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The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*

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ABSTRACT

A characteristic hallmark of *Aspergillus niger* is the formation of black conidiospores. We have identified four loci involved in spore pigmentation of *A. niger* by using a combined genomic and classical complementation approach. First, we characterized a newly isolated color mutant, *colA*, which lacked pigmentation resulting in white or colorless conidia. Pigmentation of the *colA* mutant was restored by a gene (An12g03950) which encodes a putative 4′phosphopantetheinyl transferase protein (PptA). 4′Phosphopantetheinyl transferase activity is required for the activation of Polyketide Synthases (PKSs) and/or Non-Ribosomal Peptide Synthases (NRPSs). The loci whose mutation resulted in fawn, olive, and brown color phenotypes were identified by complementation. The fawn phenotype was complemented by a PKS protein (FwnA, An09g05730), the *ovlA* mutant by An14g05350 (OlvA) and the *brnA* mutant by An14g05370 (BrnA), the respective homologs of *alb1/pksP*, *ayg1* and *abr1* in *A. fumigatus*. Targeted disruption of the *pptA*, *fwnA*, *olvA* and *brnA* genes confirmed the complementation results. Disruption of the *pptA* gene abolished synthesis of all polyketides and non-ribosomal peptides, while the naphtho-γ-pyrone subclass of polyketides were specifically dependent on *fwnA*, and funalenone on *fwnA*, *olvA* and *brnA*. Thus, secondary metabolite profiling of the color mutants revealed a close relationship between polyketide synthesis and conidial pigmentation in *A. niger*.

1. Introduction

Fungi commonly produce characteristic pigments which to some extent are species specific and therefore useful for identification purposes. Conidia of species belonging to the black aspergilli appear dark- or olive-brown to black, although some species produce spores of a lighter shade of brown (Samson et al., 2007). The apparent blackness of these aspergilli results from the combination of brown and green pigments which absorb light across the entire visible spectrum (Ray and Eakin, 1975).

Ray and Eakin (1975) first proposed that the native pigment, aspergillin with a molecular size of about 20,000 is formed from two precursor molecules. The first precursor component consists of a low-molecular weight (368) green pigment and has been identified as a hexahydroxyl pentacyclic quinoid (HPQ). The second precursor component consists of a less well characterized brown low-molecular weight (5000) melanin pigment. Although not proven in *Aspergillus niger*, the melanin pigment is expected to be synthesized via the 1,8-dihydroxynaphtalene (1,8-DHN) pathway, a conserved pathway for melanin production in aspergilli (Tsai et al., 1999; Baker, 2008). The polymerization of the two precursors is copper dependent (Ray and Eakin, 1975). *A. niger* is an important representative of the group of black aspergilli and its biotechnological relevance as a cell factory for the production of hydrolytic enzymes and organic acids has stimulated genome sequencing efforts (Pel et al., 2007, Baker, 2006) and the development of molecular and genomic tools. The genome sequence of *A. niger* and other aspergilli shows that a large part of the 1,8-DHN-melanin biosynthesis pathway is conserved among these species (Baker, 2008).

Spore color mutants are easily identified in aspergilli and have been used extensively to identify and characterize genes and their encoded proteins required for pigmentation. Examples include the

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white (*wA*) and yellow (*yA*) conidiation mutants in *Aspergillus nidulans* (Aramayo and Timberlake, 1990; Mayorga and Timberlake, 1990). Spore color mutants have also been very useful in dissecting the biosynthetic pathway of DHN-melanin in *Aspergillus fumigatus* (Tsai et al., 1997, 1999; Langfelder et al., 1998) and for studying the role of melanin in pathogenicity (Pihet et al., 2009).

The biosynthetic pathway for the production of 1,8-DHN starts with the activity of a specific polyketide synthase, designated wA in *A. nidulans* and Alb1/PksP in *A. fumigatus* (Mayorga and Timberlake, 1990; Tsai et al., 1997, 1999; Langfelder et al., 1998). Mutations in the *A. nidulans wA* gene or the *alb1*/*pksP* gene in *A. fumigatus*, results in white conidiospores lacking melanin. Spore color mutants in *A. niger* have been isolated and include fawn (*fwnA*), olive (*olvA*), brown (*brnA*), and grey (*gryA*) mutants (Pontecorvo et al., 1953; Bos et al., 1988). The *fwnA, olvA* and *brnA* loci were mapped to linkage group I (Bos et al., 1988) and predicted to be present on the right arm of chromosome 1 (Pel et al., 2007). The *gryA* mutation has not been mapped. In addition, an insertional mutagenesis screen yielded a white-spored mutant and analyses showed a insertion that disrupted a polyketide synthase gene homologous to the *wA* (Shuster and Bindel Connelley, 1999).

Both polyketide synthases (PKS) as well as non-ribosomal peptide synthases (NRPS) require posttranslational modification for activation. The modification involves transfer of a 4′phosphopantheteine moiety of coenzyme A to a conserved serine residue in all acyl carrier and peptidyl carrier domains present in PKS or NRPS, respectively. The enzymatic activity required for the activation of PKS and NRPS is possessed by a specific member of the 4′phosphopantheteinyl transferase (PPTase) family. In *A. nidulans*, mutation of one PPTase, isolated by a variety of means have been shown to be required for spore pigmentation (Han and Han, 1993; Chung et al., 1996; Kim et al., 2001), lysine biosynthesis (Márquez-Fernández et al., 2007), secondary metabolite production, including penicillin biosynthesis (Márquez-Fernández et al., 2007; Keszenman-Pereyra et al., 2003), siderophore biosynthesis (Oberegger et al., 2003), and asexual development (Márquez-Fernández et al., 2007). As mutations in the gene encoding the PPTase activity leads to a general defect in activation of PKS and NRPS it explains the effect on the wide spectrum of processes that all require activated PKS and NRPS.

The genome of *A. niger* encodes 34 PKSs and 17 NRPSs and in addition seven hybrid PKS-NRPSs (Pel et al., 2007). A recent survey of secondary metabolite and mycotoxin production in *A. niger* has further underscored the capacity of *A. niger* to produce a broad array of structurally different secondary metabolites (Nielsen et al., 2009). A link between gene(s) involved in synthesis has only been determined for a few; fumonisins, ochratoxin, a siderophore and spore pigmentation (Pel et al., 2007).

We have used spore color mutants to identify genes central to conidial pigmentation of *A. niger*. In this study, we show that a mutant producing white conidia is defective in the *pptA* gene which encodes the PKS/NRPS specific 4′phosphopantheteinyl transferase and we identify the loci defining the fawn (*fwnA*), olive (*olvA*) and brown (*brnA*) mutants by functional complementation and targeted disruption. The defined spore color mutants were finally subjected to secondary metabolite profiling, which revealed strong relationships between pigmentation and secondary metabolism in general and the naptho-γ-pyrone class of polyketides and the related heptaketide funalenone specifically.

2. Materials and methods

2.1. Strains, growth conditions, genetic and molecular techniques

The *Aspergillus* strains used in this study are listed in Table 1. *A. niger* spore color mutants were obtained from Dr. K. de Swart (Wageningen University, The Netherlands) or the Fungal Genetic Stock Centre (FGSC). Strains were grown on minimal medium (MM) (Bennett and Lasure, 1991) containing 1% (w/v) glucose as a carbon source or on complete medium (CM), containing 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract in addition to MM and glucose. When required, medium was supplemented with p-aminobenzoic acid (137 μg/ml), nicotinamine (100 μg/ml), L-tryptophan (20 mg/ml), L-arginine (20 mg/ml), L-histidine (20 mg/ml), L-leucine (20 mg/ml), methionine (30 mg/ml) or uridine (10 mM). Supplementation with siderophores was carried out by using siderophore-containing medium prepared after growth of *A. niger* strain N402 for 48 h in iron-free medium similar as described (Márquez-Fernández et al., 2007). Transformation of *A. niger* was performed as described by Meyer et al., 2010), using 40 mg lysing enzymes (Sigma, L-1412) per g wet weight of mycelium. Heterokaryons were obtained by inoculating haploid strains with complementary auxotrophies in supplemented CM (4 ml) and subsequent growth for 40–48 h at 30 °C. Mycelium was washed in 0.9% NaCl and pieces of mycelium were transferred to MM plates to select for growth of heterokaryons. As observed previously (Bos et al., 1988), heterokaryotic mycelium of complementing spore color mutants occasionally form conidiophores with black conidia indicating complementation.

Escherichia coli strain DH5α was transformed by electroporation for the propagation and amplification of cosmids. XL1-Blue was transformed using the heat shock protocol as described by Inoue et al. (1990) and used for the amplification of plasmids. Fungal chromosomal DNA was isolated as described by Meyer et al. (2010). [α-^{32}P]dCTP-labeled probes were synthesized using the Rediprime II DNA labeling system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. All molecular techniques were carried out as described (Sambrook and Russel, 2001). Sequencing was performed by ServiceXS (Leiden, The Netherlands) or at Macrogene (Seoul, South Korea).

2.2. Construction of plasmids

Plasmids to complement the spore color mutants were constructed by PCR amplification of the corresponding genes using primers listed in Supplementary Table 1 and subsequent cloning in pJet2.1 (Fermentas). Each gene was then released by digestion with *Not*I and cloned into pMA171 and/or pMA172 (Carvalho et al., 2010). The pMA171 and pMA172 vectors contain the *pyrG* or the hygromycin selection marker respectively as well as the pAMA sequence that enable autonomous replication of each plasmid in *A. niger* (Aleksenko and Clutterbuck, 1996, 1997).

The DNA sequences of several loci of the parental strain (N402) and mutants were determined by sequencing three independent clones of the different PCR products. The PCR products obtained were cloned into pJet1.2 and sequenced with appropriate primers. Sequences of the sequencing primers are available on request.

The *pptA* deletion cassette was constructed using multi-site GATEWAY technology using primers listed in Supplementary Table 1. Before transformation to AB4.1 the construct was linearised with *Bgl*I. The *fwnA, olvA and brnA* disruption cassettes were constructed by PCR amplification of 5′ and 3′ sequences flanking the corresponding coding regions using primers listed in Supplementary Table 1 and PCR amplicons were cloned of into pJet1.2. The 5'flanks were reisolated and ligated into pBluescriptKS after digestion with appropriate restriction enzymes that were introduced with the primers. The resulting plasmids were digested with XhoI and HindIII and the *A. oryzae* 1.7 kb XhoI/HindIII *pyrG* fragment from pBS-*pyrG* (Carvalho et al., 2010) was ligated into the vectors to give the final deletion constructs. A 3.1 kb XhoI–HindIII fragment from pAN7.1, containing the hygromycin resistant cassette, was used in a similar way to construct the deletion cassette with

Table 1
Strains used in this study.

Strain identifier	Relevant color allel	Genotype	Reference
N402	–	*cspA1*	Bos et al. (1988)
AB4.1	–	*cspA1, pyrG378*	van Hartingsveldt et al. (1987)
MA70.15	–	*cspA1, kusA::amdS, pyrG378*	Meyer et al. (2007)
MA169.4	–	*cspA1, kusA::DR-amdS-DR, pyrG378*	Carvalho et al. (2010)
N651	*fwnA1*	*cspA1, hisD5,pyrA5, leuA1, nicA1*	Swart et al. (2001)
A896/N531	*fwnA3*	*cspA1, pabB2, trpA1*	FGSC[a]
N647	*fwnA6*	*cspA1, argB13, nicA1*	Strain collection WUR[b]
A802/N474	*olvA1*	*cspA1, argF8*	FGSC
A89 N/6397	*brnA2*	*cspA1, metB2*	FGSC
A88 N/4583	*gryA1*	*leuB3, argE5*	FGSC
AR20.3	-	*cspA1, pyrG378*	This study
colA		*cspA1, pyrG378*	This study
colB		*cspA1, pyrG378*	This study
colC		*cspA1, pyrG378, ts color allel*	This study
colD		*cspA1, pyrG378, ts color allel*	This study
colE		*cspA1, pyrG378*	This study
colF		*cspA1, pyrG378 (osm. remediable color allel)*	This study
colG		*cspA1, pyrG378*	This study
colH		*cspA1, pyrG378*	This study
colI		*cspA1, pyrG378*	This study
colJ		*cspA1, pyrG378*	This study
colK		*cspA1, pyrG378*	This study
MGG029Δ*aamA*	*fwnA*	*cspA1, prtT, glaA::pleo, aamA::pyrG,*	Weenink et al. (2006)
MA93.1	*ΔfwnA*	*cspA1, fwnA::hygB in N402*	This study
MA100.1	*ΔfwnA*	*cspA1, kusA::amdS, fwnA::hygB, pyrG378*	This study
JP1.1	*ΔpptA*	*cspA1, pptA::AopyrG in N402*	This study
AW8.4	*ΔolvA*	*cspA1, olvA::AopyrG* in MA169.4	This study
AW6.1	*ΔbrnA*	*cspA1, brnA::AopyrG* in MA169.4	This study
AW7.1	*Δ An08g09920*	*cspA1, An08g09920::AopyrG* in MA169.4	This study
AW11.1	*ΔbrnAΔolvA*	*cspA1, brnA::AopyrG, olvA::hygB* in MA169.4	This study
AW9.1	*ΔAn08g09920ΔbrnA*	*cspA1, An08g09920::AopyrG, brnA::hygB* in MA169.4	This study
AW10.1	*Δ An08g09920ΔolvA*	*cspA1, An08g09920::AopyrG, olvA::hygB* in MA169.4	This study
MA220.1	*ΔfwnAΔolvA*	*cspA1, fwnA::hygB, olvA::AopyrG* in MA169.4	This study
MA221.1	*ΔfwnAΔbrnA*	*cspA1, fwnA::hygB,brnA::AopyrG* in MA169.4	This study

[a] Fungal Genetic Stock Centre.
[b] Wageningen University and Research centre.

the hygromycin selection marker. All plasmids used in this study are listed in Supplementary Table 2.

2.3. Complementation of the colA mutant

ColA (pyrG⁻) was transformed with a genomic cosmid library in an AMA1 containing self-replicating vector (Gems and Clutterbuck, 1993) using the *pyrG* selection marker. Growth of the *colA* mutant was reduced on MM (see Results section) which facilitated cloning of the gene. Complementation of the *colA* spore color mutant was performed by screening uridine prototrophic transformants for restored growth on MM and black conidiation. Cosmids from putative complemented black conidiating *colA* transformants were isolated using the protocol for isolation of genomic DNA (Kolar et al., 1988) and transformed to *E. coli* (DH5α) via electroporation and grown on LB-plates with ampicillin. Subsequent cosmid isolations from 40 ml of overnight cultures were performed using the small scale DNA isolation method as described by Sambrook and Russel (2001). Primers cosT7 and cosUL (Supplementary Table 1) were used for sequencing the ends of the inserts.

2.4. Melanin isolation and spectral analysis

Conidia were harvested from CM plates after seven days of incubation at 30 °C. Pigment was extracted by suspending conidia in 0.5 M NaOH and incubating the suspension for at least 8 min in a bath of boiling water. The absorption spectrum for visible light (390–750 nm) was determined for each pigment-extract to define its spore color composition.

2.5. Microscopy

Overview pictures of subaerial colonies were taken with an Olympus Camedia digital camera (C-5050 zoom) and more detailed micrographs illustrating aerial development were obtained using a Leica EZ4D stereo microscope (8–35×). Scanning electron microscope (SEM) pictures were obtained from plate grown *A. niger* strains after growth for 7 days on complete medium as described (Jørgensen et al., 2010).

2.6. Secondary metabolite analysis

Secondary metabolites were extracted from three 6 mm plugs of 8 day Yeast Extract Sucrose (YES) (Frisvad and Samson, 2004) cultures using methanol/dichloromethane/ethyl acetate (1:2:3 v/v/v) with 1% formic acid (Nielsen et al., 2009) of the wild type (N402) and pigmentation mutants (*ΔpptA*, *ΔfwnA*, *ΔolvA* or *ΔbrnA*). Analysis of the secondary metabolites was performed using HPLC–DAD–TOF mass spectrometry, using positive electrospray ionization. Separation was done on at 15–100% acetonitrile gradient on a 2 mm ID, 3 μm, 5 cm Luna C_{18} (II) column as described recently (Nielsen et al., 2009). The target ions, usually $[M+H]^+$, $[M+NH_4]^+$, $[M-H_2O+Na]^+$, and $[M+Na]^+$ were extracted with an interval of mass ± *m/z* 0.02 were used for identification of all compounds. The peak areas from the extracted ion chromatograms were extracted using QuanLynx 4.1 (Waters-Micromass, Manchester, UK) and the peak areas compared between samples. Peaks not matching the metabolite list in Nielsen et al. (2009) were matched against an internal database of approx 850 reference standards as well as the 35500 structures in Antibase2009 Antibase2010 (John Wiley and Sons, Inc.).

3. Results

3.1. Complementation analysis of A. niger spore color mutants

In *A. niger*, four different loci have been described in the literature that affect conidial color. These loci are named fawn (*fwnA*), brown (*brnA*), olive (*olvA*) and gray (*gryA*) and with the exception of *gryA*, their linkage groups had been established (Bos et al., 1988; Debets et al., 1993). In this study, we isolated eleven additional spore color mutants (named *colA* – *colK*) after UV mutagenesis that vary in their spore color from almost completely white or colorless (*colA, colI*) to fawn (*colB, colE, colF, colG, and colJ*), olive (*colD*), brown (*colH and colK*) and gray (*colC)* (Fig. 1). Two mutants showed a temperature sensitive color phenotype (*colC* and *colD*). Some of the color groups (fawn, olive and brown) obtained appear to correspond to previously described color mutants/genetic loci. At the permissive of 30 °C, spore pigmentation was black whereas at 38 °C, spores appear to be gray or olive, respectively (Fig. 1). The color of *colF* spores was fawn on normal CM medium, but on CM containing 1.2 M sorbitol, spores were black, indicating the high osmolarity conditions rescues the phenotype of the mutant (results not shown).

To test allelism between the spore color mutants, the strains carrying complementary auxotrophies were grown together on minimal medium to stimulate the formation of heterokaryotic mycelium. The formation of black conidia in heterokaryons was taken as the read out to determine complementation groups (Bos et al., 1988). The results of the complementation analysis are summarized in Table 2. The complementation analysis confirms previous studies of Bos and co-workers and resulted in the identification of five complementation groups that affect pigmentation in *A. niger*. The first group consisting of two mutants: *gryA* and *colA*. The *colA* mutant shows the most extreme spore color change, resulting in almost white or colorless conidia. As shown below in detail, the *gryA* mutant is most likely partial defective allele of the same gene. The largest group of spore color mutants (ten alleles) forms the fawn group. Noticeable from Fig. 1, there are different shades of the fawn color within this complementation group. Whereas some mutants display a light fawn color (*colB, colE, colF, colJ, fawnA1, fawnA6*) others e.g. *fawnA3* and the *fawnA* allel of MGG029 are more brownish. An additional olive mutant (*colD*) was present among our new spore color mutants. This mutant displays an olive pigmentation phenotype only when grown at 38 °C. A third complementation group consists of brown mutants. All three mutants (*brnA2*, *colH* and *colK*) produce a very characteristic brown spore color. The fourth group includes a temperature sensitive (TS) mutant. The color of *colC* mutant spores is almost black after growth at 30 °C while they are gray after growth at 38 °C. The gray color may reflect a partially active protein at the higher temperature rather than complete inactivation. Finally, a new complementation group was identified and which is represented by mutant *colI*. As shown in Fig. 1, the color of the spores produced by this mutant is similar to that of the *colA* mutant. Interestingly, the pigmentation in the *colI* mutant depends on the composition of the medium. On minimal medium the mutant produces black spores whereas on complete medium the spores are whitish. These mutants, as well as the different alleles within the five complementation groups, form a solid basis to start analysis of the genetic basis of conidial pigmentation in *A. niger*.

3.2. Complementation of colA identifies the 4′phosphopantetheinyl transferase encoding gene (pptA) as required for spore pigmentation in A. niger

On complete medium plates *colA* produces white conidia (Fig. 1). In addition, the *colA* mutant displayed reduced growth on MM (results not shown). To identify the *colA* locus, an *A. niger* cosmid library was used to complement the *colA* mutant strain. Uridine prototrophic transformants were screened for restoration of growth on MM and formation of black conidia. Two isolates of approximately 8000 transformants grew normally and with black conidia. The ability to form black spores was lost under non-selective conditions (CM with uridine), indicating that the complementation was plasmid based. Cosmids from the two complemented strains were isolated and retransformed into *colA* which confirmed the complementation by the two cosmids (pRD8.1#7 and pRD8.1#9). Restriction analysis revealed an identical restriction pattern, indicating the both cosmids contain the same genomic insert of approximately 18 kb (results not shown). Sequence analysis of the insert indicated that it included gene An12g03950 which is predicted to encode a 4′phosphopantetheinyl transferase (PptA). Mutants in the homologous *pptA* gene in *A. nidulans* include *cfwA* (cross feeding white) or *npgA* (non-pigmented) (Márquez-Fernández et al., 2007; Oberegger et al., 2003; Keszenman-Pereyra et al., 2003) displaying similar effect on spore color as observed for the *colA* mutant of *A. niger*.

3.3. Genetic and phenotypic characterization of the pptA/colA mutants

Two approaches were taken to confirm that the *colA* phenotype is due to a mutation in the *pptA* gene (An12g03950). First, the

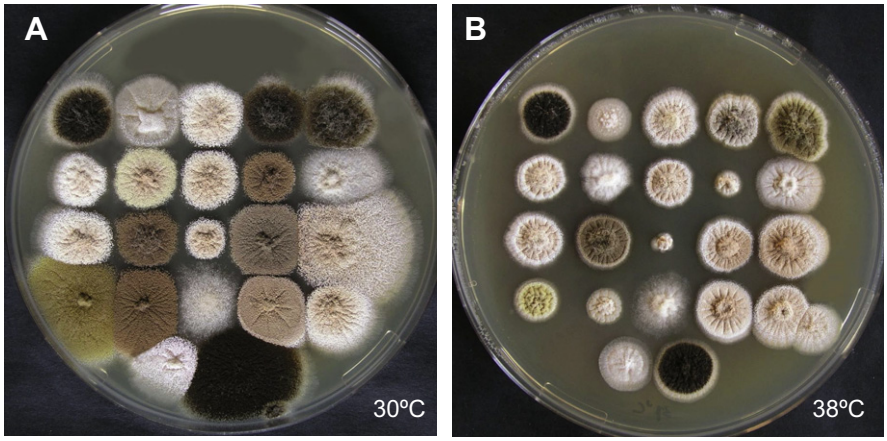

Fig. 1. Color mutants of *A. niger*. Mutants were point inoculated on complete medium plates and grown for 6 days at 30 (A) or 38 °C (B). First row from left to right are spotted: AB4.1 (wild type), *colA*, *colB*, *colC*, *colD*; second row: *colE*, *colF*, *colG*, *colH*, *colI*; third row: *colJ*, *colK*, *fwnA1*, *fwnA3*, *fwnA6*; fourth row: *olvA*, *brnA2*, *gryA*1, MGG029, *ΔfwnA*; Fifth row: *ΔpptA* and N402 wild type.

Table 2
Summary of complementation analysis.

Locus	Mutant alleles	Gene identifier	Proposed linkage group	Reference linkage group
pptA	*colA*, *gryA1*	An12g03950	n.d.[c]	
fwnA	*fwnA1*, *fwnA3*, *fwnA6*, *fawnA*, *colB*, *colC*[a], *colE*, *colF*[b], *colG*, *colJ*	An09g05730	I	Swart et al. (2001)
brnA	*brnA2*, *colH*, *colK*	An14g05370	I	Swart et al. (2001)
olvA	*olvA1*, *colD*[a]	An14g05350	I	Swart et al. (2001)
	colI[d]		n.d.[c]	

[a] Temperature sensitive mutation.
[b] High sorbitol remediable.
[c] Not determined.
[d] Spore color is dependent on medium. Minimal medium black spores, on complete medium gray spores; Mutations in underlined genes are confirmed by sequencing analysis.

predicted *pptA* coding sequence, promoter and terminator region were cloned by PCR from wt DNA into plasmid pAMA-pyrG generating plasmid pJHP2. pJHP2 was then transformed into the *colA* strain. The production of black conidia by numerous transformants generated confirmed complementation of *colA* by *pptA*. Second, we sequenced the *pptA* gene in the *colA* mutant which revealed a single point mutation (T to A) at position 738 resulting in the introduction of a stop codon (TAT, tyrosine into TAA, stop codon) (Table 3). The PptA protein in the *colA* mutant is consequently truncated from 318 to 245 amino acids. The complementation analysis (Table 2) also indicated that the previously defined gray mutant and our *colA* mutant were alleles. Sequence analysis of the *pptA* locus in the *gryA* mutant identified a single nucleotide difference (A to C) at position 183, resulting in a lysine to asparagine amino acid change at residue 61 of the PptA predicted protein (Table 3). Further studies are required to verify the critical nature of this amino acid in PptA function.

In *A. nidulans*, it has been shown that PptA activity is required for lysine and siderophore biosynthesis (Keszenman-Pereyra et al., 2003; Márquez-Fernández et al., 2007). Supplementation of MM with lysine and siderophore-containing medium (see Section 2) restored growth of the *colA* mutant on MM, indicating that also in *A. niger* the PPTase activity is required for lysine biosynthesis (activating the enzyme α-aminoadipate reductase) and Non-Ribosomal-Pepetide Synthase (NRPS)-dependent siderophore biosynthesis (not shown).

The *pptA* gene was deleted by transforming pΔ*pptA* into pAB4.1. Transformants were obtained and purified on MM supplemented with lysine and siderophore-containing medium. Transformants that produced white conidia were expected to contain the *pptA* deletion. White sporulating transformants as well as some black transformants were analyzed by diagnostic PCR and proper deletion of the *pptA* gene was confirmed by Southern blot analysis for the white sporulating strains (not shown). The importance of supplementation with lysine and siderophores to sustain growth on MM was also evaluated. Supplementation with both lysine and siderophore in the growth medium resulted in the best growth of the Δ*pptA* mutants (Fig. 2). Conidiation of the Δ*pptA* mutant was about 90% reduced compared to the wild type strain (N402), but not abolished.

3.4. Identification of loci defining the fawn, olive and brown complementation groups

Efforts to complement the other spore color mutants by transformation with the cosmid library were not successful. Therefore, candidate genes were identified by a combination of homology searches and microarray analysis. We recently performed transcriptomic analysis of submerged sporulating cultures of *A. niger* and candidate genes involved in the pigmentation of *A. niger* spores that were induced during sporulation were identified (Jørgensen et al., 2010). The candidate genes involved in the synthesis of conidial melanin based on homology or their expression profiles are listed in Supplementary Table 4. Four genes encoding the putative polyketide synthase (An09g05730), the YWA1 hydrolase homolog (An14g05350), and the multicopper oxidase homolog (An14g05370) were PCR amplified and cloned into autonomously replicating vectors to perform complementation analysis. The complementation analysis revealed that all the fawn mutants are specifically complemented by the polyketide synthase (An09g05730) gene, the olive mutants *(olvA)* are complemented by the YWA1 hydrolase homolog (An14g05350) and that the brown mutants *(brnA)* are complemented by the multicopper oxidase homolog An14g05270 gene (data not shown). Based on the complementation analysis, the gene names, *fwnA*, *olvA* and *brnA* were assigned to the respective loci (Table 2). The polyketide synthase identified (An09g05730) is the identical to the polyketide synthase that has been previously identified in the white-spored mutant from a insertional mutagenesis screen (Shuster and Bindel Connelley, 1999).

To further confirm the complementation results, alleles from representative mutants were sequenced. The results are given in Table 3. For all mutants analyzed, differences in DNA sequence were found in the different genes that were complemented by

Table 3
Sequencing results color mutant genes.

Gene	Allele	Protein length in mutant	DNA mutation	Mutation in protein sequence
pptA	*colA*	245	T to A	TAT to TAA (Tyr into stop at AA position 245)
	gryA	318 (wild type)	A to C	AAA to AAC (Lys into Asp at AA position 183)
fwnA	*fwnA3*	2153 (wild type)	AC to TT	AAC to ATT (Asp to Iso, AA position 531)
	fwnA6	1400	ctt cag ata	Deletion of tt cag a at AA 1400 frameshift
	colJ	18	G to T	GAA to TAA (GLU into stop) at position 18
olvA	*olvA*	406 (wild type)	A to G	AGG to GGG (Arg to Gly,at aa position 372)
	colD	211	T to A	TGT to TGA (Cys into stop at AA position 211)
brnA	*colH*	547 (wild type)	T to C	TGG to CGG (Trp to Arg at AA position, 79)
	colK	Unknown	Unknown	Rearrangement on the genomic locus

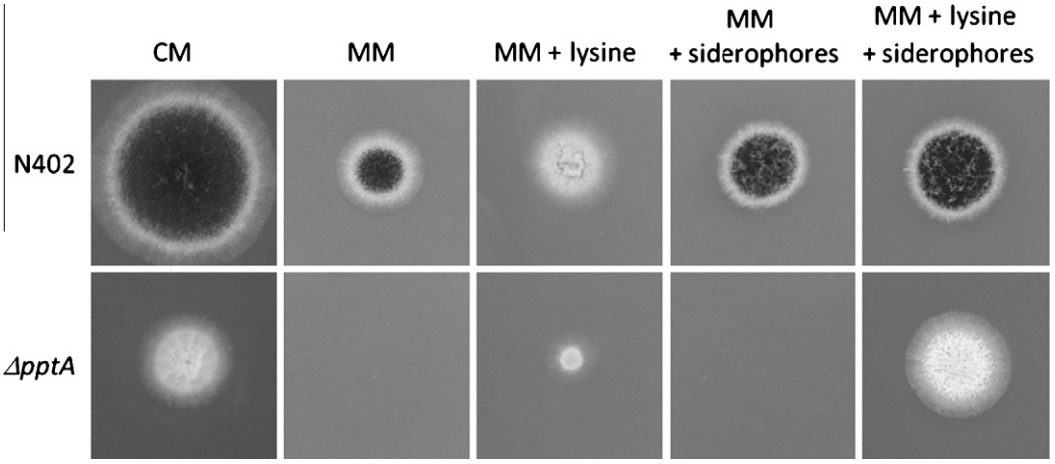

Fig. 2. Growth supplementation of the *pptA* disruption strain with lysine (10 mM) and siderophore enriched growth medium. Approximately 1×10^4 spores were point inoculated in the center of complete medium (CM) or minimal medium plates (MM) with supplements as indicated.

the different transforming vectors respectively. For the alleles that result in a frame shift or stopcodon, we can conclude that these mutation lead to an inactive gene product. For those mutants with a change in amino acid sequence, additional studies are required to prove that the amino acid substitution is relevant for its phenotype. For the *colK* mutant (representing a *brnA* allele), no PCR fragment could be amplified. Southern analysis, after digestion with *Bam*HI revealed a rearrangement of the *colK* locus as compared to the wild type (data not shown) and we assume that the rearrangement result in a non-functional *brnA* gene.

Since *A. niger* is an asexual fungus, it is difficult to remove ancillary or secondary mutations that might have been generated in other parts of the genome by UV mutagenesis. As shown in Fig. 1, many mutants generated by UV mutagenesis suffer various growth defects at 38 °C that are most likely not related to the spore color phenotype. To be able to study the effect of the spore color mutations in a clean genetic background (that is a genetic background with no other mutations), disruption constructs for the *fwnA*, *olvA* and *brnA* genes were constructed to generate null mutants. Spore color mutants were already identified on the primary transformation plates, purified and analyzed by Southern blot analysis. The spore color of the various mutants is shown in Fig. 3A, and they are identical to the mutants identified by the various mutant screens. Comparison of the Δ*pptA* and the Δ*fwnA* strain (Fig. 3A) clearly indicate that the fawn color displayed by the Δ*fwnA* spores are likely due to a second, PptA dependant PKS or perhaps even an NRPS. The conidiophores formed by the *pptA* and *olvA* deletion mutants were consistently reduced in size in comparison with the other mutants strains, and suggest that these genes are required for other developmental processes in addition to pigmentation (Fig. 3C). Conidiospores of the wild type and spore color mutants were all examined by scanning electron microscopy (Supplementary Figure 1). WT conidia show a rough ornamented surface as has been described previously (Kozakiewicz, 1989). All spore color mutants showed reduced ornamention, and the largest reduction was observed in the Δ*pptA* mutant. In this mutant background, both spores with an entirely smooth surface were observed, as well as spores in which the ornaments were reduced to small spikes. The spores with the smooth surface might represent not fully matured spores in the Δ*pptA* background.

The different spore color genes are expected to function in a linear pathway producing the black melanin. Epistasis can be studied by constructing double mutants. To determine the epistasis for the fawn, olive and brown mutants, the double mutants was constructed in all possible combination and confirmed by Southern blot analysis. As expected, *fwnA* turns out to be epistatic over *olvA* and *brnA* and *olvA* is epistatic over *brnA* (not shown).

3.5. Spectral analysis of the melanin pigments isolated from the spore color mutants

Spore color perception is influenced by many factors and the way mold colonies present their colors also affect our evaluation of conidial pigmentation. From observations with a dissection microscope it was clear that strain background, exudates, conidiation and pigmentation intensity affect the overall spore color. To obtain a more objective definition of the spore pigmentation of the color mutants we extracted the spore pigments into solution and subjected these to spectral analysis (Fig. 3B and data not shown).

The wild-type pigmentation, represented by N402 and AB4.1, consisted of two dominant features, two overlapping broad peaks with maxima around 425 and 575 nm, respectively (Fig. 3B). Together the peaks absorb light in the entire VIS-spectrum explaining the black appearance of massed *A. niger* conidia. Upon dilution of the extracted pigments from the wild-type conidia, the color appeared olive which indicates that the perceived color is dependent on the intensity of the pigment.

The green component (absorption maximum around 575 nm) and the brown component (max at 425 nm) were both FwnA-dependent and loss of this function led to an anonymous light brown or fawn, based in a broad declining absorption from the UV into the VIS spectrum (Fig. 3B). The strains in the fawn complementation group display various intensities of the fawn color, but the spectra of the isolated melanin were similar (data not shown). In the *olvA*, *brnA* and *pptA* mutants, characteristic absorption spectra were observed (Fig. 3B and data not shown). The spectrum of *coll*, the mutant defining a fifth complementation group was almost identical to the spectrum of the wild type strain, indicating that it is likely that the amount of melanin produced in these spores is reduced, but that the produced melanin is similar to the wild type produced melanin (data not shown).

3.6. Conidial pigmentation and secondary metabolism are intimately related in A. niger

The spectrum of secondary metabolites produced by *A. niger* is relatively well characterized and has recently been reviewed by Nielsen et al. (2009). After 8 days incubation on YES medium, *A. niger* N402 had formed a number of secondary metabolites among

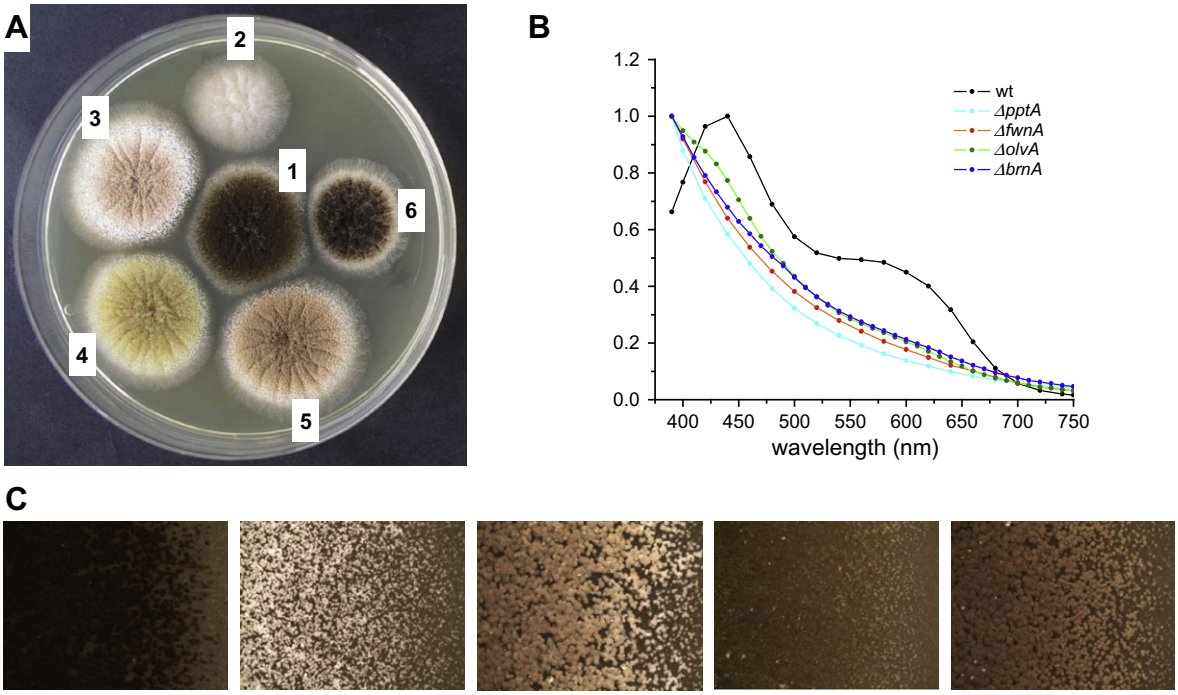

Fig. 3. Pigmentation of *A. niger* gene disruptants. A), massed conidia on colonies of (1) wt/N402, (2) *ΔpptA*, (3) *ΔfwnA*, (4) *ΔolvA*, (5) *ΔbrnA* and (6) ΔAn08g09920. B, absorption spectra (VIS) for wild type (wt) and genetically defined mutants representing each of the four complementation groups. C), conidial heads of (from left panel) wt, *ΔpptA*, *ΔfwnA*, *ΔolvA* and *ΔbrnA* after 6 days incubation on MM at 30 °C.

which major polyketide species such as the linear fumonisins and cyclic naphtho-γ-pyrones. Also the nitrogen containing compounds, pyranonigrin and tensidol B were found. No secondary metabolites were detected when the *pptA* strain had been incubated in the same conditions (Table 4). This also implies that all the listed compounds are dependent on the activity of either a polyketide or a non-ribosomal peptide synthase. A subset of polyketide metabolites were specifically affected by disruption of *fwnA*. The naphtho-γ-pyrones (Fig. 4), including the structural dimers aurasperone B, aurasperone C, and monomers, flavasperone and fonsecin were like funalenone absent in *ΔfwnA* strains (Table 4). Disruption of *olvA* and *brnA* had also distinctive effects on secondary metabolite formation. Both mutations resulted in loss of funalenone accumulation (Table 5), and flavasperone and an unidentified naphtho-γ-pyrone (TMC-256A1) were in addition absent in *ΔolvA* (Table 5).Whereas *ΔbrnA* produced wt like levels of aurasperone and other secondary metabolites listed in Table 5, *ΔolvA* also had reduced accumulation of other non-naphtho-γ-pyrone metabolites with exception of the reduced polyketides fumonisins. The naphtho-γ-pyrones are dimerized from fonsecin, heminigerone and other monomers and the ability to dimerize has often been related to spore pigment production in *Aspergillus*. The heptaketide funalenone has not been previously related to the naphtho-γ-pyrones, but seems to be related also to the black conidium color in *A. niger*. On the other hand the kotanin hexaketides are not directly related to the black conidial pigments, even though they are dimerized.

Table 4
Secondary metabolite diversity expressed by wt, *ΔfwnA* and *ΔpptA* strains after 8 days growth on YES agar.

Secondary metabolite[a]	Genotype[b]		
	wt	*ΔfwnA*	*ΔpptA*
Aspereynone	+	+	ND[c]
Undescribed polyketide ($C_{18}H_{12}O_6$, UV max 270 and 392 nm)	+	+	ND[c]
Demethylkotanin & kotanin	+	+	ND[c]
Fumonisins (B_2, B_4 and B_6)	+	+	ND[c]
Orlandin	+	+	ND[c]
Pyranonigrins	+	+	ND[c]
Tensidol B	+	+	ND[c]
Funalenone	+	ND[c]	ND[c]
Naphtho-γ-pyrones	+	ND[c]	ND[c]

[a] Secondary metabolites (Nielsen et al., 2009) detected in wildtype material.
[b] wt = MA70.15, *ΔfwnA* = MA93.1, *ΔpptA* = JP1.1.
[c] ND = not detected.

4. Discussion

In this study, a combination of molecular, genetic and genomic tools has been used to identify four genes (*pptA*/An12g03950, *fwnA*/An09g05730, *olvA*/An14g05350 and *brnA*/An14g05370) required for development of the characteristic black spore pigmentation of *A. niger*. A subsequent metabolic profiling of the corresponding four genetically defined color mutants revealed strong links to secondary metabolite formation, especially the naphtho-γ-pyrone polyketides. The four genes identified include *pptA*, which encodes for a 4′phosphopantetheinyl transferase, an activity required for activation of polyketide synthases (PKSs) and Non-Ribosomal peptide synthases (NRPSs). Supplementation studies and secondary metabolite analysis of the *pptA* mutant confirmed the pleiotrophic effect of the mutant and showed the requirement of PptA activity for lysine and siderophore biosynthesis and secondary metabolite production. Complementation and targeted deletion analysis confirmed that the fawn-colored mutants in *A. niger* are complemented by a PKS encoding gene (An09g05730) and targeted disruption showed that it is required for wt conidial pigmentation. This gene has been identified previously as a white-color mutant in a REMI-based mutagenesis screen (Shuster and Bindel Connelley, 1999). The *olvA* and *brnA* mutants were complemented by two genes that are homologs to

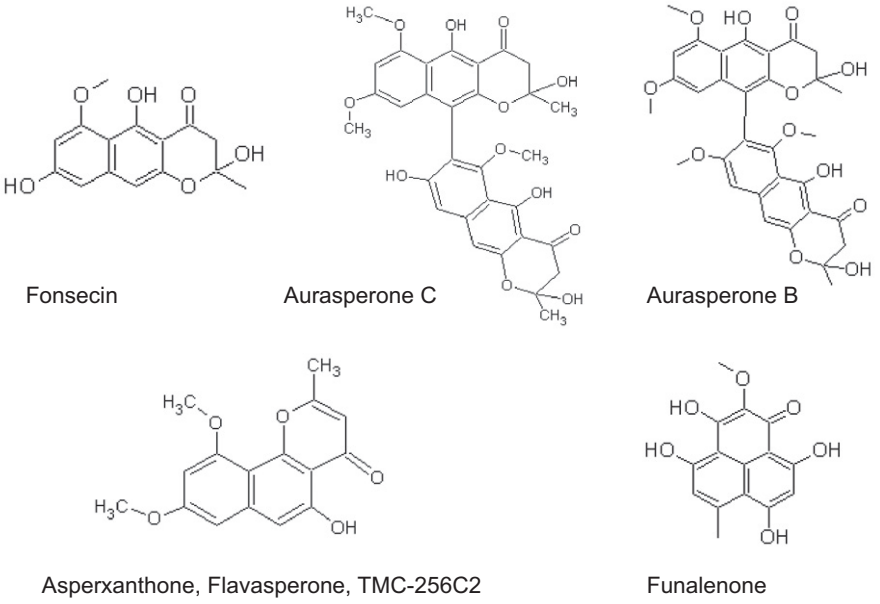

Fig. 4. Major naphtho-γ-pyrones species and funalenone formed by *A. niger* under dependence of *fwnA* (Nielsen et al., 2009 and reference herein).

Table 5
Accumulation of the *fwnA*-dependent funalenone and naphtho-γ-pyrones in *ΔolvA* and *ΔbrnA* after 8 days growth on YES agar.

Secondary metabolite[a]	Genotype[b]		
	wt	*ΔolvA*	*ΔbrnA*
Funalenone	+++[c]	ND[d]	ND
Naphtho-γ-pyrones			
TMC-256A1	+	ND	+
Flavasperone	++	ND	++
Fonsecin	++	++	++
Aurasperone B	+++	+++	+++
Aurasperone C	+++	+++	+++

[a] Compounds (Nielsen et al., 2009) detected in wildtype material.
[b] wt = MA70.15, *ΔolvA* = AW8.4, *ΔbrnA* = AW6.1.
[c] +++ is the maximum of the secondary metabolite being produced by the wt. The amount of secondary metabolite being produced was measured by a diode array detector at 210 nm and the log area (log A) of the chromatographic peaks was calculated. +++ values had a log A of 2–3.6, while ++ is approximately one log value lower (1–2.6) and + means approximately 2 log value lower (0.1–1.6).
[d] ND = not detected, the chromatographic trace was without any peaks at all at the particular retentiontime.

A. fumigatus proteins earlier described to be required for pigmentation (Tsai et al., 1999). OlvA is highly homologous to the *A. fumigatus* AygA protein. This protein has been shown to convert the heptaketide naphthopyrone (formed by the polyketide synthase) to the pentaketide 1,3,6,8-tetrahydroxynaphthalene (T4HN) (Fujii et al., 2004). BrnA is homologous to the *A. fumigatus* Abr1 protein which has high similarity to a multicopper oxidase and may have a function late in the DHN-melanin pathway catalysing oxidative polymerization or modification of the polyketide precursors (Tsai et al., 1999)

Although *A. niger* contains orthologs of some of the *A. fumigatus* genes involved in the synthesis of DHN-melanin, the genes encoding the HN-reductase, scatalone dehydratase, or the laccase have not yet been associated with specific color mutants nor identified by homology searches (Baker, 2006). Blast searches using the *A. fumigatus* HN-reductase (Arp2) and laccase (Abr2) identified several potential candidates (Baker, 2008, own results, see Supplementary Table 4). The possibility that multiple genes serve each function might hamper their identification via mutant screens. The expression profile of the different candidates were also examined in a microarray data set on *A. niger* mycelium that was cultivated under conditions known to induce massive conidiation in liquid culture (Jørgensen et al., 2010). Of the candidate genes which were differentially expressed (Supplementary Table 4), An01g13660, encoding a putative laccase increased over 230-fold during conidiation. Additional work will be required to see if An01g13660 is involved in spore pigment synthesis. The *A. niger* genome contains a single gene (An08g09920) displaying a low level of identity (E-value BlastP 7e-15) to the *A. fumigatus* scylatone dehydratase (Arp1). An08g09920 is not expressed during submerged conidiation (Supplementary Table 4). To analyze a possible role in spore pigmentation, the An08g09920 gene has been disrupted. The color of the spores of the mutants was identical to its parental strain, indicating that this gene is not required for pigmentation of the spores. (van Welzen and Arentshorst, unpublished results)

Spore pigmentation in aspergilli has been studied in most detail in *A. fumigatus*. The identification of the genes related to spore pigmentation in *A. fumigatus* was relatively straightforward because of the genomic clustering of the genes involved (Tsai et al., 1999). The color genes identified in *A. niger* are not clustered. However, the *olvA* (An14g05350) and *brnA* (An14g05370) are in close proximity and interrupted by only one predicted ORF: An14g05360. This gene encodes a hypothetical protein of 307 amino acids without any known function. Other organisms in all kingdoms do not have protein othologous to An14g05360 which makes it a unique protein for *A. niger*. Expression analysis indicates that the gene is expressed (data not shown), but not differentially expressed during submerged conidiation (Supplementary Table 3). The six genes in the pigmentation gene cluster in *A. fumigatus* are all induced during sporulation (Tsai et al., 1999). To see if our Blast-P searches had missed possible candidates which may be involved in conidial pigmentation, we examined the expression of all genes neighboring *olvA* and *brnA* (Supplementary Table 3). Of the neighboring genes only An14g05340 showed a strong induced expression during sporulation. Blast searches suggest that An14g05340 might encode a Glycosyl Hydrolase family 88 member, but its possible enzymatic function is unknown. It has yet to be determined whether this gene is involved in pigmentation.

A. niger color mutants have been used for studies of parasexual and mitotic recombination linkage maps for almost 60 years (Pontecorvo et al., 1953; Bos et al., 1988; Debets et al., 1993). The identification of the different color genes in this study (fawn (*fwnA*), brown (*brnA*) and olive (*olvA*) allows the comparison of the genetic map and the physical maps. Previously, the fawn and brown mutations have both been mapped on the same arm on chromosome I (Debets et al., 1993), but the complementation experiments described in this study clearly indicate that the complementing genes are from two different contigs (9 and 14) representing the right and left arm of chromosome I (Pel et al., 2007). As the DSM genome sequence (Pel et al., 2007) was obtained from a different *A. niger* strain (CBS513.88) used in our study (strain N402), we compared the location of the *fwnA* gene and the *brnA* gene also in our recently sequenced N402 strain (Nitsche et al., unpublished results). The comparison did not indicate a translocation event of the genomic regions containing the *brnA* and the *fwnA* loci. Also comparison of the third *A. niger* genome sequence available (ATCC) which was sequenced at the JGI/DEO genome did not suggest a translocation event in this part of the genome (Roubos and Van de Vondervoort, personal communication).

Several groups have isolated color mutants in *A. niger* (Pontecorvo et al., 1953; Bos et al., 1988, this study). Our complementation analysis reveals five complementation groups and except for *colI* multiple mutants for each group were found. Although we cannot exclude that no additional color mutants can be identified by random mutagenesis approaches, one could also argue that it is likely that additional enzymes involved in pigmentation might be redundant and therefore difficult to be isolated by random approaches. Alternatively, the color difference might be subtle and not easy to see by eye.

Another approach to study pigmentation and finally resolve the genetic elements involved is to identify the products and intermediates of the underlying pigment biosynthetic pathways which would allow prediction for the genes involved. The fact that homologous genes of central DHN-melanin pathway genes are missing in *A. niger*, combined with the observation that pigmentation is resistant to the DHN pathway inhibitor tricyclazole (Wheeler, 1983; Jørgensen, unpublished data) and that residual pigmentation is found in *ΔfwnA* void of the DHN-related naphtho-γ-pyrones all indicate that conidial pigmentation in *A. niger* is more complex than expected from the current model of the fungal DHN-melanin pathway (reviewed by Langfelder et al. (2003)). It should be noted that fonsecin is chemically closely related to YWA1 only differing by a methylation of the 1-hydroxy group. The open form of YWA1 is the precursor of T4HN and 1,8-dihydroxynahpthol in *A. fumigatus* (Fujii et al., 2004) while in *A. niger* the open form of fonsecin, if existing, would result in the formation of 1-methoxy-8-hydroxynaphtalene, which is not prone to polymerization into melanin.

A. niger ΔpptA produces nearly white conidia and no secondary metabolites of polyketide or non-ribosomal peptide origin. These phenotypes are very like those described for the same genotype in *A. nidulans* (Márquez-Fernández et al., 2007; Oberegger et al., 2003; Keszenman-Pereyra et al., 2003), as well as for *Penicillium chrysogenum* (García-Estrada et al., 2008) and *Colletrotrichum graminicola* (Horbach et al., 2009). The significant residual fawn pigmentation of the *ΔfwnA* has not been described for disruptants of orthologous genes in other Aspergilli, in which the resulting phenotype is white. Taken together this suggests that another pigment of polyketide or NRP nature is formed by *A. niger* conidia.

The polyketide funalenone was absent in the Δ*fwnA ΔolvA* and Δ*brnA* mutants indicating that funalenone might be a product of the pigmentation pathway itself and that the compound can be a potentially determinant for the of black spore pigmentation. The loss of *olvA* appeared to have pleiotropic effects on growth and conidiogenesis which would also explain reduced of accumulation of other, non-naphtho-γ-pyrone, secondary metabolites. It is possible that *olvA* encodes a function which is of structural importance to conidiogenesis, however since *ΔfwnA* sporulates abundantly and is epistatic to *ΔolvA* this explanation seems unlikely. Another hypothesis is that a resulting inability to process polyketide precursors has a negative effect on the cells and consequently on conidiogenesis. Fumonisin formation was not affected in *ΔolvA* but many other metabolites besides the naphtho-γ-pyrones were; it can be speculated that the affected metabolites are associated with the process of conidiation or conidium maturation. Besides providing a genetic basis for the conidial pigmentation in *A. niger* this study also revealed an intimate relationship between secondary metabolism and the process of conidial cell wall maturation by pigmentation.

Note added to proof

During revision of this manuscript, Chiang et al. (DOI:10.1016/j.fgb.2010.12.001) published the finding that disruption of a polyketide synthase resulted in white or colorless conidia and the inability to produce naphto-γ-pyrones in *A. niger*. The polyketide synthase in the above mentioned study (named AlbA) is identical to FwnA. There is currently little support for the suggestion by Chiang et al. that melanin biosynthesis in *A. niger* occurs via DHN-like in *A. fumigatus* and *A. nidulans*. The spore pigmentation of *A. niger* is reported to be insensitive to the HN reductase (Arp2) inhibitor tricyclazole A and there is an apparent lack of good candidate orthologs in *A. niger* of genes required for DHN-melanin biosynthesis in *A. fumigatus* (Arp1 and Arp2).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.01.005.

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