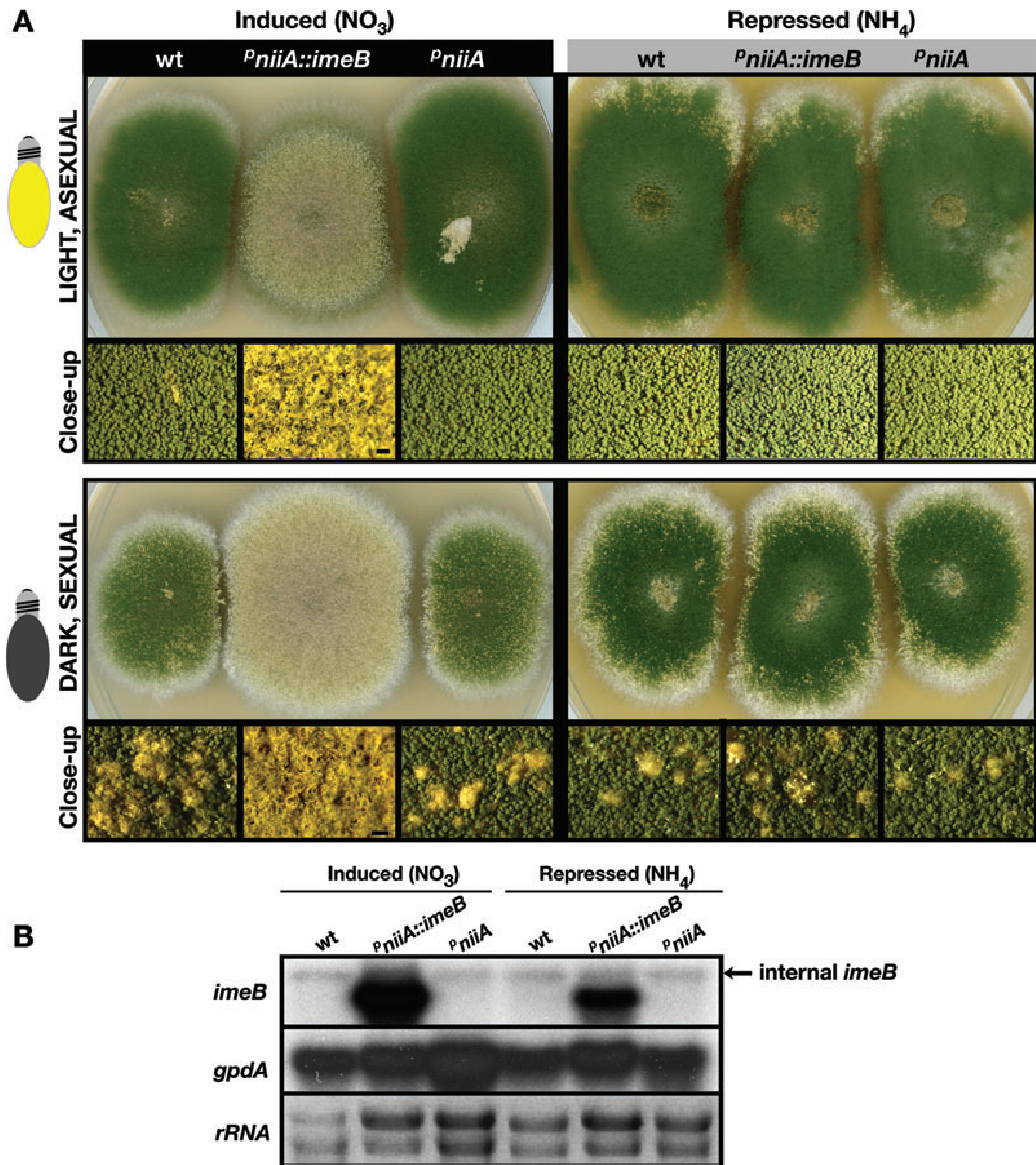

Fig. 8. Phenotypes of *imeB* overexpression.
A. A wild-type strain (AGB152), an overexpression strain (AGB447, *PniiA*::*imeB*) and a control strain carrying a *niiA* plasmid without *imeB* (AGB448) were grown at 37°C for 5 days in conditions promoting either asexual (upper panel) or sexual development (lower panel). Strains were point-inoculated (2×10^3 spores) on solid minimal media containing 10 mM Sodiumnitrate and 10 mM Ammonium-Tartrate as an inducing and a repressing nitrogen source respectively.
B. Northern analysis of the inducible expression of the *imeB* gene under the nitrate promoter. Arrow indicates the endogenous levels of *imeB* RNA. *gpdA* expression and rRNA were used as control.

© 2009 The Authors

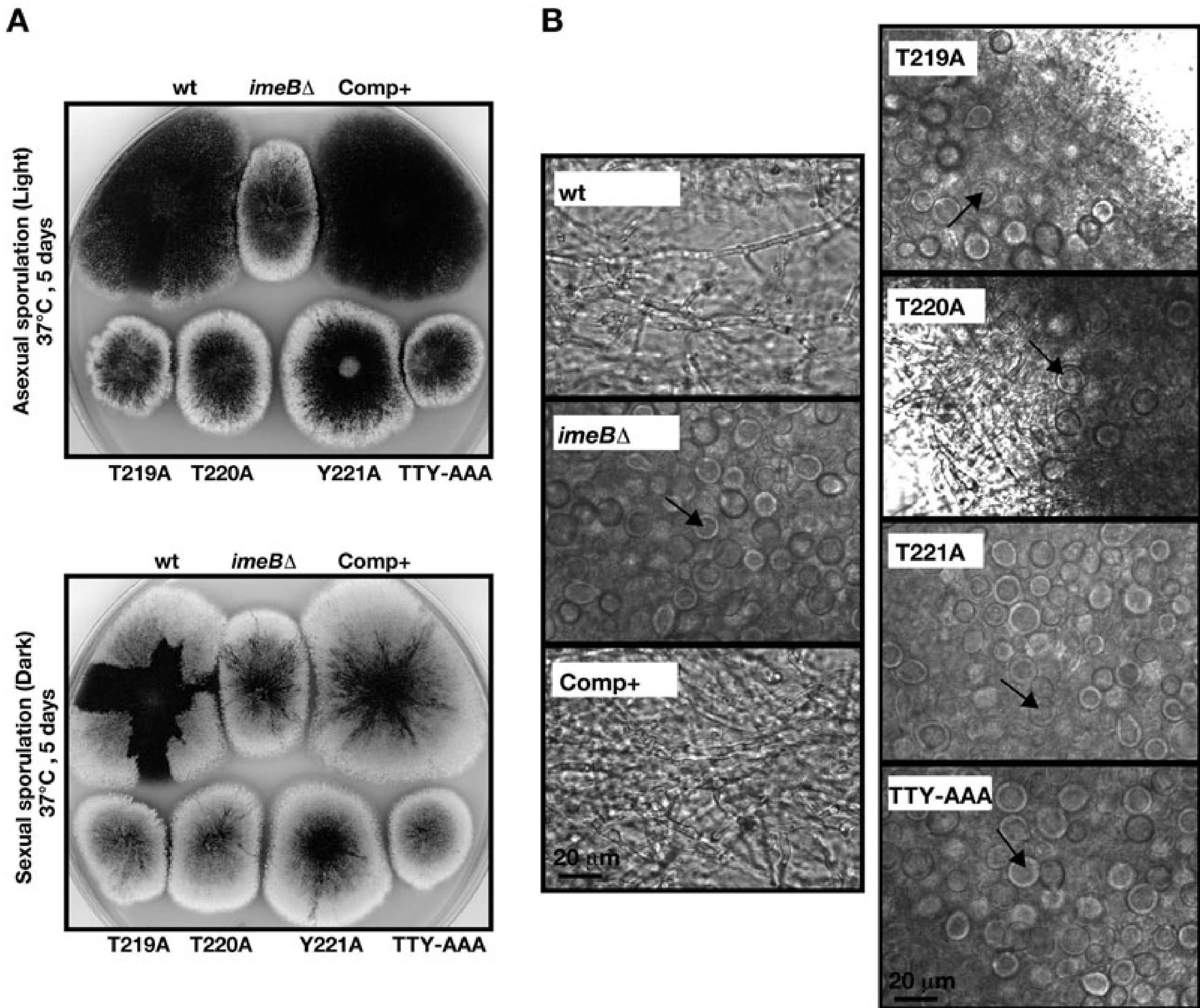

Fig. 9. Mutational analysis of the TTY motif of ImeB.
A. A wild-type strain (A4), *imeB*Δ mutant (AGB322) and the complementation strain (AGB321) carrying a wild-type *imeB* gene were point-inoculated on solid minimal medium. Additionally, strains, where single amino acids of the TTY motif were altered to alanine, $ImeB^{T219A}$ (AGB327), $ImeB^{T220A}$ (AGB328) and $ImeB^{Y221A}$ (AGB329), or where the whole TTY motif was replaced with three alanine residues $ImeB^{TTY \rightarrow AAA}$ (AGB330) were point-inoculated on the same agar plate. Strains on agar plates were propagated either asexually or sexually.
B. Strains used in (A) were cultured in liquid minimal media for 48 h. Arrows indicate Hülle cells.

with alanine, generating constructs T219A, T220A and Y221A. These mutant *imeB* versions were transformed into the *imeB* deletion strain similarly as the wild-type *imeB* gene (AGB321, complementation strain). Integration of each construct into the genome was verified by PCR amplification and sequencing.

We found that single substitutions of each amino acid of the TTY motif resulted in *imeB* versions that failed to complement the phenotype of an *imeB* deletion strain. Transformants carrying mutated *imeB* versions displayed a reduced growth like the *imeB* deletion strain (Fig. 9A) and efficiently produced Hülle cells in liquid medium (Fig. 9B). A similar effect was also observed when the whole TTY motif was replaced to AAA. A complementation of the *imeB* phenotype was only achieved when the *imeB* gene without any mutations of the TTY motif was transformed.

These data show that the TTY motif is indispensable for ImeB activity and suggest an eminent role of every single amino acid of the TTY motif, unexpectedly also for the usually variable middle amino acid of the motif, threonine-220.

Discussion

imeB is the *A. nidulans* gene showing highest homology to the yeast *IME2* gene, which encodes a meiosis-specific

© 2009 The Authors

protein kinase essential for multiple steps in the sporulation programme of yeast (Honigberg, 2004). Ime2-related proteins were identified in other fungi and in mammals. The similarity is restricted to the N-terminal parts of proteins comprising the catalytic kinase domain. In contrast, C-terminal parts show no distinct sequence conservation. In yeast, the C-terminal region apparently has a regulatory function, because a deletion of this region resulted in a hyperactive and stabilized Ime2 protein kinase (Kominami *et al*., 1993; Sari *et al*., 2008).

Our results presented here imply that ImeB is involved in the inhibition of sexual development in *A. nidulans*. Sexual development in wild-type cells mostly occurs in the dark under low-oxygen conditions. Strains lacking the *imeB* gene produce sexual Hülle cells in liquid medium and, when grown on solid media in the light, these mutant strains produce abnormally many cleistothecia, the sexual fruiting bodies containing ascospores. The number of cleistothecia of *imeB* mutants produced under conditions of illumination and aeration was nearly as high (~70%) as in the dark. Cleistothecia production also took place when plates were grown either in red or blue light (data not shown), suggesting that *imeB* mutants are impaired in response to any kind of light. Thus, our findings provide evidence that ImeB is involved in a light response pathway.

It is remarkable that not only the *imeB* deletion, but also a strong overexpression of *imeB* results in the production of high amounts of cleistothecia and a reduction of conidiospores, both in light and in dark (Fig. 8). Although this phenotype requires abnormally high ImeB levels, these data indicate that ImeB may also have a role in promoting sexual fruiting body formation, presumably at late stages. Consistent with this model, *imeB* mRNA is induced late during sexual development (Fig. 1D).

Characterization of the *imeB* deletion strain, however, implies that the primary function of ImeB is the inhibition of sexual development in the presence of light. Red light, sensed by the photoreceptor FphA (Blumenstein *et al*., 2005), induces conidiation and represses sexual development. Like in *imeB* mutants, a deletion of *fphA* caused the fungus to produce an increased number of cleistothecia in the light. To get insights into a possible connection between ImeB and FphA, we analysed development of double deletion strains. Intriguingly, combined inactivation of the *imeB* and *fphA* genes results in a significant additive phenotype. The double deletion failed to respond to any kind of light and produced large amounts of cleistothecia, irrespective of the light conditions. From these data, we propose that ImeB and FphA function in different light response pathways and cooperate to inhibit sexual development. Possibly, ImeB has a major function in response to blue light.

A light-independent induction of the sexual cycle was previously also shown for strains containing deletions of *csn* genes (Busch *et al*., 2003; Busch and Braus, 2007), encoding various subunits of the COP9 signalosome (Schwechheimer, 2004; Busch *et al*., 2007). However, in contrast to *imeB* and *fphA* mutants, the *csn* strains were blocked in development at the primordial state and therefore unable to produce mature cleistothecia.

Previous findings have revealed that VeA is a further pivotal factor involved in light-mediated regulation of development (Mooney and Yager, 1990; Kim *et al*., 2002). A *veA* deletion strain fails to produce any fruiting bodies, whereas *veA* overexpression leads to differentiation of Hülle cells and even cleistothecia in liquid cultures (Kim *et al*., 2002). VeA acts as a positive regulator of sexual development. The observation that intracellular localization of VeA is light-dependent, preferentially cytoplasmic in the light and nuclear in the dark (Stinnett *et al*., 2007), provides compelling evidence that regulated transport of VeA is important for the light response pathway. In the nucleus, VeA forms a trimeric complex with VelB and LaeA, a regulator of secondary metabolism, and co-ordinates light-dependent development with secondary metabolism (Bayram *et al*., 2008b). VeA interacts also with the red light sensor FphA and the two putative blue light photoreceptors LreA and LreB (Purschwitz *et al*., 2008).

Nuclear localization of neither VeA nor VelB is affected in *imeB* deletion strains (data not shown). To analyse possible genetic interactions between *veA* and *imeB*, we attempted to get a double mutant. However, no progeny with both mutations was obtained, possibly because the combination of mutations results in a synthetic lethal phenotype. However, when the *imeB* deletion was introduced into a *veA1* strain background, expressing a truncated *veA* protein (Kim *et al*., 2002), only a small number of cleistothecia were produced, suggesting that fruiting body formation in the *imeB* mutant is dependent on the function of VeA.

The characterization of the *velvet* protein complex clearly demonstrated that regulation of development and production of secondary metabolites are co-ordinated events (Bayram *et al*., 2008b). Deletion of either *veA* and *velB* results not only in an impaired sexual development, but also abolishes production of the mycotoxin ST. In contrast, deletion of *laeA*, the third *velvet* component, resulted in strains impaired in ST production, while development was not affected (Bok and Keller, 2004; Keller *et al*., 2006). LaeA encodes a putative methyltransferase and is directly involved in epigenetic regulation of the large ST gene cluster.

Our findings showing that *imeB* mutants are also unable to produce significant levels of ST provide compelling evidence that ImeB is a further important factor required for controlling or modulating the co-ordination of development and mycotoxin production. While expression

© 2009 The Authors

of *laeA* is not affected by an *imeB* deletion, we found that mRNAs of *stcU*, a gene of the ST gene cluster, is reduced to marginal levels. Expression of the transcriptional activator of the ST gene cluster, *aflR*, is reduced to about 50% and thereby less affected than the *stcU* gene.

Up to present, it is unknown how ImeB may regulate transcription of the ST gene cluster. Further experiments will be required to test whether LaeA and/or AflR may be direct targets of this protein kinase and how such post-translational modifications may affect the activity of these regulators of ST expression.

RosA is a further factor previously identified as an inhibitor of sexual development. When *rosA* is absent, Hülle cell formation was observed in liquid medium, as well as an increase in cleistothecia number under conditions of low glucose levels and darkness (Vienken *et al*., 2005). Fruiting bodies were not produced in the light, indicating that RosA is not involved in light response, but may rather sense the availability of nutrients. There is no evidence that ImeB is also involved in sensing glucose, because the number of cleistothecia is not increased in *imeB* mutants when glucose levels were decreased (data not shown). We constructed *imeB*Δ *rosA*Δ double knock-out strains, which do not show any additive phenotype in liquid culture, but an increase in the number of sexual structures on the plates (Fig. S4). ImeB and RosA seem to transmit different signals, resulting in a similar response, the repression of sexual development. Such a response may include the transcriptional downregulation of genes required for sexual development. Consistent with this model, positive regulators of development were found to be elevated in both *imeB* (Fig. 7) and *rosA* mutants (Vienken *et al*., 2005). The effects are more distinct in the *rosA* mutants, possibly because RosA is a transcription factor directly regulating transcription of these genes.

ImeB belongs to a family of non-classical MAP kinases, with representatives found from yeast to mammals (Fig. 1). The TXY motif is conserved in all members and its relevance for a dual phosphorylation was demonstrated for mammalian ICK (Fu *et al*., 2006) and Crk1 of *U. maydis* (Garrido *et al*., 2004). In this dimorphic plant pathogenic fungus, Crk1 is important for sexual development, particularly because it is required for cell fusion during the mating process. This kinase has also been implicated in pathogenicity, because mating and pathogenicity are closely linked in this fungus. Furthermore, *crk1* deletion mutants display a reduction in filamentation. Crk1 activity requires a dual phosphorylation of the conserved TXY motif and this modification was shown to be dependent on the MAP kinase kinase Fuz7 (Garrido *et al*., 2004).

We showed that the TXY motif of ImeB, the sequence element TTY, is essential for function of this protein. Any single substitution obviously resulted in inactive ImeB versions. TTY is unusual among the motifs found in the family of MAP kinases and may, in principle, enable a triple phosphorylation. Intriguingly, replacement of the middle threonine residue with the structurally similar alanine residue results in an inactive protein kinase. Further investigations will be required to demonstrate phosphorylation of this motif and to identify the responsible protein kinase.

Characterization of yeast Ime2, *A. nidulans* ImeB and *U. maydis* Crk1 demonstrated that all proteins have important functions in the regulation of sexual development. However, it is noticeable that these functions appear to be clearly different. While Crk1 is mainly needed for the mating process, Ime2 activity promotes progression through the meiotic cell division leading to haploid spores. In contrast, *imeB* mutants displayed no discernible defects in mating, heterokaryon formation, meiosis or spore formation, but fail to repress sexual development. The functional diversity of these kinases may explain why an *imeB* cDNA expressed in yeast fails to complement the meiosis defect of a yeast *ime2* mutant (data not shown). Cross-species function is possibly impaired, because Ime2 contains the sequence TAY as a MAPK consensus motif (Fig. 1A). Indeed, a substitution of TTY with TAY results in a non-functional ImeB protein (Fig. 9).

It is remarkable that within the phylum of the ascomycota, the related kinases Ime2 and ImeB have obviously acquired opposing functions with respect to sexual development. What may be the reason for these differences in functions of related kinases? In this regard, it should be mentioned that construction of phylogenetic trees of protein kinases revealed that the Ime2 family appeared early in evolution, before cyclin-dependent kinases (Krylov *et al*., 2003). It was suggested by the authors that Ime2 could have been a regulator of meiosis before the appearance of cyclin-dependent kinases. During evolution, yeast Ime2 may have retained a main function as auxiliary kinase for cyclin-dependent kinases regulating meiosis. In yeast cells, there is no need for a kinase to balance different developmental programmes or for light response. In contrast, adjusting development to different environmental conditions is of fundamental importance for *A. nidulans*. In this organism, ImeB could have lost its importance for meiosis during evolution, but instead could have acquired a function as developmental regulator. Thus, Ime2 and ImeB may represent an example for related proteins, which have evolved in a divergent manner, fitting to the demands of the respective genus.

Experimental procedures

Strains and culture conditions

Aspergillus nidulans strains used in this study are listed in Table 1. The FGSCA4 and TNO2A3 strains served as wild-

© 2009 The Authors

Table 1. Strains used in this study.

Strain	Genotype	Source
FGSC A4	Glasgow wild-type	FGSC
TNO2A3	*nkuA∆, pyroA4, pyrG89, veA1*	Nayak *et al.* (2006)
AGB152	*pyroA4, pyrG89, veA+*	Busch *et al.* (2003)
AGB165	*pabaA1, pyrG89, yA2*	This study
AGB320	*imeB∆::ptrA*	This study
AGB321	*imeB∆::ptrA* transformed with pME3293	This study
AGB322	AGB320 backcrossed with FGSC A4	This study
AGB325	AGB152 transformed with pME3295: *^{p}alcA::egfp::imeB, pyrG, pyroA4*	This study
AGB326	AGB320 transformed with pME3296: *imeB∆::ptrA,^{p}gpdA::natR-^{p}mutA::sgfp*	This study
ABG327	AGB320 transformed with pME3297 (T219-ALA): *imeB1*	This study
AGB328	AGB320 transformed with pME3298 (T220-ALA): *imeB2*	This study
AGB329	AGB320 transformed with pME3299 (Y221-ALA): *imeB3*	This study
AGB330	AGB320 transformed with pME3300 (TTY-ALA): *imeB4*	This study
AGB445	TNO2A3 containing *^{p}gpdA::intron::mrfp::h2A, pyrG*	This study
AGB446	AGB 445 transformed with *^{p}imeB::imeB::sgfp::^{p}gpdA::natR::imeB^t* fusion construct	This study
AGB447	AGB152 transformed with pME3191: *^{p}niiA::imeB::niiA^t, pyrG, pyroA4*	This study
AGB448	AGB152 transformed with pME3160 (empty *niiA* plasmid)	This study
AGB449	SAB2 originated double knockout, *fphA∆::argB, imeB∆::ptrA*	This study
AGB450	*pyrG89; pyroA4; rosA∆::argB, imeB∆::ptrA*	This study
AGB452	*nkuA∆, pyroA4, pyrG89, veA1, imeB∆::ptrA*	This study
SAB2	*fphA∆::argB*	Blumenstein *et al.* (2005)
SKV8	*pyrG89; pyroA4; rosA∆::argB*	Vienken *et al.* (2005)

type for the *imeB* deletion. AGB152 and AGB445 were used for overexpression and localization of GFP fusion proteins. Plasmids were reproduced in *Escherichia coli* DH5α and MACH-1 (INVITROGEN). *E. coli* strains were grown as described elsewhere (Bayram *et al.*, 2008b). *A. nidulans* growth media were used as given in detail (Bayram *et al.*, 2008b). Only additional selection agent phleomycin (InvivoGen; 30 μg ml^{-1}) was used in this study. 10 mM ammonium or nitrate was used as nitrogen source in inducing medium. For asexual development of *A. nidulans* strains, plates were grown in the light (90 μWm^2). Sexual development of *A. nidulans* strains was induced by wrapping plates with parafilm, covering them with aluminium foil, and incubation in complete dark. For development-specific RNA isolation, *A. nidulans* liquid cultures were pre-grown for 20 h. Subsequently mycelia were harvested with miracloth (Calbiochem) and transferred to solid media. Transferred plates were induced either for asexual or sexual development.

DNA and RNA manipulations

Escherichia coli strains were chemically transformed according to Mandel and Higa (1970). For *A. nidulans,* protoplast-mediated transformation was conducted (Punt and van den Hondel, 1992). For PCR experiments, standard protocols (Saiki *et al.*, 1985) were applied using the MWG-Biotech primus96 cycler. *Taq*, *Pfu* (MBI Fermentas), *KOD* (Novagen), Platinum-*Taq* (Invitrogen) or *Phusion* (Finnzymes) were used as thermostable polymerases. Sequencing was conducted in the Göttingen Genomics Laboratory. Genomic DNA and RNA isolations, probe labelling and hybridization experiments were carried out as described formerly (Bayram *et al.*, 2008a,b). Sequences were analysed and aligned with the Lasergene software *DNAstar*.

Plasmid constructions

All plasmids used in this work are summarized in Table S1. Oligonucleotides are listed in Table S2. Plasmids employed for *A. nidulans* manipulations were constructed as follows:

Deletion of imeB *and complementation.* The wild-type *imeB* locus was deleted by homologous gene replacement. The deletion construct pME3292 was generated by stepwise cloning of the fragments *imeB* promoter (5 UTR; OZG38/F39), *ptrA*-cassette (from pTRII Takara; OZG40/OZG41) and *imeB* terminator (3 UTR; F42B/F43B) into the vector pBluescript KS (Invitrogen). The deletion cassette was excised from pME3292 with KpnI and transformed into the FGSCA4 strain. A Southern analysis was applied to verify the disruption by using a DNA probe that had been amplified with the primers Fat15/Fat16 and that hybdridized to the 5′ untranslated region of *imeB*. For complementation, a 5.2 kb fragment comprising the *imeB* promoter (1.6 kb), *imeB* open reading frame and *imeB* terminator (1.3 kb) was amplified (Fat17b/Fat18b) with Platinum *Taq* DNA polymerase (Invitrogen). The PCR product was digested with EcoRI and cloned into the EcoRI-linearized pAN8-1 (Mattern *et al.*, 1988) vector ($phleo^R$). The created *imeB* complementation construct, pME3293, was transformed into the *imeB* mutant strain yielding AGB321.

Overexpression of imeB. For overexpression under *niiA* promoter, the *imeB* open reading frame was amplified (Fat19/Fat20) with *Phusion* polymerase from a cDNA library (Krappmann *et al.*, 2006) and inserted into the PmeI site of the vector pME3160 (Bayram *et al.*, 2008b). The *niiA* overexpression vector contains the auxotrophy marker *pyrG* for selection and the *A. nidulans* nitrate source inducible *niiA*

© 2009 The Authors

promoter. The *niiA-imeB* construct, pME3191, was transformed into the *pyrG* mutant strain AGB152 yielding AGB447. As a control, pME3160 was also transformed into AGB152 strain to create AGB448.

N-terminal tagging of imeB *with e-gfp expressed from the inducible alcA promoter.* The *imeB* open reading frame was amplified from an *A. nidulans* cDNA library. The primers F21gfp containing an AscI site at the 5′ end and F22gfp having a PacI site at the 3′ end were used for amplification. N-terminal tagging was achieved by digesting the plasmid pCMB17apx with AscI-PacI, thus opening the vector behind the *alcA* promoter. Subsequent cloning of the AscI-PacI-digested PCR product into the opened vector generated the plasmid pME3295. The *imeB*-e*gfp* construct was transformed into the *pyrG* mutant strain AGB152 yielding the strain AGB325.

Chromosomal C-terminal tagging of imeB *with sgfp (under native promoter).* The C-terminal region of *imeB* gene including the second intron was amplified with *imeB*GFP1/3, and 3 UTR region of *imeB* was amplified with primers *imeB*GFP4/6. Then, these two fragment as well as *sgfp*::*natR* cassette (PCR amplicon) were fused by fusion PCR (*imeB*GFP2/5). Final amplicon was used for transformation into AGB445, which later created AGB446. Homologous recombination event was confirmed by Southern blot (Fig. S1C and D).

Generation of ^{p}mutA::sgfp*.* The plasmid pME3296, carrying the GFP-tagged mutanaseA promoter, was created by blunt ending the vector Mut(p)-GFP (Vienken *et al*., 2005) with *Ecl*136II and subsequent ligation of a ^{p}*gpdA-natR* module. The ^{p}*gpdA-natR* module was amplified by using Fat42gpdA/Fat43gpdA primers with KOD polymerase to produce blunt-ended PCR products. The plasmid pME3296 was transformed into the *imeB* mutant strain ABG320. The *imeB* mutant strain bearing the ^{p}*mutA*::*sgfp* construct was named AGB326.

Mutations of the TTY motif. A two-step fusion PCR was applied to generate mutations in the TTY motif using the high-fidelity *Phusion* polymerase (Finnzymes). Tyrosin-219 was exchanged by alanine using the primers F17b/OZGImeB (T1-ALA) for the first fragment and OZGImeB (TTYterm)/F18b for the second fragment that were fused in a PCR reaction. The resulting product was ligated in an EcoRI-digested pAN8-1 vector (pME3297). The same procedure was conducted to get constructs with substitutions of tyrosine-220 [pME3298; used primers: F17b, OZGImeB (T2-ALA), OZGImeB (TTYterm) and F18b] and of threonine-221 [pME3299; used primers: F17b, OZGImeB (Y-ALA), OZGImeB (TTYterm) and F18b] with alanine, as well as substitution of the whole TTY motif [pME3300; used primers: F17b, OZGImeB (TTY-ALA), OZGImeB (TTYterm) and F18b] with three alanine residues. All constructs were transformed into the *imeB* mutant strain AGB320. Transformants were selected on phleomycin plates. Strains were additionally sequenced for the presence of the corresponding point mutations.

Light sources and irradiation measurements

Photobiological experiments were performed as previously described (Bayram *et al*., 2008a).

Microscopy

Light or fluorescence microscopy experiments were performed as given previously (Bayram *et al*., 2008b).

Mycotoxin and TLC analysis

Samples (1.5 cm diameter disc with fungal samples and agar together) were collected after asexual and sexual developmental induction. The fungal samples were cut into tiny pieces and shaken in 3 ml ddH2O with glass beads (450 r.p.m.) at 4°C for 30 min, and then 3 ml chloroform was added to extract ST from the aqueous phase. Chloroform containing mixture was further shaken (450 r.p.m.) at 4°C for 30 min and centrifuged at 1000 r.p.m. at 4°C for 10 min. The 2 ml chloroform containing ST was collected after centrifugation, and dried in vacuum (Rotavapor, Bühl, Germany). The dried extracts were resuspended in 50 μl of chloroform or methanol, and 10 μl was separated in hexane : ethyl acetate (4:1) or chloroform : acetone (4:1) on TLC plates (Macherey and Nagel, Germany). ST was visualized after spraying the TLC plates with a 20% $AlCl_3$ solution in ethanol (95% v/v) and drying at 75°C for 5–10 min. The plates were photographed under UVA-light (366 nm) and images were analysed by winCATS ver.1.4.4 software (Camag, Switzerland).

Acknowledgements

We thank Sybille Traupe and Verena Grosse for excellent technical assistance and Armgard Janczikowski for electron microscopy. We are also grateful to Sven Krappmann for helpful comments on the manuscripts and to Reinhard Fischer for providing plasmids and *fphA*Δ and *rosA*Δ strains. This work was supported by grants to G.H.B. of the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the Volkswagen-Stiftung.

References

Abe, H., and Shimoda, C. (2000) Autoregulated expression of *Schizosaccharomyces pombe* meiosis-specific transcription factor Mei4 and a genome-wide search for its target genes. *Genetics* **154:** 1497–1508.

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998) Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* **62:** 35–54.

Bayram, O., Biesemann, C., Krappmann, S., Galland, P., and Braus, G.H. (2008a) More than a repair enzyme: *Aspergillus nidulans* photolyase-like CryA is a regulator of sexual development. *Mol Biol Cell* **19:** 3254–3262.

Bayram, O., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., *et al.* (2008b) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320:** 1504–1506.

© 2009 The Authors

Benjamin, K.R., Zhang, C., Shokat, K.M., and Herskowitz, I. (2003) Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. *Genes Dev* **17:** 1524–1539.

Blumenstein, A., Vienken, K., Tasler, R., Purschwitz, J., Veith, D., Frankenberg-Dinkel, N., and Fischer, R. (2005) The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. *Curr Biol* **15:** 1833–1838.

Bok, J.W., and Keller, N.P. (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* **3:** 527–535.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., *et al.* (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65:** 663–675.

Braus, G.H., Krappmann, S., and Eckert, S. (2002) Sexual development in ascomycetes: fruit body formation in *Aspergillus nidulans*. In *Molecular Biology of Fungal Development*. Osiewacz, H.D. (ed.). New York: Marcel Dekker, pp. 215–244.

Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., *et al.* (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc Natl Acad Sci USA* **93:** 1418–1422.

Busch, S., and Braus, G.H. (2007) How to build a fungal fruit body: from uniform cells to specialized tissue. *Mol Microbiol* **64:** 873–876.

Busch, S., Eckert, S.E., Krappmann, S., and Braus, G.H. (2003) The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*. *Mol Microbiol* **49:** 717–730.

Busch, S., Schwier, E.U., Nahlik, K., Bayram, O., Helmstaedt, K., Draht, O.W., *et al.* (2007) An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. *Proc Natl Acad Sci USA* **104:** 8089–8094.

Champe, S.P., and el-Zayat, A.A. (1989) Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *J Bacteriol* **171:** 3982–3988.

Champe, S.P., Rao, P., and Chang, A. (1987) An endogenous inducer of sexual development in *Aspergillus nidulans*. *J Gen Microbiol* **133:** 1383–1387.

Coppin, E., Debuchy, R., Arnaise, S., and Picard, M. (1997) Mating types and sexual development in filamentous ascomycetes. *Microbiol Mol Biol Rev* **61:** 411–428.

Dirick, L., Goetsch, L., Ammerer, G., and Byers, B. (1998) Regulation of meiotic S phase by Ime2 and a Clb5,6-associated kinase in *Saccharomyces cerevisiae*. *Science* **281:** 1854–1857.

Dutton, J.R., Johns, S., and Miller, B.L. (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *EMBO J* **16:** 5710–5721.

Eckert, S.E., Hoffmann, B., Wanke, C., and Braus, G.H. (1999) Sexual development of *Aspergillus nidulans* in tryptophan auxotrophic strains. *Arch Microbiol* **172:** 157–166.

Fernandes, M., Keller, N.P., and Adams, T.H. (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol Microbiol* **28:** 1355–1365.

Fischer, R. (2002) Conidiation in *Aspergillus nidulans*. In *Molecular Biology of Fungal Development*, vol. 1. Osiewacz, H.D. (ed.). New York: Marcel Dekker, pp. 59–86.

Fu, Z., Larson, K.A., Chitta, R.K., Parker, S.A., Turk, B.E., Lawrence, M.W., *et al.* (2006) Identification of yin-yang regulators and a phosphorylation consensus for male germ cell-associated kinase (MAK)-related kinase. *Mol Cell Biol* **26:** 8639–8654.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.J., Wortman, J.R., Batzoglou, S., *et al.* (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438:** 1105–1115.

Garrido, E., and Perez-Martin, J. (2003) The *crk1* gene encodes an Ime2-related protein that is required for morphogenesis in the plant pathogen *Ustilago maydis*. *Mol Microbiol* **47:** 729–743.

Garrido, E., Voss, U., Muller, P., Castillo-Lluva, S., Kahmann, R., and Perez-Martin, J. (2004) The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. *Genes Dev* **18:** 3117–3130.

Han, K.H., and Han, K.Y., Yu, J.H., Chae, K.S., Jahng, K.Y., and Han, D.M. (2001) The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. *Mol Microbiol* **41:** 299–309.

Hepworth, S.R., Friesen, H., and Segall, J. (1998) NDT80 and the meiotic recombination checkpoint regulate expression of middle sporulation-specific genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **18:** 5750–5761.

Honigberg, S.M. (2004) Ime2p and Cdc28p: co-pilots driving meiotic development. *J Cell Biochem* **92:** 1025–1033.

Jinno, A., Tanaka, K., Matsushime, H., Haneji, T., and Shibuya, M. (1993) Testis-specific mak protein kinase is expressed specifically in the meiotic phase in spermatogenesis and is associated with a 210-kilodalton cellular phosphoprotein. *Mol Cell Biol* **13:** 4146–4156.

Keller, N., Bok, J., Chung, D., Perrin, R.M., and Keats Shwab, E. (2006) LaeA, a global regulator of Aspergillus toxins. *Med Mycol* **44** (Suppl.)**:** 83–85.

Keller, N.P., Turner, G., and Bennett, J.W. (2005) Fungal secondary metabolism – from biochemistry to genomics. *Nat Rev Microbiol* **3:** 937–947.

Kim, H., Han, K., Kim, K., Han, D., Jahng, K., and Chae, K. (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genet Biol* **37:** 72–80.

Kominami, K., Sakata, Y., Sakai, M., and Yamashita, I. (1993) Protein kinase activity associated with the IME2 gene product, a meiotic inducer in the yeast *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* **57:** 1731–1735.

Krappmann, S., Jung, N., Medic, B., Busch, S., Prade, R.A., and Braus, G.H. (2006) The *Aspergillus nidulans* F-box protein GrrA links SCF activity to meiosis. *Mol Microbiol* **61:** 76–88.

Krylov, D.M., Nasmyth, K., and Koonin, E.V. (2003) Evolution of eukaryotic cell cycle regulation: stepwise addition of regulatory kinases and late advent of the CDKs. *Curr Biol* **13:** 173–177.

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd,

Lubertozzi, D., and Keasling, J.D. (2006) Marker and promoter effects on heterologous expression in *Aspergillus nidulans*. *Appl Microbiol Biotechnol* **72:** 1014–1023.

Mandel, M., and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53:** 159–162.

Matsushime, H., Jinno, A., Takagi, N., and Shibuya, M. (1990) A novel mammalian protein kinase gene (mak) is highly expressed in testicular germ cells at and after meiosis. *Mol Cell Biol* **10:** 2261–2268.

Mattern, I.E., Punt, P.J., and Van den Hondel, C.A. (1988) A vector of *Aspergillus nidulans* conferring phleomycin resistance. *Fungal Genet Newsl* **35:** 25.

Miller, K.Y., Wu, J., and Miller, B.L. (1992) StuA is required for cell pattern formation in *Aspergillus*. *Genes Dev* **6:** 1770–1782.

Mitchell, A.P., Driscoll, S.E., and Smith, H.E. (1990) Positive control of sporulation-specific genes by the IME1 and IME2 products in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10:** 2104–2110.

Miyata, Y., Akashi, M., and Nishida, E. (1999) Molecular cloning and characterization of a novel member of the MAP kinase superfamily. *Genes Cells* **4:** 299–309.

Mooney, J.L., and Yager, L.N. (1990) Light is required for conidiation in *Aspergillus nidulans*. *Genes Dev* **4:** 1473–1482.

Mooney, J.L., Hassett, D.E., and Yager, L.N. (1990) Genetic analysis of suppressors of the *veA1* mutation in *Aspergillus nidulans*. *Genetics* **126:** 869–874.

Muro-Pastor, M.I., Gonzalez, R., Strauss, J., Narendja, F., and Scazzocchio, C. (1999) The GATA factor AreA is essential for chromatin remodelling in a eukaryotic bidirectional promoter. *EMBO J* **18:** 1584–1597.

Nayak, T., Szewczyk, E., Oakley, C.E., Osmani, A., Ukil, L., Murray, S.L., *et al.* (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. *Genetics* **172:** 1557–1566.

Nierman, W.C., May, G., Kim, H.S., Anderson, M.J., Chen, D., and Denning, D.W. (2005) What the Aspergillus genomes have told us. *Med Mycol* **43** (Suppl. 1)**:** S3–S5.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., *et al.* (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* **10:** 885–892.

Pöggeler, S., Nowrousian, M., and Kück, U. (2006) Fruiting-Body Development in Ascomycetes. In *The Mycota I Growth, Differentiation and Sexuality*. Fischer, K. (eds). Heidelberg: Springer-Verlag, pp. 325–355.

Punt, P.J., and van den Hondel, C.A. (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Meth Enzymol* **216:** 447–457.

Purschwitz, J., Muller, S., Kastner, C., Schoser, M., Haas, H., Espeso, E.A., *et al.* (2008) Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. *Curr Biol* **18:** 255–259.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350–1354.

Sari, F., Heinrich, M., Meyer, W., Braus, G.H., and Irniger, S. (2008) The C-terminal region of the meiosis-specific protein kinase Ime2 mediates protein instability and is required for normal spore formation in budding yeast. *J Mol Biol* **378:** 31–43.

Schindler, K., and Winter, E. (2006) Phosphorylation of Ime2 regulates meiotic progression in *Saccharomyces cerevisiae*. *J Biol Chem* **281:** 18307–18316.

Schwechheimer, C. (2004) The COP9 signalosome (CSN): an evolutionary conserved proteolysis regulator in eukaryotic development. *Biochim Biophys Acta* **1695:** 45–54.

Shinkai, Y., Satoh, H., Takeda, N., Fukuda, M., Chiba, E., Kato, T., *et al.* (2002) A testicular germ cell-associated serine-threonine kinase, MAK, is dispensable for sperm formation. *Mol Cell Biol* **22:** 3276–3280.

Stinnett, S.M., Espeso, E.A., Cobeno, L., Araujo-Bazan, L., and Calvo, A.M. (2007) *Aspergillus nidulans* VeA subcellular localization is dependent on the importin alpha carrier and on light. *Mol Microbiol* **63:** 242–255.

Todd, R.B., Hynes, M.J., and Andrianopoulos, A. (2006) The *Aspergillus nidulans rcoA* gene is required for *veA*-dependent sexual development. *Genetics* **174:** 1685–1688.

Togawa, K., Yan, Y.X., Inomoto, T., Slaugenhaupt, S., and Rustgi, A.K. (2000) Intestinal cell kinase (ICK) localizes to the crypt region and requires a dual phosphorylation site found in map kinases. *J Cell Physiol* **183:** 129–139.

Vienken, K., Scherer, M., and Fischer, R. (2005) The Zn(II) 2Cys6 putative *Aspergillus nidulans* transcription factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submersed culture. *Genetics* **169:** 619–630.

Wei, H., Scherer, M., Singh, A., Liese, R., and Fischer, R. (2001) *Aspergillus nidulans* alpha-1,3 glucanase (mutanase), *mutA*, is expressed during sexual development and mobilizes mutan. *Fungal Genet Biol* **34:** 217–227.

Yoshida, M., Kawaguchi, H., Sakata, Y., Kominami, K., Hirano, M., Shima, H., *et al.* (1990) Initiation of meiosis and sporulation in *Saccharomyces cerevisiae* requires a novel protein kinase homologue. *Mol Gen Genet* **221:** 176–186.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2009 The Authors