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

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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, sporulation 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 (Matsushima 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 VeIB 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 AfIR (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
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).

**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 32P 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.
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 (12–72 h). mRNA levels of the control gene (12 and 24 h) as well as under conditions inducing sexual development (12–72 h) gradually increased after transfer of mycelium to solid medium. Northern hybridization with an *imeB* DNA probe confirmed the replacement of *imeB* by *sgfp*, expressed from the native *imeB* promoter (Fig. 2B). This fully functional *imeB::sgfp* fusion protein is also targeted to the nucleus. Therefore, ImeB protein is a predominantly nuclear protein, which is not affected in its localization by illumination.

**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). *ImeB::sgfp* fusion protein 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 conidia 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).
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 pmutA::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
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 veA 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.

**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 imeBΔ (AGB322) and the complementation strain (AGB321) were point-inoculated (2 × 10³ 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 mm²). Five independent 10 mm² squares were counted under a stereomicroscope. Vertical bars indicate standard deviations. C. Comparison of growth of a wild-type (FGSCA4) and the imeBΔ strain on solid minimal media. Strains were point-inoculated (2 × 10³ spores) and colony size was measured (in cm).
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 μWm²), red light (30 μWm²) and dark conditions. In white light, the imeB strain produced about 70% of cleistothecia compared with dark...
Fig. 5. Phenotype of an imeB deletion in the veA1 and in the fphA strain background.
A. Comparison of the cleistothecia production of imeBΔ 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 mm² squares were counted under a stereomicroscope. Vertical bars indicate standard deviations.
C. A wild-type (FGSC A4), the imeBΔ (AGB322), fphAΔ (SAB2) and fphAΔ/imeBΔ (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).
(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 sterigmatocystin 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 VeB-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). 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 reversely induced 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.

**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*, *veB*, *stuA* and *nsdD* genes (Fig. 7). *veA* (Kim et al., 2002) and *veB* (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*, *veB* 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
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 endogenous promoter was replaced by the niiA promoter (Muro-Pastor et al., 1999). The niiA promoter is induced by nitrate and repressed by ammonium. The niiA::imeB construct was introduced ectopically into the A. nidulans wild-type strain AGB152 as a single copy.

A wild-type strain, the niiA::imeB strain and a control strain carrying only 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 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 gpdA mRNA levels (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 cleistothecia 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...
Fig. 8. Phenotypes of imeB overexpression.
A. A wild-type strain (AGB152), an overexpression strain (AGB447, pnieA::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 x 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.
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
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 (Kominiari 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 conidia, 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 stage 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 VeB 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 VeIB 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.
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, Hüüle 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 imeBΔ rosAΔ double knockout 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 meioitic 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 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-

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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 (Invitrogen) was used for overexpression and localization of GFP fusion proteins. Plasmids employed for *A. nidulans* manipulations were constructed as follows:

### Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSC A4</td>
<td>Glasgow wild-type</td>
<td>FGSC</td>
</tr>
<tr>
<td>TNO2A3</td>
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<td>Busch et al. (2003)</td>
</tr>
<tr>
<td>AGB165</td>
<td><em>pabaA</em>, <em>pyrG</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB320</td>
<td><em>imeB</em>::<em>ptrA</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB321</td>
<td><em>imeB</em>::<em>ptrA</em> transformed with pME3293</td>
<td>This study</td>
</tr>
<tr>
<td>AGB322</td>
<td>AGB320 backcrossed with FGSC A4</td>
<td>This study</td>
</tr>
<tr>
<td>AGB325</td>
<td>AGB152 transformed with pME3295::<em>alcA</em>:sgfp::<em>imeB</em>, <em>pyrG</em>, <em>pyrA</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB326</td>
<td>AGB320 transformed with pME3296::<em>imeB</em>::<em>ptrA</em>::<em>gpdA</em>::natF::mutA*::sgfp</td>
<td>This study</td>
</tr>
<tr>
<td>AGB327</td>
<td>AGB320 transformed with pME3297 (T219-ALA): <em>imeB</em></td>
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</tr>
<tr>
<td>AGB328</td>
<td>AGB320 transformed with pME3298 (T220-ALA): <em>imeB</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB329</td>
<td>AGB320 transformed with pME3299 (Y221-ALA): <em>imeB</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB330</td>
<td>AGB320 transformed with pME3300 (TTY-ALA): <em>imeB</em></td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
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<td>AGB446</td>
<td>AGB 445 transformed with <em>imeB</em>:<em>imeB</em>:sgfp::<em>gpdA</em>:natF::<em>imeB</em> fusion construct</td>
<td>This study</td>
</tr>
<tr>
<td>AGB447</td>
<td>AGB152 transformed with pME3191::<em>nlIV</em>:::<em>imeB</em>:::<em>nlIV</em>, <em>pyrG</em>, <em>pyrA</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB448</td>
<td>AGB152 transformed with pME3160 (empty <em>nlIV</em> plasmid)</td>
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</tr>
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<td>AGB449</td>
<td>SAB2 originated double knockout, <em>fphA</em>:::<em>argB</em>, <em>imeB</em>:::<em>ptrA</em></td>
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</tr>
<tr>
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<tr>
<td>AGB452</td>
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<td>This study</td>
</tr>
<tr>
<td>SAB2</td>
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<td>Blumenstein et al. (2005)</td>
</tr>
<tr>
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<td>Vienken et al. (2005)</td>
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</tr>
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<td>AGB152</td>
<td>AGB445 transformed with pME3292 (T219-ALA): <em>imeB</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB320</td>
<td>AGB320 transformed with pME3298 (T220-ALA): <em>imeB</em></td>
<td>This study</td>
</tr>
<tr>
<td>AGB325</td>
<td>AGB320 transformed with pME3295::<em>imeB</em>::<em>ptrA</em>::<em>niiA</em></td>
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<td>AGB326</td>
<td>AGB320 transformed with pME3296::<em>imeB</em>::<em>ptrA</em>::<em>gpdA</em>::natF::mutA*::sgfp</td>
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</tr>
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</tr>
<tr>
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<td>AGB320 transformed with pME3300 (TTY-ALA): <em>imeB</em></td>
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<tr>
<td>AGB445</td>
<td>TNO2A3 containing <em>gpdA</em>:intron:*mrp&quot;:<em>h2A</em>, <em>pyrG</em></td>
<td>This study</td>
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<tr>
<td>AGB446</td>
<td>AGB 445 transformed with <em>imeB</em>:<em>imeB</em>:sgfp::<em>gpdA</em>:natF::<em>imeB</em> fusion construct</td>
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<tr>
<td>AGB447</td>
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<td>AGB152 transformed with pME3160 (empty <em>nlIV</em> plasmid)</td>
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<td>AGB449</td>
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<td>This study</td>
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<td>AGB450</td>
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<tr>
<td>AGB452</td>
<td><em>rhoA</em>, <em>pyroA</em>, <em>pyrG</em>, <em>veA</em>, <em>imeB</em>:::<em>ptrA</em></td>
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<tr>
<td>SAB2</td>
<td><em>fphA</em>:::<em>argB</em></td>
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<td><em>pyrG</em>, <em>pyrA</em>:::<em>argB</em></td>
<td>Vienken et al. (2005)</td>
</tr>
</tbody>
</table>

**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* (Fermantas), *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 pBlue-script KS (Invitrogen). The deletion cassette was excised from pME3292 with Kpnl 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 hybridized 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., 1998) vector (*phleoR*). The created *imeB* complementation construct, pME3293, was transformed into the *imeB* mutant strain yielding AGB321.

**Overexpression of *imeB*.** For overexpression under *nlIV* promoter, the *imeB* open reading frame was amplified (Fat19/Fat20) with *Phusion* polymerase from a cDNA library (Krapmann et al., 2006) and inserted into the Pmel site of the vector pME3160 (Bayram et al., 2008b). The *nlIV* overexpression vector contains the auxotrophy marker *pyrG* for selection and the *A. nidulans* nitrate source inducible *nlIV*.
The pmutA::sgfp 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 Ascl 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 Ascl-PacI, thus opening the vector behind the alcA promoter. Subsequent cloning of the Ascl-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 imeBGFPP1/3, and 3 UTR region of imeB was amplified with primers imeBGFPP4/6. Then, these two fragment as well as sgfp::natR cassette (PCR amplicon) were fused by fusion PCR (imeBGFPP2/5). 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 nmtA::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 EcolI and subsequent ligation of a *gpdA-natR* cassette. The *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 AGB320. The imeB mutant strain bearing the nmtA::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) to 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 (TYY-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 development 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% AlCl₃ 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**

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**References**


Supporting information

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

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