hymA* (hypha-like metulae), a new developmental mutant of *Aspergillus nidulans

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Asexual fruiting body development in *Aspergillus nidulans* requires a precise spatial and temporal coordinated expression of many genes. Insertional mutagenesis was used to isolate and characterize a new mutant of *A. nidulans* in which hyphal growth was slightly reduced and conidiophore development was specifically blocked at the metula stage. In contrast to the uninucleate metulae of the wild-type, in the mutant these structures were elongated, multinucleate and septate. Further differentiation and production of phialides by a budding-like process was not observed. The mutant metulae thus resembled hyphae rather than metulae and the gene was therefore named *hypha-like metulae* (*hymA*). The *hymA* gene was mapped to linkage group VI. The integrated vector was rescued with border sequences from the integration site. The border sequences were used to isolate a cosmid from a wild-type library which was subcloned to a 5 kb fragment able to complement the mutation *in trans*. This fragment encoded a 1.8 kb transcript expressed in hyphae and throughout development. It is proposed that *hymA* is involved in budding processes, and is required for the formation of metulae and for their further differentiation.

Keywords: *Aspergillus nidulans*, filamentous fungi, asexual development, budding, insertional mutagenesis

INTRODUCTION

The life cycle of the filamentous fungus *Aspergillus nidulans* consists of a sexual and an asexual part. The asexual reproductive pathway has been used as a model system to study the molecular genetic control of morphogenesis in lower eukaryotes for more than 30 years (Clutterbuck, 1969; Timberlake, 1990, 1991, 1993; Clutterbuck & Timberlake, 1992). During asexual development of *A. nidulans*, hyphae differentiate after acquisition of developmental competence and specialized multicellular spore-forming structures, called conidiophores, are formed. First, a hyphal cell develops into a foot cell, which elongates into a stalk, about 100 µm in length. The stalk swells at the tip to a vesicle, which then produces two uninuclear cell types, metulae and phialides. The formation of metulae and phialides is accompanied by transition from hyphal growth to a budding-like proliferation. Phialides continuously bud off spores (conidia);

many thousand conidia are produced by one conidiophore.

Martinelli & Clutterbuck (1971) mutagenized *A. nidulans* and compared the number of auxotrophs with the number of developmental mutants. From this ratio it was estimated that the number of developmental genes is at least 150. Strains with a defect in vegetative growth and in developmental processes were not considered. In an alternative approach, the appearance of differentially expressed transcripts during asexual development was studied; from these data the number of involved genes was re-estimated to be 1200 (Timberlake, 1980; Clutterbuck & Timberlake, 1992). Genes expressed in hyphae and in conidiophores were neglected. Subsequent deletion studies revealed that some of these genes, discovered by reverse genetics, were dispensable for conidiophore production and had a very subtle or no phenotype under laboratory conditions (Aramayo *et al.*, 1989).

More than 12 developmental genes of *A. nidulans* have been analysed at the molecular level. Some of these are transcriptional activators, including *flbD*, *brlA*, *abaA* and

Abbreviation: DAPI, 4,6-diamidino-2-phenylindole.

Table 1. *A. nidulans* strains used in this study

Strain	Genotype	Source
FGSC26	<i>biA1; veA1</i>	Fungal Genetics Stock Center, Kansas, USA
GR95	<i>suA1 adE20, yA2, adE20; acrA1; galA1; pyroA4; facA303; sB3; nicB8; riboB2, veA1</i>	J. Clutterbuck, Glasgow, UK
RMSO11	<i>pabaA1, yA2, ΔargB::trpCΔB; veA1, trpC801</i>	Stringer <i>et al.</i> (1991)
GR5	<i>pyrG89; wA3; pyroA4; veA1</i>	G. May, Houston, TX, USA
G623	<i>biA1; nicC10; veA1</i>	J. Clutterbuck, Glasgow, UK
SMK8	<i>pyrG89; wA3; pyroA4; hymA::pMK1; veA1</i>	Transformation of GR5 with pMK1
K5	<i>pabaA1; pyroA4; hymA::pMK1; veA1</i>	Progeny of SMK8 × RMSO11
SIK5	<i>pabaA1; pyroA4; hymA::pMK1; veA1</i>	Progeny of K5 × GR95
SIK8	<i>pabaA1, yA2; ΔargB::trpCΔB; veA1</i>	Progeny of SIK5 × RMSO11
SAA1	<i>yA2, pabaA1; ΔargB::trpCΔB; abaA1</i>	A. Andrianopoulos, Melbourne, Australia
SR1	<i>pyrG89; wA3; pyroA4; apsB6; veA1</i>	This work
SRF30	<i>pabaA1, yA2, wA3; ΔargB::trpCΔB; pyroA4; apsA::pyr4; veA1</i>	Fischer & Timberlake (1995)

probably *wetA*, which form a linear signal-amplifying pathway (Clutterbuck, 1969; Boylan *et al.*, 1987; Adams *et al.*, 1988; Sewall *et al.*, 1990; Marshall & Timberlake, 1991; Prade & Timberlake, 1993; Andrianopoulos & Timberlake, 1994; Wieser & Adams, 1995). Others, including *medA* and *stuA*, act as modifiers in the main cascade (Miller *et al.*, 1991, 1992; Gems & Clutterbuck, 1994). In addition, a set of structural genes, including *wA*, *yA*, *dewA* and *rodA*, were identified as targets of the transcription factors (Aramayo & Timberlake, 1990, 1993; Mayorga & Timberlake, 1990, 1992; Stringer *et al.*, 1991; Stringer & Timberlake, 1994). Finally, genes such as *apsA* or *apsB* couple vegetative functions (e.g. nuclear migration) to developmental processes (Clutterbuck, 1994; Fischer & Timberlake, 1995). Mutants defective in one of these developmental genes display an abnormal conidiophore phenotype, but in most cases vegetative growth is not affected.

In *fluffy* mutants, such as *flbA* or *flbD*, hyphae produce cotton-like aerial mycelia but fail to produce further developmental structures. The *fluffy* genes have therefore been recognized as 'early genes' in the developmental process, leading to activation of the transcriptional activator *brlA*. In *bristle* mutants (*brlA*), development stops after the formation of stalks. The zinc-finger protein BrlA directly activates the transcription factor AbaA, a leucine-zipper protein with an ATTS/TEA DNA-binding motif. In *abacus* mutants (*abaA*), phialides are elongated and septate; they fail to differentiate and to produce spores. In *wet* mutants (*wetA*), conidia are formed but are unstable and lyse after a short time. The function of the modifier genes *medusa* (*medA*) and *stunted* (*stuA*) is less well established. Defects in some structural genes such as *white* (*wA*), *yellow* (*yA*), *rodletless* (*rodA*) and *dew* (*dewA*) lead to abnormalities in pigmentation or cell wall morphogenesis. *aps* mutants (*apsA* and *apsB*) produce metulae which remain anucleate and thus do not proceed with the developmental programme. In addition to the genes mentioned, many more components are expected to be necessary for the production of conidiophores.

In this paper we describe the isolation of a new developmental mutant with a specific block in conidiophore development at the metula stage, possibly due to a defect in the transition from hyphal to budding-like growth.

METHODS

***Aspergillus nidulans* strains, *Escherichia coli* plasmids and growth conditions.** All *A. nidulans* strains used in this study are listed in Table 1. Supplemented minimal and complete media for *A. nidulans* were prepared as described by Käfer (1977). Standard genetic techniques for strain construction were used, essentially as described by Käfer (1977). *A. nidulans* colonies were incubated on complete agar medium until the hyphae of two different strains had grown together. A small agar piece from this region was transferred to minimal agar medium, where only the heterokaryon could grow. For induction of the sexual cycle, plates were sealed with tape and incubated for 2 weeks until the fruit bodies had formed.

E. coli strains, and the constructed plasmids and cosmids used in this study, are listed in Table 2.

Molecular techniques. Standard DNA transformation procedures for *A. nidulans* (Yelton *et al.*, 1984) and *E. coli* (Sambrook *et al.*, 1989) were used. In *A. nidulans* transformation experiments *Kpn*I (20 U) was added, which increased the transformation efficiency. Genomic DNA was extracted from the fungus essentially as described by Timberlake & Marshall (1989). RNA was isolated with TRIzol (Gibco BRL), according to the manufacturer's protocol. DNA and RNA analyses (Southern and Northern hybridization) were performed as described by Sambrook *et al.* (1989).

For plasmid rescue, 20 µg SIK5 genomic DNA was digested to completion with *Xho*I, purified with the Wizard Minipreps DNA purification system (Promega) and religated in a 200 µl volume overnight at 16 °C. Competent *E. coli* XL1-blue cells (New England Biolabs) were electrotransformed with 2 µl of the ligation mixture. The rescued plasmid was used to isolate the border sequences of the integration site.

Staining and microscopy. For examination of conidiophores by fluorescence microscopy, microscope slides were point-inocu-

Table 2. *E. coli* plasmids and cosmids used in this study

Plasmid/ cosmid	Construction	Source
pRG1	Contains <i>pyr4</i> gene from <i>Neurospora crassa</i>	Waring <i>et al.</i> (1989)
pMK1	Contains <i>pyr4</i> gene with pBluescript KS(−) as vector	This work
pMK2	Rescued plasmid out of genomic DNA from <i>A. nidulans</i> strain SIK5; incomplete pMK1 with border sequences of the integration site	This work
pMK4	5 kb <i>Xho</i> I fragment containing the complete <i>hymA</i> gene in pBluescript KS(−)	This work
pDC1	<i>A. nidulans argB</i> gene in pIC20R	Aramayo <i>et al.</i> (1989)
CMK1	Cosmid, PUI as vector, with a 40 kb insert of genomic <i>Aspergillus</i> DNA, which contains the complete <i>hymA</i> gene and the <i>argB</i> gene as selection marker	B. Miller, Idaho, USA; this work

lated, covered with a thin film of medium (solidified with 0.7% agarose), and incubated at 37 °C for 1–2 d in a Petri dish with 25 ml medium, which did not cover the microscope slide but made contact with the agarose film (Clutterbuck, 1994; Fischer & Timberlake, 1995). During the fixation procedure with 8% (v/v) formaldehyde in PME buffer (50 mM PIPES, pH 6.0; 25 mM EGTA, pH 8; 5 mM MgSO₄) the colonies came off the slide, floated on the surface and were transferred to several washes in PME and followed by staining with 0.1 µg ml^{−1} DAPI or Calcofluor in PME with 0.1% Triton X-100 added. Fluorescence microscopy was performed with a Zeiss Axiophot microscope with the appropriate filter combination and a 3 CCD INTAS Seescan video camera (INTAS, Göttingen, Germany).

For scanning electron microscopy, colonies grown on plates were transferred with a piece of agar into 5% (v/v) glutaraldehyde for fixation. After several washes with water, the pieces were transferred to ethylene glycol monoethyl ether and incubated overnight at room temperature. They were then transferred to water-free acetone and critical-point dried. The samples were then sputter-coated with gold and observed in a Hitachi S-530 scanning electron microscope.

RESULTS

Transformation of *A. nidulans* with plasmids frequently leads to non-homologous integration of the vector into the genome, thereby disrupting the gene at the integration locus. This phenomenon, often a disadvantage in specific knock-out experiments which are dependent on homologous integration events, was used as a method to mutagenize *A. nidulans*. The advantages of this approach are the potential for disrupting any gene and the tagging of the integration site by the transformed plasmid. Consequently, this method allows quick access to the gene of interest (Fig. 1) (Kahmann *et al.*, 1995). The approach is particularly suitable for studying developmental genes, because loss-of-function mutations in these genes are usually not lethal.

Isolation of a new developmental mutant

In a transformation experiment of the uracil and uridine auxotrophic but conidiating *A. nidulans* strain GR5 with the *pyr4*-containing plasmid pMK1, approximately 500 *ura*⁺ transformants were obtained. The majority of

the transformants produced conidiophores like the wild-type. In some strains conidiation was markedly reduced and only a few did not conidiate at all. The phenotypes of the latter group were frequently unstable after restreaking. However, we were able to colony-purify one stable aconidial strain, named K5, with a slightly reduced growth rate (Fig. 2a). This strain was subsequently crossed to a wild-type and the progeny were analysed. Conidiating and non-conidiating colonies were found at a ratio of 1:1. This suggested that the defect was due to a single gene. However, Southern blot analysis revealed that the genome of the strain harboured several integrations of the plasmid (result not shown). Therefore three sequential crosses of progeny mutants with wild-type strains were performed. Finally, we obtained a strain, SIK8, with a single integration (result not shown). The mutant phenotype (Fig. 3b) still was inherited in a Mendelian manner. In order to test whether in SIK8 an unknown gene had been hit, the strain was crossed to several known *A. nidulans* developmental mutants. In all cases hyphae of different strains fused and formed stable heterokaryons with nuclei of both parent strains. All heterokaryons conidiated, suggesting cross-complementation of the mutations. In addition, among the progeny analysed after sexual spore formation, wild-type colonies were found as expected for a non-linked gene. These results, together with the phenotypic characteristics, suggested that in the mutant an unknown gene was disrupted; this gene was named *hymA* (hypha-like metulae).

Phenotype of the *hymA* mutant

The *hymA* mutant strain was compared to an *A. nidulans* wild-type strain with respect to hyphal growth and conidiophore development using phase-contrast, fluorescence and scanning electron microscopy.

The mycelia of mutant strains were more branched than wild-type mycelia, leading to the compact colony phenotype of *hymA* strains (Fig. 2b, c). Other morphological or cell biological abnormalities were not observed in mutant hyphae. However, a dramatic difference between mutant and wild-type was found in conidiophore development.

Fig. 1. Scheme of insertional mutagenesis and rescue of the integrated plasmid.

Fig. 2. (a) Colonies of wild-type *A. nidulans* (FGSC26) and mutant strain K5 grown on complete medium for 2 d at 37 °C. (b, c) Colony margins of the wild-type (b) and the mutant strain (c). Bars, 1 cm (a) and 500 µm (b; same magnification in c).

A. nidulans wild-type conidiophores consist of five different cell types (see Introduction), which are sequentially produced during the differentiation process. The pigmented asexual spores lead to green-coloured colonies. In contrast, colonies of SIK8 appeared brown on agar plates, because differentiation of the mutant conidiophores stopped after formation of a stalk, a vesicle and metulae. The latter were elongated in comparison to wild-type metulae, which are defined by a precise cell length and width (Fig. 3a, b). The metula-structures in the SIK8 mutant were quite variable in length (10–20 µm). The phenotype was independent of the growth medium. Interestingly, some older metulae produced a spore-like structure at their tips, comparable to conidia generated by hyphae in liquid media when grown under nutrient limitation or after forced expression of *brlA* (Marhoul & Adams, 1995; Skromne *et al.*, 1995). SIK8 conidiophores were also compared to conidiophores of *aps* mutants, in which development is also blocked at the metula stage, and to *abaA* mutants, which have a defect in phialide differentiation. However, in *aps* and *aba* strains the metulae are essentially like the wild-type in morphology (Fig. 3c, d).

To characterize the SIK8 phenotype further, conidiophores of wild-type, SIK8 and *apsA* strains were analysed with respect to nuclear distribution and septum formation (Figs 4 and 5). Whereas wild-type and *aba* metulae contained a single nucleus (Fig. 4a, d) and *aps* metulae were anucleate (Fig. 4c), in SIK8 the metulae contained several nuclei and were compartmentalized by septa at different positions (Figs 4b and 5a–c). In some cases, a cell wall very close to the vesicle surface was observed (Fig. 5b). Several compartments were multinucleate. Thus, the metulae of SIK8 resembled hyphae rather than true metulae (hence the gene name *hymA*: hypha-like metulae). The idea was supported by the study of double mutants.

Fig. 3. Scanning electron microscopic analysis of (a) a wild-type, (b) a *hymA*, (c) an *apsA* and (d) an *abaA* mutant conidiophore of *A. nidulans*. Bar, 5 μ m (a–c) and 10 μ m (d).

Fig. 4. Nuclear distribution in conidiophores of (a) a wild-type, (b) a *hymA*, (c) an *apsA* and (d) an *abaA* mutant of *A. nidulans*. Nuclei were visualized by DAPI fluorescence microscopy. Bar, 5 μ m. M, metulae; P, phialide.

Fig. 5. Comparison of (a–c) different *hymA* conidiophores with (d) a wild-type *A. nidulans* conidiophore. Cell walls were stained with Calcofluor. (b) Sometimes a septum was found close to the vesicle (arrowhead). (b, c) In older *hymA* conidiophores spore-like structures were observed at the tip of the hypha-like metulae (arrowhead in c). Bar, 10 μ m.

Analysis of double mutants

hymA and *apsB* mutant strains specifically stop development at the metula stage, whereas *abaA* mutants fail to differentiate their phialides (Sewall *et al.*, 1990; Clutterbuck, 1994). Double mutants between *hymA* and *abaA*, and between *hymA* and *apsB*, were constructed and phenotypically studied using phase-contrast, DAPI and Calcofluor fluorescence microscopy (results not shown).

The *hymA/abaA* double mutant displayed a *hymA* phenotype, indicating that the action of *hymA* was required before production of abacus structures. Surprisingly, *hymA/apsB* double mutants also showed a *hymA* conidiophore type, with all metulae structures nucleate. This result suggested that in the absence of the *hymA* function, 'normal' metulae could not be formed. Instead hypha-like 'metulae' arose from the vesicles.

Isolation of the *hymA* gene

In order to isolate the *hymA* wild-type gene advantage was taken of the mutagenesis method in which the vector marked its integration site (Fig. 1). Although the restriction enzyme *KpnI* was added in the transformation experiment, the integration event did not occur via this site and could not be explained in Southern blot analyses (results not shown). This suggested a non-homologous integration. In order to rescue the integrated plasmid, genomic DNA from SIK8 was isolated and restricted with *XhoI*. After religation of the restriction fragments pMK2 was recovered in *E. coli*. To detect the border sequences of the integration site of SIK8 in pMK2 this plasmid was compared with pMK1. pMK2 was analysed by restriction enzyme digestion and hybridization of the fragments in a Southern blot with pMK1. DNA fragments visible after ethidium bromide staining in the gel but not detectable with the probe were isolated. In a triple digest of pMK2 with *KpnI*, *XhoI* and *EcoRV* a 1 kb fragment was isolated with these attributes. In order to isolate the complete wild-type copy of *hymA* the 1 kb fragment was used to screen a wild-type cosmid library of *A. nidulans* (PUI library, kindly provided by B. Miller, Moscow, ID, USA). Of 50000 clones, 12 hybridized to the specific probe and all 12 clones contained the same 50 kb cosmid, CMK1. To test this cosmid for complementation of a *hymA* mutant strain, SIK8 was transformed with CMK1; this resulted in 35 transformants, 33 of which conidiated like the wild-type. This high frequency of complementation suggested that the cosmid was ectopically integrated and hence contained the entire *hymA* gene. This was confirmed by crosses of five transformants with wild-type *A. nidulans* strains in which colonies with *hymA* phenotypes could be recovered. The complementing activity was finally localized on a 5 kb *XhoI* restriction fragment. With this fragment as a probe, a 1.8 kb transcript was detected in wild-type RNA. In *hymA* mutant strains the 1.8 kb transcript was shifted to 1.5 kb, indicating that this mRNA was encoded by the *hymA* gene and disrupted by the vector (Fig. 6). The *hymA* transcript was detected in hyphae before and after acquisition of developmental competence, as well as in developing conidiophores (result not shown). Since in *hymA* mutant strains a shorter mRNA was still detectable it remains to be shown whether the observed phenotype is the phenotype of the null mutant. However, the truncated transcript also contained vector sequences, indicating that a significant part of *hymA* transcript is missing in the mutant.

Mapping of the *hymA* gene

The 5 kb *XhoI* restriction fragment was also used as a probe to identify the corresponding cosmids in an ordered cosmid library, kindly provided by R. Prade and J. Arnold (Athens, GA, USA) (Xiong *et al.*, 1996). The fragment hybridized to the three overlapping cosmids SL19F08, RL11H09 and RL30F06 localized at the very end of the left arm of chromosome VI (information available on the Internet: http://fungus.genetics.uga.edu:5080/Physical_Maps.html). The next mapped gene in the

Fig. 6. RNA analysis of *A. nidulans* wild-type (WT) and *hymA* mutant strain SIK5. Total RNA (50 µg) was denatured with glyoxal at 50 °C and separated on a 1% (w/v) agarose gel. After transfer to a nylon membrane the blot was probed with the 5 kb *XhoI* fragment, labelled with ³²P. The signal was obtained after 9 d exposure.

vicinity of these cosmids is *orlA* and the closed marker to *orlA* on the genetic map of *A. nidulans* is *nicC*, 8 cM from *orlA*. However, linkage between *hymA* and *nicC* was not observed in a genetic analysis.

DISCUSSION

Asexual development in *A. nidulans* is defined through the onset of transcription of a number of regulatory as well as structural genes. Regulation of gene function is thereby triggered at the transcriptional or post-transcriptional level (Timberlake, 1993). In addition, genes required for vegetative growth and during development at specific stages have been recognized as important components (Fischer & Timberlake, 1995; Goldman & Morris, 1995; Morris *et al.*, 1995). We have isolated a novel gene, *hymA*, required for hyphal growth and differentiation. Mutation of *hymA* by insertional mutagenesis slightly reduced vegetative growth in comparison to wild-type and led to a specific block in conidiophore development after formation of metulae. Further differentiation and the production of phialides and spores was not observed. Phenotypic studies of *hymA* mutant strains revealed that the metulae resembled hyphae rather than true metulae.

Two possible functions of *hymA* are suggested. First, the pleiotropic changes of conidiophore morphology in *hymA* strains could be explained by *hymA* being involved in differentiation of metulae. *hymA* could be a transcriptional activator, responsible for the expression of metulae-specific genes. A failure of metula maturation would lead

to hypha-like structures. Thus the function of *hymA* in metulae would be comparable to the function of *abaA* in phialides (Andrianopoulos & Timberlake, 1994). However, the reduction of growth rate and the presence of the *hymA* transcript throughout the entire life cycle could not be accounted for entirely by this hypothesis.

Secondly, a molecular function of *hymA* is suggested from the analysis of a double mutant between an *apsB* and a *hymA* strain. In *apsB* strains metulae are anucleate and in *hymA* strains they are nucleate. Therefore it would be expected that the double mutant *hymA/apsB* would produce anucleate metulae. However, the double mutant displayed a *hymA* phenotype. This result could be explained if *aps* (or wild-type) strains generate metulae by a different mechanism to the hypha-like metulae in *hymA* strains. In wild-type conidiophores a transition from hyphal to budding growth occurs as metulae are formed. *hymA* could be involved in this budding process. Hence, *hymA* could encode a protein necessary for e.g. bud site selection, like *BEM1* in *Saccharomyces cerevisiae* (Leberer *et al.*, 1995). Another possibility for the molecular function of *hymA* in budding-like processes could be a regulatory one. *hymA* could be involved in the transition from hyphal to budding growth as a transcriptional activator of budding specific genes. The *hymA* gene of *A. nidulans* might thus be related to genes of *S. cerevisiae* involved in the transition from budding to pseudohyphal growth (Gimeno *et al.*, 1992; Fink & Gimeno, 1994; Fink *et al.*, 1994). The requirement for *hymA* in hyphal growth still remains unexplained. In either case, *hymA* as a structural component or as a regulator, the absence of bud formation could explain why hypha-like structures would grow out of the vesicle. Because these structures do not differentiate into metulae the developmental programme cannot proceed.

Further molecular analysis of the *hymA* gene is needed to elucidate its molecular functions in vegetative growth and during asexual development.

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