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Fungal Genetics and Biology 139 (2020) 103377

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Identification of SclB, a $Zn(II)_2Cys_6$ transcription factor involved in sclerotium formation in *Aspergillus niger*

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ARTICLE INFO

Keywords:
Fungal development
Secondary metabolites
Sexual development
Sclerotia
Indoloterpenes
Aurasperones

ABSTRACT

Certain *Aspergillus* species such as *Aspergillus flavus* and *A. parasiticus* are well known for the formation of sclerotia. These developmental structures are thought to act as survival structures during adverse environmental conditions but are also a prerequisite for sexual reproduction. We previously described an *A. niger* mutant (*scl-2*) which formed sclerotium-like structures, suggesting a possible first stage of sexual development in this species. Several lines of evidence presented in this study support the previous conclusion that the sclerotium-like structures of *scl-2* are indeed sclerotia. These included the observations that: (i) safranin staining of the sclerotia-like structures produced by the *scl-2* mutant showed the typical cellular structure of a sclerotium; (ii) metabolite analysis revealed specific production of indoloterpenes, which have previously been connected to sclerotium formation; (iii) formation of the sclerotium-like structures is dependent on a functional NADPH complex, as shown for other fungi forming sclerotia. The mutation in *scl-2* responsible for sclerotium formation was identified using parasexual crossing and bulk segregant analysis followed by high throughput sequencing and subsequent complementation analysis. The *scl-2* strain contains a mutation that introduces a stop codon in the putative DNA binding domain of a previously uncharacterized $Zn(II)_2Cys_6$ type transcription factor (An08g07710). Targeted deletion of this transcription factor (*sclB*) confirmed its role as a repressor of sclerotial formation and in the promotion of asexual reproduction in *A. niger*. Finally, a genome-wide transcriptomic comparison of RNA extracted from sclerotia versus mycelia revealed major differences in gene expression. Induction of genes related to indoloterpene synthesis was confirmed and also let to the identification of a gene cluster essential for the production of aurasperones during sclerotium formation. Expression analysis of genes encoding other secondary metabolites, cell wall related genes, transcription factors, and genes related to reproductive processes identified many interesting candidate genes to further understand the regulation and biosynthesis of sclerotia in *A. niger*. The newly identified SclB transcription factor acts as a repressor of sclerotium formation and manipulation of *sclB* may represent a first prerequisite step towards engineering *A. niger* strains capable of sexual reproduction. This will provide exciting opportunities for further strain improvement in relation to protein or metabolite production in *A. niger*.

1. Introduction

Aspergillus niger is an industrially important filamentous fungus which is only known to reproduce asexually through production of conidiospores. Since it is widely used as a host for the production of homologous and heterologous proteins and other important compounds such as organic acids (Pel et al., 2007; de Vries et al., 2017; Cairns et al., 2019), the discovery of a sexual cycle in *A. niger* would not only give

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https://doi.org/10.1016/j.fgb.2020.103377
Received 7 December 2019; Received in revised form 7 February 2020; Accepted 14 February 2020
Available online 03 April 2020

more insights into the biology of the species, but also provide further opportunities for strain improvement via exploitation of sexual crossing (Ashton and Dyer, 2016).

Sexual reproduction in fungi requires the presence of many specific genes in the genome including those with roles in environmental perception, mating and fruiting body development. Over 75 genes have been shown to be required for sexual development in *Aspergillus* species (Dyer and O'Gorman, 2012). In addition the ability to form sclerotia is considered to be an important prerequisite for sex in certain species of filamentous fungi with fruiting bodies formed either directly on, or developing within, sclerotia (Smith et al., 2014). Sclerotia are compact mycelial masses with characteristic hardened, thick walls and a less dense stroma. They play a role in dormancy, enabling survival of adverse environmental conditions (Smith et al., 2014). Several environmental factors have been shown to be correlated with the production of sclerotia, such as absence or presence of light, growth medium composition, oxygen availability and temperature (Rai et al., 1967; Agnihotri, 1969; Bennett et al., 1978; Frisvad et al., 2014). For example, oxidative stress caused by reactive oxygen species (ROS) induces sclerotial differentiation in several basidiomycete and ascomycete species (Georgiou et al., 2006; Li et al., 2012). In the case of the genus *Aspergillus*, the formation of sclerotia is required for sexual reproduction in members of the section *Flavi* (which includes economically important species such as *A. flavus, A. parasiticus* and *A. oryzae*) and section *Nigri* (which includes *A. niger*), in which fruit bodies known as cleistothecia develop in a stroma (a mass or matrix of vegetative hyphae) within sclerotia (Dyer and O'Gorman, 2011, 2012). This differs from the majority of aspergilli (such as the model organism *A. nidulans*), where cleistothecia develop direct without the prior need for formation of sclerotia.

There is evidence that the timing and balance between asexual and sexual development in *Aspergillus* species is regulated by hormone-like oxylipins, known as precocious sexual inducer (psi) factors. The production of these linoleic and oleic acid derived secondary metabolites is dependent on three *ppo* (psi factor producing oxygenase) biosynthetic genes in *A. nidulans* (Tsitsigiannis et al., 2004a, 2004b, 2005) and *A. fumigatus* (Garscha et al., 2007). The *A. flavus* genome contains homologues of the *A. nidulans ppoA, ppoB* and *ppoC* genes together with an additional *ppoD* gene (Brown et al., 2009), while homologues of *ppoA, ppoC* and *ppoD* have been identified in *A. niger* (Wadman et al., 2009). Deletion of *ppo* genes altered the balance between conidiation and sclerotium formation in *A. flavus*, with *ppoA* and *ppoC* having antagonistic roles (Brown et al., 2009). By contrast, deletion of *ppoA* and *ppoD* did not affect conidiation and oxylipin production in *A. niger* (Wadman et al., 2009). In addition, two basic helix-loop-helix (bHLH) transcription factors, EcdR and SclR, have been identified to be involved in sclerotium formation in *A. oryzae*. Based on the opposing phenotypes of strains deleted for or overexpressing *ecdR* or *sclR*, together with protein interaction studies, it was shown that these two transcription factors probably act as a heterodimer which results in mutual inhibition of function. Higher expression of *sclR* results in sclerotium formation, whereas higher expression of *ecdR* favours asexual reproduction. Thus, the relative ratio of SclR vs EcdR determines a developmental decision towards either sclerotium formation (low EcdR/high SclR) or asexual development (high EcdR/low SclR) (Jin et al., 2011a, 2011b. Meanwhile, the light/dark response velvet protein VeA is required for sclerotial development in *A. parasiticus* and *A. flavus* (Calvo et al., 2004, Cary et al., 2007). Finally, a calcineurin-response protein CrzA and a zinc-finger putative stress response protein MsnA have been shown to have roles in the development of sclerotia of *A. flavus* and *A. parasiticus* (Chang, 2008; Chang et al., 2012). Beyond this, little is known of the genetic control of sclerotial production in *Aspergillus* species. However, a series of other genes have been shown to be involved in formation of sclerotia in other ascomycetes, notably in *Sclerotinia* and *Botrytis* species (Li and Rollins, 2010; Li et al., 2012; Zhang et al., 2016; Li et al., 2018; Xu et al., 2018), and studies have identified possible orthologous genes involved in sclerotial formation in *S. sclerotiorum, B. cinerea, A. flavus* and *A. oryzae* (Li and Rollins, 2009; Amselem et al., 2011).

It has recently been shown that sclerotium formation could be induced in certain isolates of *A. niger* by growing them on medium with raisins, other fruits, or rice (Frisvad et al., 2014). Sclerotium formation in these isolates was accompanied by the production of sclerotium-associated metabolites, mainly aflavinin-type apolar indoloterpenes. Production of aflavinins was also specifically found in *A. flavus* sclerotia and these metabolites were suggested to protect the dormant structures from fungivorous insects (Wicklow, 1988; Gloer, 1995).

We previously reported the formation of sclerotium-like structures in a conidiation-affected UV-mutant of *A. niger* (Jørgensen et al., 2011a). In the present study, we use a combination of bulk segregant analysis and whole genome sequencing to identify the mutation in the *scl-2* mutant responsible for the reduced asexual sporulation and formation of putative sclerotium seen in this mutant. This combined approach has been shown to be a powerful tool to identify classical mutations in sexual filamentous fungi such as *Neurospora crassa* (e.g. McCluskey et al., 2011; Pomraning et al., 2011; Gonçalves et al., 2019) and we used a similar approach to characterize a non-acidifying mutant in *A. niger* (Niu et al., 2016). Transcriptome analysis of the putative sclerotia and vegetative mycelium revealed sclerotium-specific expression of genes related to secondary metabolism and cell wall biosynthesis, reflecting a fundamental change of strategy from trophic growth to dormant survival. The identification of a putative repressor of sclerotium formation in *A. niger*, here termed SclB, provides new opportunities for engineering *A. niger* strains capable of sexual reproduction.

2. Materials and methods

2.1. Strains, culture conditions and microscopy

A. niger strains used in this study are listed in Table 1. Strains were grown on either solidified (2% agar) or liquid minimal medium (MM) or complete medium (CM). MM contains 7 mM KCl, 8 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$ and spore trace elements solution, pH5.5 (Arentshorst et al., 2012). When required, plates or medium were supplemented with 10 mM uridine or 2.5 μg/ml nicotinamide. For secondary metabolite analysis, strains were grown on CYA or YES medium as previously described (Kildgaard et al., 2014; Grijseels et al., 2016). Plates were incubated at 30 °C in continuous light or complete darkness as indicated. SEM-pictures were taken as previously described (Jørgensen et al., 2010) and safranin staining was performed according to Xiaoke and Shunxing (2005).

2.2. Molecular techniques and strain constructions

Restriction and ligation enzymes were obtained from Thermo Scientific and used according to manufacturer's instructions. PCR reactions were performed using Phire Hot Start II DNA polymerase or Phusion DNA polymerase (Thermo Scientific). All primers used in this study are listed in Supplementary Table 1. Sequencing was performed by Macrogen. Transformation and chromosomal DNA isolation of *A. niger* was done according to Arentshorst et al. (2015a). Southern blot analysis was performed according to Sambrook and Russell (2001). α-^{32}P-dCTP-labelled probes were synthesized using the Decalabel DNA labelling kit (Thermo Scientific), according to the manufacturer's instructions.

To delete the *sclB* gene, a split marker approach was used (Arentshorst et al., 2015a). The *sclB* 5′ flanking region (955 bp) was PCR amplified using primers sclAP1f-NotI and sclAP2r and genomic DNA from *A. niger* strain N402 as template. The *sclB* 3′ flanking region (647 bp) was PCR amplified using primers sclAP3f and sclAP4r-NotI. *sclB-AOpyrG* split marker fragments were created by fusion PCR [see Arentshorst et al. (2015a) for details about primers and selection

Table 1
Strains used in this study.

Strain	Genotype	Description	Reference
N402	*cspA1*	derivative of ATCC9029 (N400, NRRL 3, CBS 120.49)	Bos et al. (1988)
AB4.1	*pyrG-*	*pyrG-* derivative of N402	van Hartingsveldt et al. (1987)
MA70.15	*kusA::amdS, pyrG-*	*ku70* derivative of AB4.1	Meyer et al. (2007)
MA169.4	*kusA::DR-amdS-DR pyrG-*	curable *ku70* derivative of AB4.1	Carvalho et al. (2010)
MA100.1	*kusA::amdS, fwnA::hygB, pyrG-*	*fwnA* deletion strain of MA70.15	Jørgensen et al. (2011a)
TJ1.2 (Scl-2)	*scl-2, kusA::amdS, fwnA::hygB, pyrG-*	UV mutant derived from MA100.1	Jørgensen et al. (2011b)
MA146.2	*scl-2, pAB4.1(A. niger pyrG* containing plasmid)	TJ1.2 complemented with *A. niger pyrG*	Jørgensen et al. (2011b)
MA192.3	*scl-2, pAB4.1-fwnA*	TJ1.2 complemented with *A. niger pyrG* and *fwnA*	This study
MA145.1	*scl-2, noxA::pyrG*	*ΔnoxA* in TJ1.2	This study
MA144.1	*scl-2, noxR::pyrG*	*ΔnoxR* in TJ1.2	This study
JN6.2	*cspA1, olvA::AopyrG, nicB::hygB*	*ΔolvA, ΔnicB* of MA169.4	Niu et al. (2016)
AR206	*cspA1/cspA1,fwnA/fwnA::hygB,olvA/ olvA::AopyrG, pyrG/pyrG378, nicB/ nicB::hygB*	diploid TJ1.2 × JN6.2	This study
MA336.1	*ΔsclB::pyrG*	*ΔsclB* in MA169.4	This study
MA337.1	*ΔfwnA::hygB, ΔsclA::pyrG*	*ΔsclB* in MA100.1	This study
MA338.1	*scl-2, pAB4.1-sclA*	TJ1.2 complemented with *A. niger pyrG* and *sclB*	This study
TS51.5, TS51.9	An03g05440::*AopyrG* in TJ1.2	ΔAn03g05440 in TJ1.2	This study
TS58.4, TS58.5	An03g05440::*AopyrG* in MA100.1	ΔAn03g05440 in MA100.1	This study

marker template]. These *sclB-AOpyrG* split marker fragments were transformed into *A. niger* strains MA169.4 (*kusA::DR-amdS-DR, pyrG*$^-$) and MA100.1 (*kusA::amdS, ΔfwnA::hygB,pyrG*$^-$), resulting in strains MA336.1 (*kusA::DR-amdS-DR, ΔsclA::AOpyrG*) and strain MA337.1 (*kusA::amdS, ΔsclA::AOpyrG, ΔfwnA::hygB*) (Table 1). Deletion of the *sclB* gene was confirmed by Southern blot analysis (data not shown). Deletion cassettes to inactivate the *noxA* and *noxR* genes in *scl-2* were described earlier (Kwon et al., 2011). Correct deletion of *noxA* and *noxB* was confirmed by Southern blot analysis (Supplemental Fig. 1).

For complementation of the *scl-2* mutant, the complete *sclB* ORF (An08g07710), including 1000 bp upstream region and 650 bp downstream region, was PCR amplified using primers sclAP1f-NotI and sclAP4r-NotI and genomic DNA from *A. niger* strain N402 as template. The resulting PCR product (3,346 bp) was ligated into PCR cloning vector pJet1.2 (Thermo Scientific) and sequenced. The whole complementing *sclA* fragment was isolated with *Not*I and ligated into the *Not*I opened *pyrG* targeting vector pMA334 (Arentshorst et al., 2015b). The resulting plasmid, pMA363, was digested with *Asc*I to release the final *pyrG* targeted *sclB* complementation fragment, and this fragment was transformed to the *scl-2* mutant. Correct integration of the *sclB* complementation fragment at the *pyrG* locus was confirmed by Southern blot analysis (Supplemental Fig. 2).

The gene An03g05440 was deleted with a similar approach compared to *sclB*. The 5′ flanking region (855 bp) was PCR amplified using primers 868 and 869 and genomic DNA from *A. niger* strain N402 as template. For the 3′ flanking region (935 bp) the primers 870 and 871 were used. Split marker fragments containing part of the *AOpyrG* were generated by fusion PCR and transformed into *A. niger* strains TJ1.2 (*scl-2, kusA::amdS, ΔfwnA::hygB,pyrG*$^-$) and MA100.1 (*kusA::amdS, ΔfwnA::hygB,pyrG*$^-$). The resulting strains were TS51.5 (*scl-2, kusA::amdS, ΔfwnA::hygB,* An03g05440::*AOpyrG*) and TS58.4 (*kusA::amdS, ΔfwnA::hygB,* An03g05440::*AOpyrG*). Deletion was confirmed by Southern blot analysis (Supplemental Fig. 3).

2.3. Secondary metabolite analysis

Secondary metabolites were analysed and extracted from samples taken from CYA or YES agar plates as described previously (Grijseels et al., 2016). Strains were grown at 30 °C for 7–14 days in biological duplicates. Sclerotia were collected using a toothpick, washed with water and smashed prior to extraction. Extracts were stored at −20 °C prior to analysis. Samples were then analysed by liquid chromatography-high resolution mass spectrometry on Agilent 1290 infinity UHPLC (Agilent Technologies, Torrence, CA) equipped with an Agilent Poroshell 120 phenyl-hexyl column (250 mm × 2.1 mm, 2.7 μm particles), running an acidic water/ACN gradient. This was coupled to an Agilent 6545 Q-TOF-MS equipped with an ESI source and operated in positive polarity and sampling *m/z* 50–1700 in full scan and auto MS/MS mode (Kildgaard et al., 2014). Compounds were then identified by MS/HRMS spectra and retention time (Kildgaard et al., 2014), and peaks integrated using Agilent Quant Analysis 6.0 as described by Nielsen and Larsen (2015).

2.4. Bulk segregant analysis and whole genome sequencing

Parasexual crossing of haploid strains TJ1.2 (*scl-2, ΔfwnA::hygB, pyrG*$^-$) and JN6.2 (*olvA::pyrG, nicB::hygB*), was performed as previously described (Niu et al., 2016; Arentshorst and Ram, 2018). Haploid segregants (fawn- or olive-colored sectors) were purified and genotypically analysed for conidial spore color, *pyrG* and *nicB* auxotrophies, and the ability to form sclerotia. From 203 purified segregants, 147 mutants formed sclerotia. A total of 28 fawn and 43 olive sclerotia-forming segregants were selected, and equal amounts of fresh weight mycelium (100 mg each) was pooled for isolation of genomic DNA. In addition, genomic DNA of both the parental strains TJ1.2 and JN6.2) was isolated. Genomic DNA from parental strains and the segregant pools was further purified using Macherey-Nagel NucleoBond Xtra columns before DNA sequencing.

Illumina Paired-End sequencing was performed by ServiceXS (Genome Scan, Leiden, The Netherlands) as previously described (Niu et al., 2016). To reach sufficient sequence depth for the pools, 22 GB of sequence data for the pools (~600× coverage) and about 3 GB of sequence data was obtained for the parental strains (80× coverage). Image analysis, base calling and quality check was performed with the Illumina data analysis pipeline RTA v1.13.48 and/or OLB v1.9/CASAVA v1.8.2. Based on the mapped reads, variants in the sample data were detected when compared to the reference genome of *A. niger* ATCC1015 (http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Aspni5) and between the samples using an in-house SNP pipeline v3.2 (ServiceXS). Validated variants had to be found in one location in at least one sample consistently for a frequency of 0.7 or higher, in at least 20 overlapping reads (minimum coverage) with no quality filtering, before it was reported as a SNP. The combined pool sample was processed with a minimal variant frequency of 0.3. For each SNP it was verified whether the SNP was in a predicted protein encoding region using the *A. niger* 3.0 genome at JGI using the SNP coordinates and given in Supplementary Table 2. Genome sequences of TJ1.2, JN6.2 and segregants are available upon request.

2.5. Transcriptome analysis

To identify genes specifically expressed in sclerotia, the *scl-2* mutant was grown for 8 days in darkness on a polycarbonate filter placed on top of a complete medium plate. Sclerotia were then taken from this plate using tweezers and RNA was isolated. For comparison, RNA was isolated from mycelium that had not produced sclerotia from the exact same plate. RNA extraction of the two samples, which we will refer to as the 'sclerotium sample' and the 'mycelial sample', respectively, was performed by grinding the fungal biomass in liquid nitrogen with pestle and mortar and subsequent RNA extraction using Trizol. Following extraction, RNA was further purified on NucleoSpin RNA II columns (Machery-Nagel), including a DNase I digestion step. RNA was eluted in 60 µl of MilliQ water. RNA quantity and quality were determined on a Nanodrop spectrophotometer, and integrity was tested on an Agilent 2100 Bioanalyser. The spectrum generated by the Agilent Bioanalyser was visually inspected for possible RNA degradation and contamination with genomic DNA to ensure good sample quality. Affymetrix microarray analyses for biological duplicate samples for both conditions were performed at ServiceXS (Genome Scan, Leiden, The Netherlands) as previously described (Jørgensen et al., 2010). The transcriptomic data set and normalized expression values are given in Supplementary Table 3. Identification of differentially expressed genes was performed as described by Schachtschabel et al. (2013). Gene Ontology (GO) enrichment analysis for differentially expressed gene sets was performed using Fisher's exact Test Gene Ontology annotation tool (FetGOat) (Nitsche et al., 2012).

3. Results

3.1. Sclerotia formation in the scl-2 mutant

We previously isolated a developmental *A. niger* mutant, *scl-2* with an altered growth phenotype (Jørgensen et al., 2011b). Vegetative growth was not affected in this mutant, but the development of conidiophores was severely reduced both on plates and in liquid cultures compared to the MA100.1 Δ*fwnA* parent (Fig. 1 and Jørgensen et al. (2011a)). Incubation on complete medium agar plates (see materials and methods for details) for eight days in the dark resulted in the formation of sclerotia-like initials near the centre of the colony and the formation of a ring of conidiophores at the colony edge by *scl-2* (Fig. 1). Formation of sclerotia was dependent on darkness as incubation in continues light abolished their formation (Fig. 1). The *scl-2* mutant was isolated in a *fwnA* mutant background in which the *fwnA* gene, encoding a polyketide synthase required for the biosynthesis of conidial melanin and naphtho-γ-pyrones (Jørgensen et al, 2011b), was deleted. Sclerotia formation was not dependent on the absence of the *fwnA* gene as reintroduction of *fwnA* restored the ability to form black conidiospores but the *fwnA* complemented *scl-2* strain (MA192.3) still continued to produce sclerotia in the dark (Fig. 1).

Formation of sclerotia-like structures in the *scl-2* mutant was found to be dependent on the conidial inoculation method, being most abundant when spores were point inoculated in the centre of an agar plate. If the conidial inoculum was spread in a dense carpet or as perpendicular streaks, sclerotia formation was less obvious. Inoculation-dependent effects have also been observed for sclerotial formation in *A. flavus* (Brown et al., 2009). Sclerotia formation was also dependent on the media composition, because only cultivation on complete medium resulted in production of sclerotia. After six days of incubation in the dark, immature sclerotia were visible on plates (Fig. 2A). In time, the sclerotia matured and become hardened. The mature sclerotia (1–2 mm in size) could be easily removed from the mycelia using tweezers. Scanning electron microscopy analysis of 12 day old sclerotia showed that *scl-2* sclerotia are often lobed and appear to consist of aggregated hyphal structures (Fig. 2B). Safranin stained cross sections of the sclerotia formed by the *scl-2* strain showed the typical organization of a sclerotium with an outside layer of thick walled hyphal cells (Fig. 2C). The cells of the sclerotium-like structures in *A. niger* were not darkly pigmented as seen in some other fungi (e.g. *Aspergillus alliaceus* species, Frisvad et al., 2019) but whitish even after reintroduction of the *fwnA* polyketide synthase (Fig. 1). Upon further incubation the sclerotia became more brownish. Frisvad et al. (2014) also reported the formation of white sclerotia in other strains of *A. niger*. The stromal matrix of the *scl-2* strain, the site in which cleistothecia will develop in sexual fungi,

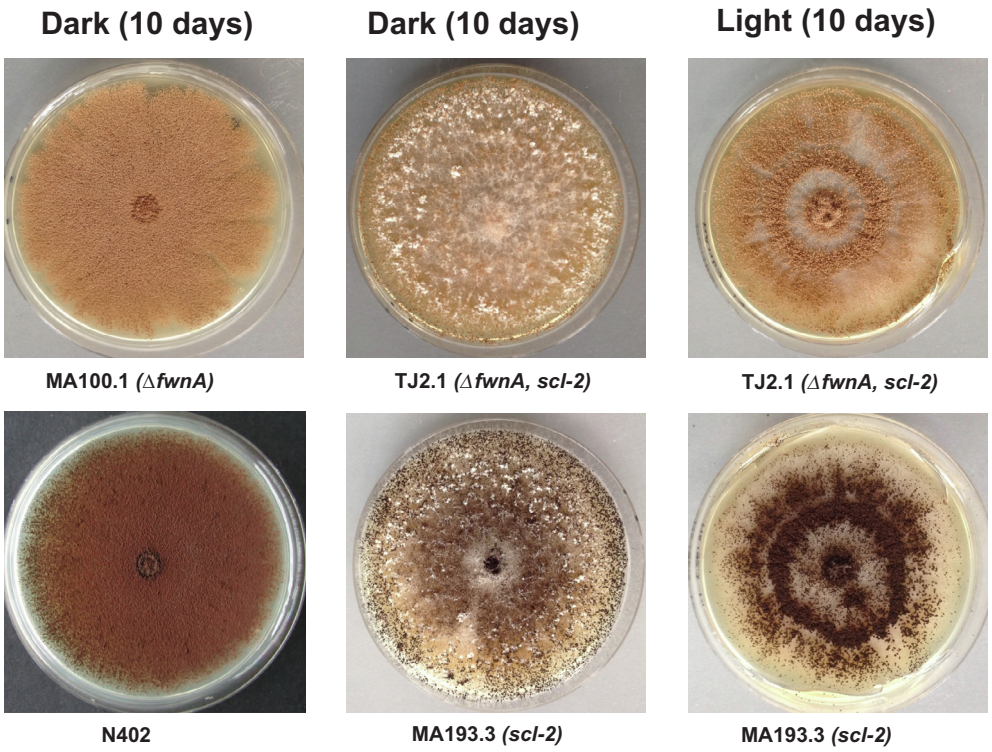

Fig. 1. Formation of sclerotia in the *scl-2* mutant requires darkness. The top row shows growth following incubation in continuous light or darkness of the Δ*fwnA* parental strain and *scl-2* derivative strain. The bottom row shows growth following incubation in continuous light or darkness of the N402 (black conidiating) wild-type compared to a *scl-2* derivative strain MA193.3 in which the *fwnA* polyketide synthase (required for the black melanin spore color) has been restored. Spores were point inoculated and grown for 10 days at 30 °C.

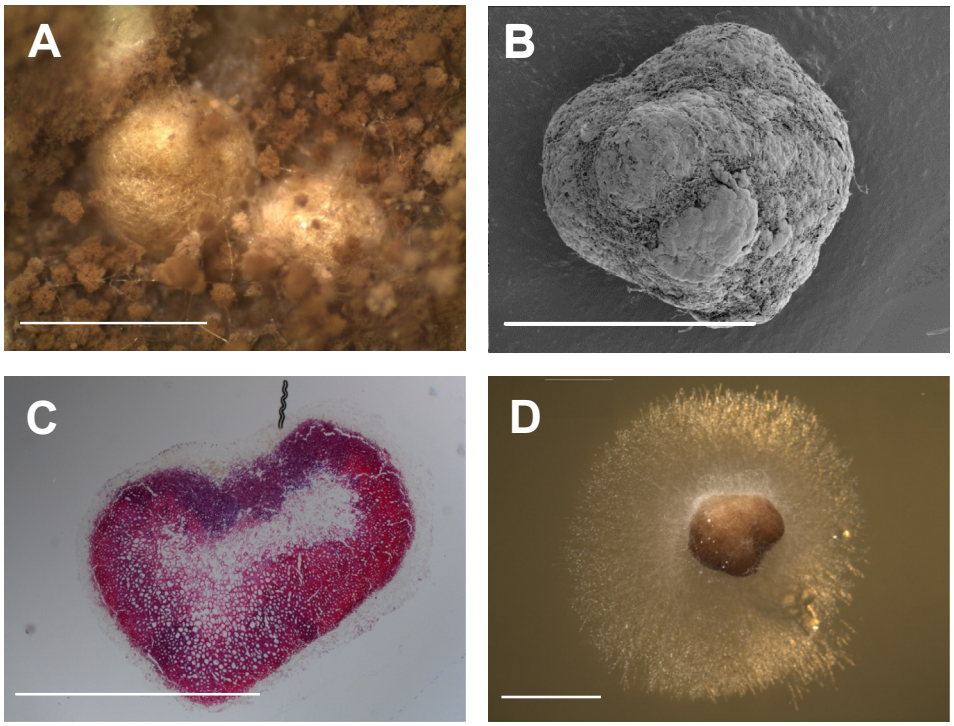

Fig. 2. Morphology of sclerotia formed in the *scl-2* mutant of *A. niger*. (A) Six day old sclerotia surrounded by conidiophores. (B) Scanning electron microscope image of 12 day old sclerotium. (C) Transection of sclerotium stained with Safranin. (D) Outgrowth of mycelium from a 28 day old brown and hardened sclerotium when transferred to a fresh medium plate. Scale bar is 2 mm.

consists of less densely packed hyphal cells referred to as pseudoparenchymatous hyphae (Dyer and O'Gorman, 2012) (Fig. 2C). These sclerotium-like structures were found to be viable, as shown by the fact that when sclerotia were washed thoroughly with 0.9% NaCl + Tween 80 (0.01%) to remove conidia and then incubated for 24 h at 30°C, there was resulting outgrowth of hyphal cells from them (Fig. 2D).

3.2. Secondary metabolite analysis in the scl-2 mutant

Previous work has indicated that sclerotium development in *A. niger* is associated with the production of indoloterpenes (Frisvad et al., 2014). Twelve day old putative sclerotia from the *scl-2* mutant in both the Δ*fwnA* background (TJ1.2) as well as in the *fwnA*$^+$ strain (MA192.3) were therefore analysed for the presence of secondary metabolites as previously described (Nielsen and Larsen, 2015). The chromatogram of metabolites extracted from sclerotia of the *scl-2* strain showed the presence of at least three different indoloterpenes (14,25-dihydroxy-10,23-dihydro-aflavinine, hydroxyaflavinine, and 10,23-dihydro-24,25-dehydro-21-oxo-aflavinine, respectively) (Supplementary Fig. 4). In addition to the indoloterpenes, aurasperones (member of the naphtho-γ-pyrone class of secondary metabolites) were detected in sclerotia of the Δ*fwnA scl-2* strain. This was surprising, as we and others had previously shown that production of naphtho-γ-pyrone class compounds requires the *fwnA* polyketide synthase (Jørgensen et al., 2011b, Chiang et al., 2011). The presence of the malformin C and small amounts of nigragillin, was also detected in sclerotia. In addition to these secondary metabolites, several other secondary metabolites, including fumonisin B2/B4, kotanin (and its precursors demethylkotanin and orlandin), fungisporin A, pyranonigrin A, nigerazine and funalenone, were also detected in sclerotia.

3.3. Formation of sclerotia-like structure in scl-2 is dependent on the NADPH complex

Previous studies in sclerotium-producing fungi such as *Sclerotinia sclerotiorum* and *Botrytis cinerea* have shown that the NADPH oxidase complex that produces ROS are involved in sclerotium formation (Kim et al., 2011, Segmüller et al., 2008). To further obtain evidence that the structures formed by the *scl-2* strain were indeed sclerotia, we determined whether the formation of the sclerotia-like structures in *scl-2* was dependent on the NADPH complex by deletion of the *noxA* (encoding the putative NADPH oxidase) and *noxR* (encoding the NADPH oxidase regulator NoxR/p67) genes (Kwon et al., 2011). As shown in Fig. 3, formation of the sclerotia-like structures was abolished in the *scl-2 noxA* and *scl-2 noxR* double mutants.

3.4. Genetic characterization of the scl-2 mutant

To obtain haploid segregants for bulk segregant analysis, TJ1.2 (*scl-2, ΔfwnA, pyrG*) was crossed to JN6.2 (*olvA, nicB*) to form diploid strain AR206. This diploid strain sporulated normally and did not produce sclerotia indicating that the *scl-2* mutation is recessive (data not shown). To obtain a collection of segregants that display the *scl-2* phenotype, AR206 was grown in the presence of benomyl, resulting in haploidization and the formation of sectored colonies (Fig. 4). In total, 203 segregants were purified and tested for their ability to form sclerotia and the presence of other markers (spore colour and *pyrG* and *nicB* auxotrophies). Of the 203 segregants, a total of 147 mutants formed sclerotia. Genomic DNA was isolated and pooled from 76 sclerotia-forming strains (28 fawn coloured and 48 olive coloured) to obtain the DNA for the segregant pool. In addition, genomic DNA of both the parental strains (TJ1.2 and JN6.2) was also isolated and sequenced. To reach sufficient sequence depth for the pools, 22 GB of sequence data for the segregant pool (600× coverage) and about 3 GB of sequence data was obtained for the parental strains (80× coverage).

Because UV mutagenesis mainly induces point mutations, we performed a SNP/indel analysis to determine the genetic basis of the *scl-2* mutant phenotype. Comparison of the genome sequence of *scl-2* and JN6.2 identified 56 SNPs (Supplemental Table 2). The genomic DNA of the pool of segregants was subsequently analysed to identify SNPs conserved in the pool. In theory, SNPs unlinked with the sclerotia-forming phenotype have a 50% chance of being present, while a SNP responsible for the phenotype should be 100% conserved within the pool. SNPs physically linked to the SNP responsible for the phenotype could also have a high percentage chance of conservation. As shown in Supplemental Table 2, variants of all the SNPs were present in the pool

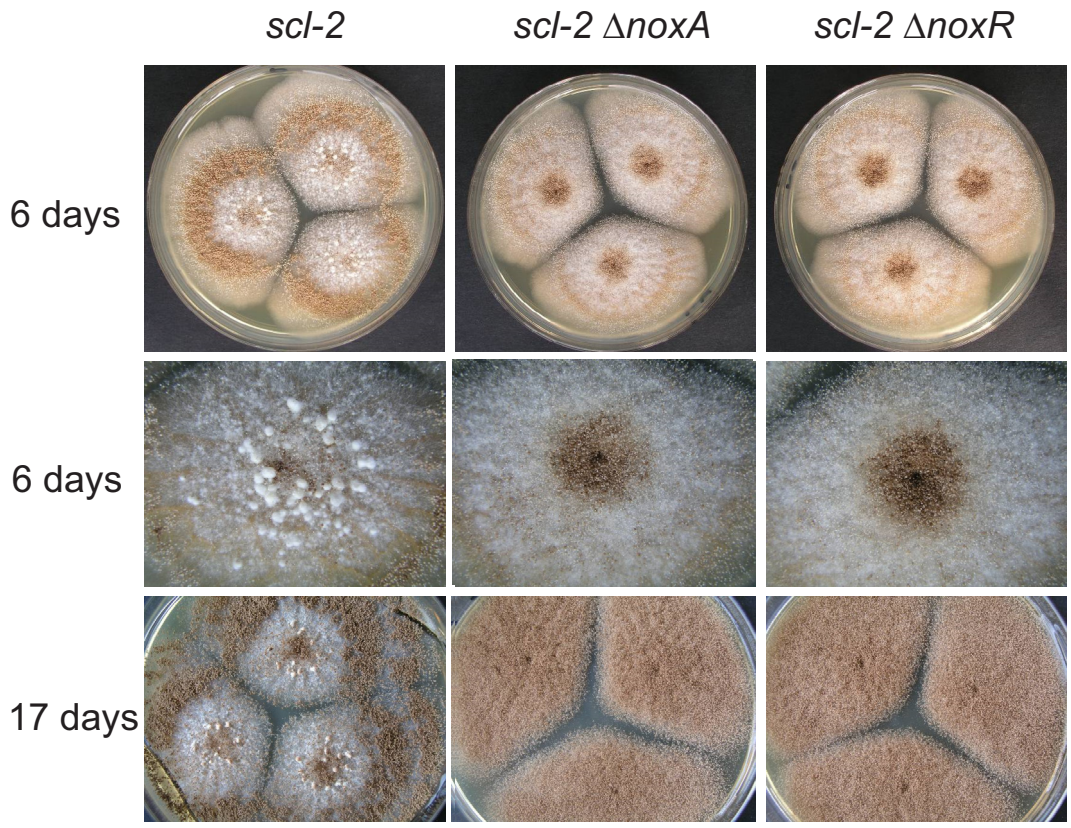

Fig. 3. Sclerotium formation in the *scl-2* mutant is dependent on the NADPH oxidase complex. Left hand column shows growth of the *scl-2* parent, compared to *scl*-2Δ*noxA* and *scl*-2Δ*noxR* (with deletions of genes encoding subunits of the NADPH oxidase complex). Strains were point inoculated and grown in the dark for 6 or 17 days,

of segregants, but only two SNPs on the right arm of chromosome 8 showed high conservation. The SNP at position 8-2; 1,853,323 was 100% conserved in the pool, whilst the SNP at position 8-2; 2,297,744 was 99.15% conserved. This suggested that the SNP at position 8-2-1853323 is responsible for the phenotype. The SNP at position 8-2-1853323 was linked to the phenotype but the 99.15% frequency suggests that in the pool of segregants a single segregant is present which contains the wild type variant. This segregant is likely the result of a mitotic cross over event during haploidization.

Significantly, the 100% conserved mutation at position 8-2; 1,853,323 was found to be present in the coding region of a gene encoding a putative $ZnII_2Cys_6$ transcription factor of 548 amino acids (An08g07710). The mutation (A to T) introduces a stop codon (AAA to TAA; Lysine (K) to STOP) at amino acid position 194 of the protein. The resulting stop codon is located in the N-terminal part of the protein and is located in the central part of the Zn-finger domain. The mutation is likely to result in a null phenotype.

To confirm whether the SNP identified in An08g07710 was responsible for the sclerotium-forming phenotype, experiments involving targeted deletion and complementation experiments were performed. As shown in Fig. 5, the deletion of An08g07710 resulted in a sclerotium-forming phenotype in both the *fwnA*$^+$ and Δ*fwnA* backgrounds (Fig. 5D and E). Introducing the An08g07710 gene at the *pyrG* locus complemented the sclerotium-forming mutant and restored the growth and sporulation phenotype of the wild-type strain. Proper deletion and complementation was confirmed by Southern blot analysis

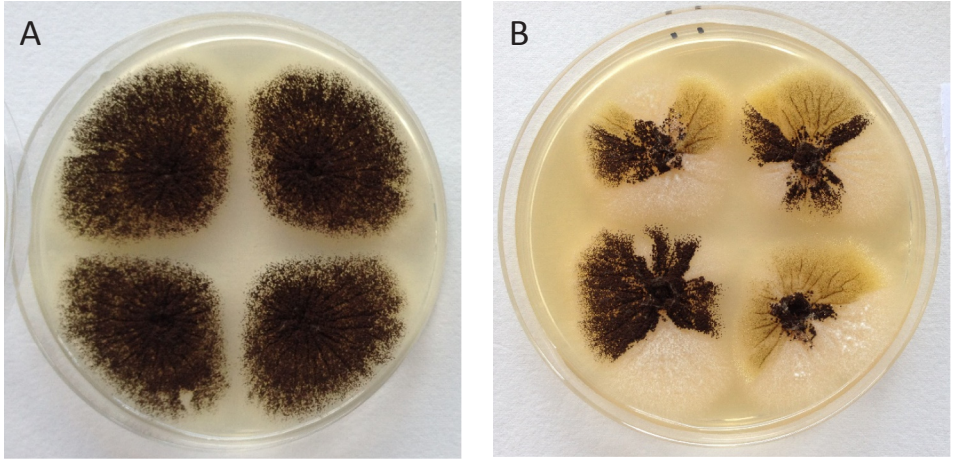

Fig. 4. Instability of the diploid state of *A. niger* in the presence of benomyl. A) Diploid strain A206 point inoculated on complete medium. B) Diploid strain A206 point inoculated on complete medium containing 0.4 ug/ml benomyl. The formation of sectors in which the mutant color marker is visible indicates that these sectors are haploid.

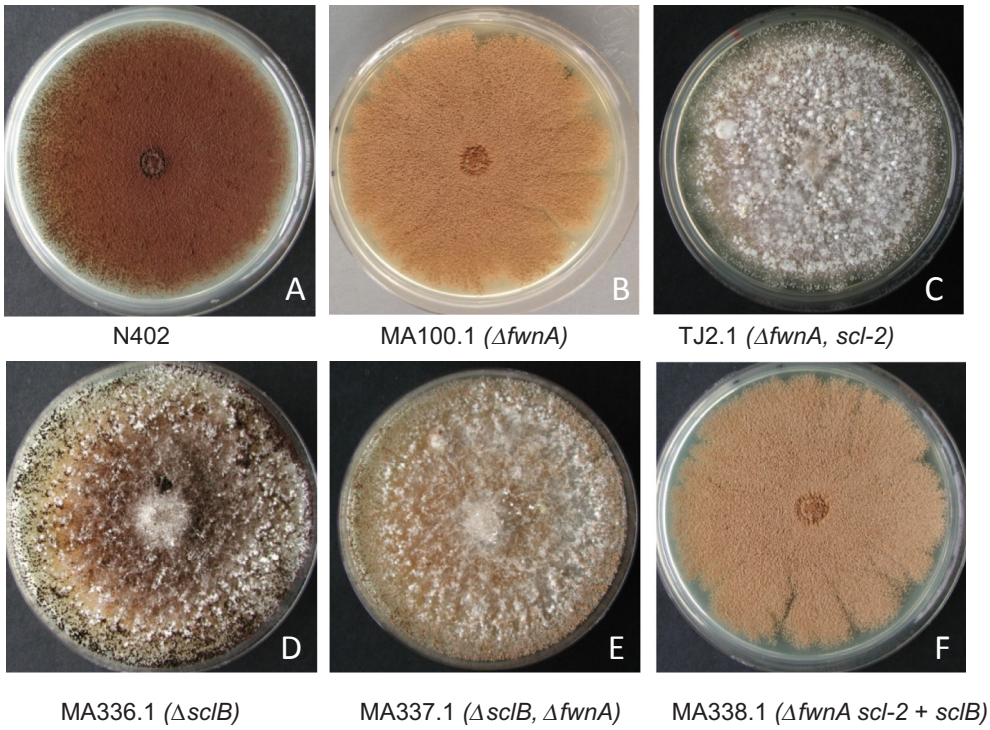

Fig. 5. The sclerotial phenotype of the *scl-2* mutant is caused by a mutation in the ZnII(2)Cys6 transcription factor SclB. Upper row (panels A, B, C) shows parental (N402 and MA100.1) and original *scl-2* mutant. Lower row (panels D, E and F) shows that integration of the *sclB* gene into the *scl-2* background complements the hypersclerotial phenotype of *scl-2* (D), whilst targeted deletion of *sclB* results in the formation of sclerotia (E, F).

(Supplementary Figs. 1 and 2). Thus, there was clear evidence that the nonsense mutation in the ZnIICys6 finger binding domain of An08g07710 in the *scl-2* mutant is responsible for the sclerotial phenotype. We refer to this transcription factor as SclB, to indicate the derivation from the *scl-2* strain. BlastP searches with SclB showed that the SclB transcription factor has clear orthologous transcription factors (best bi-directional hits) in the *Eurotiales* (*Aspergillus* and *Penicillium* spp.). BlastP hits with homologous TFs from the *Sordariales* (*Neurospora* and *Magnaporthe* spp.), and *Hypocreales* (*Trichoderma* and *Fusarium* spp.), showed only homology in the $Zn(II)_2Cys_6$ domain and not outside of this DNA binding region. PsortII analysis (https://psort.hgc.jp/) indicated likely nuclear targeting of SclB.

3.5. Genome-wide expression analysis in sclerotia compared to vegetative hyphae

To identify specific genes or processes related to sclerotium formation, a genome-wide expression study was conducted using *A. niger* Affymetrix GeneChips. RNA from two sclerotial and two vegetative mycelial samples of the *scl-2* mutant were isolated and used for microarray analysis. Normalized expression data for the four microarrays is provided in Supplementary Table 3. Genome-wide expression analysis revealed major differences in gene expression profiles. 3,778 genes were found to be differentially expressed between the two conditions (q-value < 0.005) (Supplementary Table 3). 1900 genes were more highly expressed in sclerotia, while expression of 1878 different genes was higher in mycelium samples (Supplementary Table 3).

For more insight into the processes affected in the sclerotia, a GO-enrichment analysis was made of genes that were significantly higher expressed in sclerotia using FetGOat (Nitsche et al., 2012) based on a stringent statistical cut-off (q-value < 0.005) and a fold-change in expression > 3.0 (Supplementary Table 3). These criteria identified a set of 608 genes up-regulated in sclerotia, which encompassed 12 biological processes (Table 2 and Supplementary Table 4) and included genes associated with three terminal node terms, namely inositol phosphate biosynthetic processes, sporocarp development, and tryptophan metabolic processes. A similar GO enrichment analysis for genes highly expressed in vegetative growth but low in sclerotia resulted in the identification of 606 genes encompassing 32 biological processes including 9 terminal nodes (Table 3 and Supplementary Table 5). Six of these terminal nodes contain genes encoding glycosylhydrolases.

In additional to the FetGOat analysis, genes related to secondary metabolism, sexual reproduction, transcription factors and cell wall biosynthesis were examined in more detail (Supplementary Tables 6–12) as follows.

3.6. Secondary metabolism

Differences in expression of secondary metabolism genes relating to indoloterpene synthesis, polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), and hybrid PKS-NRPS were detected between the sclerotial and vegetative mycelial samples.

Regarding indoloterpene synthesis, sclerotia of *A. niger* and other Section *Nigri* species have previously been shown to contain aflavinine-type indoloterpenes (Frisvad et al., 2014), with production likely to be specific to sclerotium formation (Nielsen et al., 2009). Orthologs of four genes required for the synthesis of the indoleterpene paspaline in *Penicillium paxilli*, namely a GGPP-synthase (*paxG*), a FAD-dependent monooxygenase (*paxM*), a membrane protein (*paxB*) and a prenyl transferase (*paxC*) (Saikia et al., 2006), were identified in *A. niger*. Expression analysis revealed that two putative *paxG* homologues (An03g00860 and An11g02500) showed strong induction in sclerotia (Table 4 and Supplemental Table 6). Greatly increased expression of *paxM* (An11g02480), *paxB* (An01g07680 and An11g02490) and *paxC* (An03g00850) homologues was also seen in sclerotia (Table 4). Interestingly, three genes adjacent to the *paxC-paxG* cluster were also highly expressed in sclerotia (Table 4). Homologues of other genes in the *pax* cluster were not specifically induced in sclerotia (Supplemental Table 6). Other genes with possible roles in biosynthesis of indoloterpenes were also identified based on homology to genes for pimaradiene biosynthesis in *A. nidulans* (Bromann et al., 2012). Upregulation of a putative HMG-CoA reductase (An04g00610), an elongation factor homolog (An01g09610), two short-chain dehydrogenase homologues (An12g09700 and An18g01750), and a PbcR homologue (An18g03950) was observed in sclerotia of the *scl-2* mutant (Supplemental Table 6).

Table 2
Enriched GO terms (terminal nodes) in sclerotia up-regulated genes.

Gene ID	Description	Myc	Scl	FC	FDR	BPs (terminal node)
An14g04590	Ortholog(s) have inositol heptakisphosphate kinase activity (ScKCS1)	117.6	386.9	3.3	7.1E-06	inositol phosphate biosynthetic process
An08g06320	Ortholog(s) have phospholipase C activity (ScPLC1)	82.4	496.4	6.0	1.2E-05	inositol phosphate biosynthetic process
An18g04520	Ortholog(s) have inositol tetrakisphosphate 3-kinase activity (ScARG82)	86.9	437.4	5.0	9.0E-06	inositol phosphate biosynthetic process
An01g07260	Ortholog(s) have anthranilate phosphoribosyl transferase activity	930.7	6839.8	7.3	6.9E-07	fruiting body
An08g06080	Ortholog(s) have anthranilate synthase activity	515.2	2395.5	4.6	2.9E-06	fruiting body
An14g06010	Ortholog(s) have chorismate mutase activity	169.7	1187.5	7.0	3.2E-06	fruiting body
An14g01820	strong similarity to hypothetical cell wall protein binB	573.0	2533.4	4.4	5.9E-06	fruiting body
An04g05880	Putative dioxygenase	483.9	4281.9	8.8	4.9E-07	fruiting body
An01g10540	BrlA transcription factor	22.7	208.6	9.2	6.7E-07	fruiting body
An14g02970	Ortholog(s) have protein histidine kinase activity. red light photoreceptor activity	45.1	142.0	3.2	1.8E-05	fruiting body
An08g06080	Ortholog(s) have anthranilate synthase activity	515.2	2395.5	4.6	2.9E-06	tryptophan metabolic process
An02g06820	Ortholog(s) have pyruvate decarboxylase activity	433.2	3586.3	8.3	1.7E-06	tryptophan metabolic process
An01g07260	Ortholog(s) have anthranilate phosphoribosyl transferase activity	930.7	6839.8	7.3	6.8E-07	tryptophan metabolic process

Regarding other secondary metabolism genes, the genome of *A. niger* has been reported to contain 34 PKS, 17 NRPS and seven hybrid PKS-NRPS encoding genes, most of which are located in clusters (Pel et al., 2007). Four PKS-encoding genes (An02g09430, An03g05440, An11g09720, An12g07070) were up-regulated in sclerotia. Interestingly, An03g05440 is part of a secondary metabolite cluster and the entire cluster was induced (Table 5). Genes adjacent to An11g09720 were also induced (Supplemental Table 7). Sclerotia of the *scl-2* mutant were shown to contain aurasperones, members of the naphtho-γ-pyrone class of secondary metabolites. Previous studies in *A. niger* have shown that the production of naphtho-γ-pyrones was dependent on the PKS gene *fwnA* (Jørgensen et al, 2011b; Chiang et al., 2011). Given that An03g05440 exhibited similarity to *fwnA* (45% identity and 62% similarity in amino acid sequence) we hypothesised that production of aurasperones might be dependent on induction of An03g05440. Deletion of An03g05440 in the *scl-2* mutant and subsequent analysis of secondary metabolites indeed revealed the absence of detectable amounts of aurasperones in sclerotia while other metabolites (indoloterpenes and fumonisins) were still found (Supplementary Fig. 5). Two genes [An01g06890 (NRPS) and An01g06930 (PKS)] involved in fumonisin biosynthesis (Pel et al., 2007) were significantly downregulated in sclerotia, as were the majority of genes in the same cluster (Table 6). A second gene cluster comprising An03g00640-An03g00690 involved in production of a currently unknown metabolite was also expressed at a lower level in sclerotia compared to mycelium (Table 6).

Expression of five NRPS encoding genes was up-regulated in sclerotia (Supplemental Table 7). An12g07230 showed a very high expression value but has not yet been associated with production of a particular secondary metabolite. An adjacent gene of unknown function, An12g07220, was also strongly upregulated during sclerotia formation (Table 5). Meanwhile, some genes adjacent to the NRPS-encoding genes An03g06010, An12g02840, An02g00840 were also induced during sclerotium formation, indicating likely upregulation of gene clusters (Supplemental Table 7).

3.7. Sexual reproduction

Certain genes with known roles in sexual reproduction showed differential expression in sclerotia of the *scl-2* mutant (Supplemental Table 8). Two pheromone signalling-related genes, a putative a-factor pheromone efflux pump (An04g03690) and a putative pheromone processing enzyme (An16g01860) showed increased expression. In addition, two dioxygenases (*ppoA* and *ppoC*) and two transcription factors related to development (*A. niger* homologs of FlbD and BrlA) were also upregulated in sclerotia. The function of FlbD has not yet been investigated in *A. niger,* but the BrlA protein in *A. niger* seems to have similar functions as in *A. nidulans* and *A. fumigatus* (Krijgsheld et al., 2013, van Munster et al., 2015, Ojeda-López et al., 2018). However, the majority of sex-related genes showed no obvious upregulation, and there was even downregulation of An04g07400, a Zn $(II)_2Cys_6$ transcription factor homologous to Pro1 essential for sexual development in *Sordaria macrospora* (Masloff et al., 1999) (Supplemental Table 8).

3.8. Transcription factors

The *A. niger* genome contains at least 657 genes encoding transcription factors (TFs) (Pel et al., 2007). A total of 68 TFs were significantly upregulated in sclerotia whilst 57 TFs were downregulated (Supplemental Tables 9 an 10). Among the highest up-regulated TFs were An03g05500 and An12g02040, both encoding $Zn(II)_2Cys_6$ transcription factors. Interestingly, An03g05500 is located in a putative secondary metabolite cluster including the PKS An03g05440 responsible for the synthesis of aurasperone, as well as other highly induced adjacent genes (Table 5). Upregulation of An14g02540, which encodes a TF of the basic helix-loop-helix family of TFs, was also observed (Supplemental Table 9). Significantly, this gene is the orthologue of SclR, a TF from *A. oryzae,* which, when overexpressed, promotes sclerotium formation (Jin et al., 2011a,b). By contrast, the *A. niger* TF homologous to EcdR (An04g01250) was not differentially expressed in the *scl-2* mutant.

Downregulated TFs included the $Zn(II)_2Cys_6$ transcription factor FumR in the fumonisin gene cluster (Supplemental Table 10). Downregulation of several TFs required for the production of extracellular enzymes was also observed, including the protease activator PrtT, the starch degrading enzymes activator AmyR, the putative cellulase/mannanase regulator ManR and the xylanase regulator XlnR. The downregulation of these transcription factors is consistent with the observed downregulation of many extracellular enzymes (Table 3). Targets of the PrtT protease activator (Punt et al., 2007; Schachtschabel et al., 2013) were also down regulated in sclerotia (Supplemental Table 11).

3.9. Cell wall biosynthesis

Expression analysis of genes encoding cell wall biosynthetic enzymes (Pel et al., 2007) revealed interesting patterns and indications of an altered cell wall composition of sclerotia (Supplemental Table 12). Most striking was the strong induction of a putative alpha-1,3-glucan synthase (*agsD*) and down-regulation of an alpha-1,3-glucan processing enzyme (*agtC*) and genes putatively involved in the synthesis of galactosaminogalactan [the latter identified based on their similarity to homologues from *A. fumigatus* (Bamford et al., 2015)]. In addition, certain cell wall biosynthesis genes were found to be specific for vegetative hyphae or for sclerotia. For example, the *gelB* and *gelD* genes, both putative 1,3-beta-glucanosyltransferases, were expressed in vegetative hyphae, but not in sclerotia (Supplemental Table 12). BgtA and BgtE are putative beta-1,3-glucanosyltransferases which were expressed

Table 3
Enriched GO terms (terminal nodes) in sclerotia down-regulated genes.

Gene ID	Description	Myc	Scl	FC	FDR	BPs (terminal node)
An01g03340	strong similarity to xyloglucan-specific *endo*-beta-1,4-glucanase	170,3	51,1	0,3	9,0E−06	Cellulose metabolic process
An07g09330	cellulose 1,4-beta-cellobiosidase cbhA - *Aspergillus niger*)	137,8	19,3	0,1	1,0E−06	Cellulose metabolic process
An14g02760	endoglucanase A eglA - *Aspergillus niger*)	217,6	15,9	0,1	1,4E−06	Cellulose metabolic process
An16g06800	strong similarity to endoglucanase eglB - *Aspergillus niger*	233,5	56,1	0,2	1,3E−05	Cellulose metabolic process
An01g11660	1,4-beta-D-glucan cellobiohydrolase B cbhB - *Aspergillus niger*	876,5	35,6	0,0	1,6E−05	Cellulose metabolic process
An01g11670	strong similarity to *endo*-beta-1,4-glucanase A	95,8	17,9	0,2	0,0012	Cellulose metabolic process
An12g01210	strong similarity to peptide transport protein	2.757,9	112,5	0,0	2,6E−07	Dipeptide transport
An13g03680	strong similarity to allantoate permease	1.079,4	100,1	0,1	1,4E−06	Dipeptide transport
An12g03550	strong similarity to allantoate permease	888,4	56,3	0,1	5,6E−07	Dipeptide transport
An12g05010	acetyl xylan esterase (aceA - *Aspergillus niger*)	430,2	16,5	0,0	4,2E−06	Xylan catabolic process
An03g00940	*endo*-1,4-beta-xylanase A precursor xynA - *Aspergillus niger*)	348,2	22,6	0,1	1,8E−07	Xylan catabolic process
An01g00780	xylanase xynB - *Aspergillus niger*)	270,4	26,6	0,1	4,1E−07	Xylan catabolic process
An14g04200	rhamnogalacturonase rhgB - *Aspergillus niger*	72,5	19,8	0,3	5,2E−06	Pectin catabolic process
An09g02160	rhamnogalacturonan acetyl esterase rgaeA - *Aspergillus niger*	833,4	30,0	0,0	3,2E−06	Pectin catabolic process
An12g00950	rhamnogalacturonase rhgA - *Aspergillus niger*	125,6	28,4	0,2	0,00011	Pectin catabolic process
An01g11520	polygalacturonase pgaI - *Aspergillus niger*	48,1	10,6	0,2	3,6E−05	Pectin catabolic process
An02g04900	endopolygalacturonases pgaB - *Aspergillus niger*	359,5	38,4	0,1	1,5E−06	Pectin catabolic process
An15g02300	arabinofuranosidase B abfB *Aspergillus niger*	677,7	32,3	0,0	2,4E−07	Pectin catabolic process
An04g06920	extracellular alpha-glucosidase aglU - *Aspergillus niger*	6.443,7	1.067,3	0,2	1,6E−06	Starch metabolic process
An14g04200	rhamnogalacturonase rhgB - *Aspergillus niger*	72,5	19,8	0,3	5,2E−06	Starch metabolic process
An11g03340	acid alpha-amylase - *Aspergillus niger*	3.032,5	14,7	0,0	2,5E−07	Starch metabolic process
An04g06910	transcriptional regulator amyR - *Aspergillus niger*	1.203,3	139,4	0,1	6,6E−07	Starch metabolic process
An12g00950	rhamnogalacturonase rhgA - *Aspergillus niger*	125,6	28,4	0,2	0,00011	Starch metabolic process
An01g11520	polygalacturonase pgaI - *Aspergillus niger*	48,1	10,6	0,2	3,6E−05	Monosaccharide metabolic process
An07g03570	strong similarity to sorbitol utilization protein sou2	617,0	86,9	0,1	3,9E−06	Monosaccharide metabolic process
An14g04200	rhamnogalacturonase rhgB - *Aspergillus niger*	72,5	19,8	0,3	5,2E−06	Monosaccharide metabolic process
An03g00940	*endo*-1,4-beta-xylanase A precursor (xynA) - *Aspergillus niger*	348,2	22,6	0,1	1,8E−07	Monosaccharide metabolic process
An03g00960	(1,4)-beta-D-arabinoxylan arabinofuranohydrolase axhA - *Aspergillus niger*	1.015,5	18,5	0,0	1,3E−07	Monosaccharide metabolic process
An02g09090	strong similarity to the mutarotase enzyme	1.103,7	235,1	0,2	3,0E−06	Monosaccharide metabolic process
An09g01190	endo 1,5-alpha-arabinanase abnA - *Aspergillus niger*	585,2	37,6	0,1	2,3E−06	Monosaccharide metabolic process
An15g02300	arabinofuranosidase B abfB *Aspergillus niger*	677,7	32,3	0,0	2,4E−07	Monosaccharide metabolic process
An04g02670	strong similarity to NADPH-dependent aldehyde reductase -	1.315,1	187,3	0,1	1,6E−06	Monosaccharide metabolic process
An11g01120	strong similarity to aldehyde reductase	463,6	67,3	0,1	2,5E−06	Monosaccharide metabolic process
An12g00950	rhamnogalacturonase rhgA - *Aspergillus niger*	125,6	28,4	0,2	0,00011	Monosaccharide metabolic process
An02g11150	alpha-galactosidase (aglB) - *Aspergillus niger*	1.236,7	201,2	0,2	1,7E−06	Monosaccharide metabolic process
An09g00260	alpha-galactosidase C aglC - *Aspergillus niger*	234,4	27,2	0,1	1,2E−05	Monosaccharide metabolic process
An03g06550	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*	16.399,6	327,9	0,0	7,9E−08	Monosaccharide metabolic process
An02g04900	endopolygalacturonases pgaB - *Aspergillus niger*	359,5	38,4	0,1	1,5E−06	Monosaccharide metabolic process
An01g00780	xylanase xynB *Aspergillus niger*	270,4	26,6	0,1	4,1E−07	Monosaccharide metabolic process
An07g09330	cellulose 1,4-beta-cellobiosidase cbhA *Aspergillus niger*	137,8	19,3	0,1	1,0E−06	Glucan catabolic process
An03g05290	similarity to glucan 1,3-beta-glucosidase BGL2	157,9	17,3	0,1	7,1E−07	Glucan catabolic process
An03g06550	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*	16.399,6	327,9	0,0	7,9E−08	Glucan catabolic process
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - *Aspergillus niger*	876,5	35,6	0,0	1,6E−05	Glucan catabolic process
An01g11670	strong similarity to *endo*-beta-1,4-glucanase A eglA - *Emericella nidulans*	95,8	17,9	0,2	0,0012	Glucan catabolic process
An16g07040	similarity to beta-1,3-glucanosyltransferase BGT1	2.249,8	96,4	0,0	5,6E−07	Glucan catabolic process
An04g09420	strong similarity to neutral amino acid permease mtr - *Neurospora crassa*	612,6	40,2	0,1	1,2E−06	Neutral amino acid transport
An16g07680	strong similarity to neutral amino acid permease mtr - *Neurospora crassa*	312,2	68,1	0,2	1,1E−05	Neutral amino acid transport
An03g05360	strong similarity to neutral amino acid permease mtr - *Neurospora crassa*	379,3	29,5	0,1	5,6E−07	Neutral amino acid transport
An14g07130	strong similarity to neutral amino acid permease mtr - *Neurospora crassa*	91,1	26,7	0,3	9,5E−05	Neutral amino acid transport
An14g02720	strong similarity to neutral amino acid permease mtr - *Neurospora crassa*	647,9	112,3	0,2	1,0E−06	Neutral amino acid transport
An15g07550	strong similarity to neutral amino acid permease Mtr - *Neurospora crassa*	87,6	28,7	0,3	2,1E−05	Neutral amino acid transport
An08g08840	strong similarity to glutamate decarboxylase 1	1.845,3	316,6	0,2	2,0E−06	Dicarboxylic acid metabolic process
An12g00160	strong similarity to malate dehydrogenase homolog mae1 -	430,7	66,0	0,2	1,6E−06	Dicarboxylic acid metabolic process
An11g02170	strong similarity to fumarylacetoacetate hydrolase fahA -	1.064,7	207,6	0,2	1,7E−06	Dicarboxylic acid metabolic process
An07g05830	strong similarity to formamidase fmdS - *Aspergillus nidulans*	620,4	46,1	0,1	2,5E−07	Dicarboxylic acid metabolic process
An03g01140	strong similarity to oxalate decarboxylase (APOXD)	1.070,6	178,9	0,2	5,4E−05	Dicarboxylic acid metabolic process
An02g05540	strong similarity to kynurenine/alpha-aminoadipate aminotransferase -	578,1	186,4	0,3	1,2E−05	Dicarboxylic acid metabolic process
An11g02160	strong similarity to maleylacetoacetate isomerase maiA -	200,1	55,6	0,3	2,9E−05	Dicarboxylic acid metabolic process

either exclusively in sclerotia or vegetative hyphae, respectively. The Chr-family in *A. niger* consists of 7 members of which one is specifically expressed in sclerotia (*chrA*), whereas two other members were only expressed during vegetative growth (*chrB* and *chrD*) (Supplemental Table 12). Also two glucanases (ExgA and BxgB) were specifically expressed in sclerotia. Finally, a putative chitin deacetylase encoded by An12g04480 was strongly induced in sclerotia indicating the possibility of the formation of chitosan by the de-acetylation of chitin in sclerotia.

The expression of genes encoding putative cell wall proteins was also compared. Several genes were identified that were specific for vegetative growth or sclerotia, indicating that cell wall mannoprotein composition varies between sclerotia and vegetative hyphae (Supplemental Table 12). The only GPI-anchored cell wall protein to have been analysed in detail in *A. niger* is CwpA (Damveld et al., 2005), which was found to be expressed in vegetative hyphae, but not in sclerotia. No differences in expression of any genes encoding hydrophobins was observed (Supplemental Table 12).

3.10. Sclerotia-specific gene expression

For *A. niger*, a large collection of microarray data is publicly available dating back from 2007 onwards, encompassing transcriptomic studies conducted by many groups (Paege et al., 2016, Schäpe et al., 2019). We used this large collection of microarrays (over 155 different

Table 4
Expression analysis of gene related to terpene biosynthesis in *A. niger*.

Gene ID*	Description	Mean Hyphae	Mean Sclerotia	FC S vs H	qValue
An11g02480	Orthologs have salicylate hydroxylase activity (paxM-like)	19.5	2259.1	116.1	6.02E−08
An11g02490	paxB homolog	19.8	204.7	10.4	3.46E−07
An11g02500	Orthologs have geranylgeranyl pyrophosphate synthetase activity PaxG-like	21.6	97.1	4.5	1.14E−04
An11g02510	similarity to cytochrome P450 monooxygenase TRI4 - *Myrothecium roridum*	17.0	2111.1	124.3	4.88E−08
An03g00830	weak similarity to intestinal mucin MUC2 - *Homo sapiens*	20.8	57.7	2.8	6.73E−05
An03g00840	strong similarity to cDNA clone an_3113 - *Aspergillus niger*	28.0	325.1	11.6	1.02E−05
An03g00850	Orthologs have prenyl transferase activity paxC - *Penicillium paxilli*	40.3	8306.5	206.2	6.02E−08
An03g00860	Orthologs have geranylgeranyl pyrophosphate synthetase activity	17.2	9528.1	554.6	2.13E−08
An03g00870	strong similarity to hypothetical protein An12g10520 – *A.niger*	25.5	402.8	15.8	6.04E−07
An01g07680	paxB homolog	14.7	3.639.5	247.7	5.67E−08

Table 5
Secondary metabolite gene clusters higher expressed in sclerotia compared to hyphae.

Gene ID*	Description	Mean Hyphae	Mean Sclerotia	FC S vs H	qValue
An12g07220	weak similarity to antibacterial glycoprotein achacin - *Achatina fulica*	17.9	10984.7	614.8	2.13E−08
An12g07230	strong similarity to actinomycin synthetase III acmC - *Streptomyces chrysomallus*	25.7	1436.8	56.0	7.16E−08
An03g05420	strong similarity to nitrogen metabolic repression regulator protein hNmrr *H. sapiens*	15.8	3059.8	193.7	1.53E−07
An03g05430	strong similarity to O-methyltransferase omtA - *Aspergillus parasiticus*	29.1	1796.7	61.8	3.59E−07
An03g05440	strong similarity to polyketide synthase alb1 - *Aspergillus fumigatus*	12.8	585.9	45.7	1.6E−07
An03g05450	similarity to zeaxanthin epoxidase ABA2 - *Nicotiana plumbaginifolia*	17.2	626.5	36.4	1.65E−07
An03g05460	strong similarity to cytochrome P450 monooxygenase avnA - *Aspergillus parasiticus*	21.8	3829.8	175.5	8.37E−08
An03g05470	strong similarity to hypothetical protein SCD69.03 - *Streptomyces coelicolor*	47.6	7123.6	149.6	1.85E−06
An03g05480	similarity to O-methyltransferase omtA - *Aspergillus parasiticus*	18.7	7301.3	390.0	2.13E−08
An03g05490	strong similarity to maleylacetate reductase macA - *Rhodococcus opacus*	30.2	1189.7	39.4	3.69E−06
An03g05500	similarity to Zinc finger II protein ORF13 patent WO200037629-A2 - *Aspergillus terreus*	29.7	1618.4	54.5	1.22E−07
An03g05510	hypothetical protein	19.3	881.9	45.7	7.58E−08

growth conditions) to identify genes that were uniquely expressed in sclerotia. In total 83 genes were defined as sclerotium-specific (Supplemental Table 13). These include some of the aforementioned genes such as the aurasporene gene cluster An03g05420-An03g05510 and a chitin deacetylase, and several other genes that are highly and specifically expressed in sclerotia but for which the function is unknown and requires additional functional analysis.

4. Discussion

Sclerotia are considered as dormancy structures, important for fungal survival during environmental extremes such as changes in temperature, pH and water availability. They are produced by phylogenetically and ecologically diverse fungi and it has been suggested that the ability to form sclerotia has evolved on at least 14 different occasions in the fungal kingdom (Smith et al., 2014). For certain fungal species, the formation of sclerotia is in addition an essential prerequisite for sexual reproduction, notably in *Sclerotinia* and some *Botrytis* and *Aspergillus* species (Li et al, 2012; Zhang et al., 2016; Dyer and O'Gorman, 2011).

Sclerotia are produced by members of six different taxonomic sections of the genus *Aspergillus* (Dyer and O'Gorman, 2011). Mature sclerotia exhibit differences in morphology such as in size, shape and colour (Abu El-Souod et al., 2017). Sclerotia may simply act as dormancy structures and/or have been considered to represent vestiges of sexual reproduction in species that have become asexual (Raper and Fennell, 1965; Geiser et al., 1998; Dyer and Kück, 2017). However, given the correct environmental conditions, cleistothecia-containing ascospores may develop internally within sclerotia. In homothallic (self-fertile) species such as *Petromyces alliaceus* sexual development

Table 6
Secondary metabolite gene clusters lower expressed in sclerotia compared to hyphae.

Gene ID*	Description	Mean Hyphae	Mean Sclerotia	FC S vs H	qValue
An01g06820	strong similarity to fatty acid omega-hydroxylase (P450foxy) *F. oxysporum*	1426.5	49.6	28.8	2.88E−07
An01g06830	similarity to ketosphinganine reductase Tsc10p – *S. cerevisiae*	1973.2	41.3	47.8	8.01E−08
An01g06840	strong similarity to acid-CoA ligase Fat2p – *S. cerevisiae*	3389.8	82.9	40.9	1.81E−07
An01g06850	similarity to 4-hydroxybutyrate dehydrogenase - *Alcaligenes eutrophus*	5023.9	170.7	29.4	1.25E−07
An01g06860	strong similarity to hypothetical Fum9p protein - *Gibberella moniliformis*	5400.6	94.8	57.0	7.08E−08
An01g06870	strong similarity to hypothetical protein Fum8p - *Gibberella moniliformis*	3461.7	88.6	39.1	8.37E−08
An01g06880	similarity to dihydroflavonol 4-reductase BAA12723.1 - Rosa hybrid cultivar	3503.9	93.1	37.6	1.45E−07
An01g06890	similarity to hypothetical peptide synthetase pesA - *Metarhizium anisopliae*	5038.2	184.7	27.3	1.11E−07
An01g06900	weak similarity to transcription activator amyR – *A. oryzae*	1259.1	26.5	47.5	8.4E−07
An01g06910	strong similarity to cytochrome P450 CYP94A1 - *Vicia sativa*	197.7	25.8	7.7	4.57E−06
An01g06920	strong similarity to multidrug resistance protein ABCC2 - *Homo sapiens*	3097.0	366.7	8.4	1.27E−06
An01g06930	strong similarity to polyketide synthase FUM5 - *Gibberella moniliformis* (PKS)	3107.4	148.1	21.0	1.36E−07
An03g00640	similarity to neutral amino acid permease mtr – *N. crassa* [truncated ORF]	3039.3	49.9	60.9	4.54E−07
An03g00650	similarity to AM-toxin synthetase AMT - *Alternaria alternate* (NRPS)	95.8	16.9	5.7	1.13E−05
An03g00660	similarity to taurine dioxygenase tauD – *E. coli*	3390.7	26.1	129.8	1.08E−07
An03g00670	similarity to D-nopaline dehydrogenase nos – *A. tumefaciens*	428.7	30.1	14.2	8.49E−07
An03g00680	strong similarity to multidrug resistance protein FNX1 – *S. pombe*	474.7	20.0	23.7	3.33E−07
An03g00690	hypothetical protein	4423.0	42.5	104.2	8.01E−08

proceeds without the need for a mating partner (McAlpin and Wicklow, 2005), whereas in heterothallic (self-sterile) species such as *A. flavus*, *A. parasiticus* and *A. terreus* sexual development requires the presence of complementary *MAT1-1* and *MAT1-2* mating-type partners (Horn et al., 2009, 2014; Arabatzis and Velegraki, 2013).

The formation of cleistothecia and thereby discovery of a sexual cycle in the presumed heterothallic *A. niger* would require the identification of compatible *MAT1-1* and *MAT1-2* strains. Natural strains of *A. niger* are almost exclusively found to contain the *MAT1-1* locus (Pel et al., 2007). The scarcity and lack of compatible *MAT1-1* and *MAT1-2* strains in *A. niger* has so far prevented finding conditions that could lead to cleistothecia formation (Darbyshir, Houbraken, Samson, Baker, Susca, van de Vondervoort and Dyer, unpublished results). Nevertheless, Frisvad et al. (2014) recently found that sclerotium formation could be induced in some strains of *A. niger* by growth on Czapek yeast autolysate (CYA) agar to which raisins or other fruits had been added. It was also noted that non-sclerotium forming *A. niger* strains could be triggered to produce sclerotia by storing conidia at −18 °C for at least three weeks prior to inoculation on CYA and fruit growth plates. These observations were highly significant as they indicated the potential for sexual reproduction in the supposedly asexual *A. niger*, given that related members of the section *Nigri* which undergo sexual reproduction (such as *A. japonica*, *A. tubingensis* and *A. sclerotiocarbonarius*) all form sclerotia within which cleistothecia later develop after an extended period of incubation (Rajendran and Muthappa, 1980; Horn et al., 2013; Darbyshir et al., 2013). However, not all strains of *A. niger* tested by Frisvad et al. (2014) formed sclerotia. Indeed, the strain used in our study N402 (a derivative of N400) does not develop sclerotia on CYA plates amended with raisins even after freezing of conidia (Frisvad, unpublished data). It was perhaps surprising then that a UV-mutant of N402 was obtained by Jørgensen et al. (2011a) which appeared to produce sclerotia-like structures with concomitant reduced levels of asexual sporulation. Work was therefore undertaken in the present study to evaluate first whether the structures seen in this *scl-2* strain were indeed sclerotia, second to determine the genetic basis of sclerotial production, and finally to use transcriptome analysis to determine changes in gene expression linked to production of sclerotia.

4.1. Confirmation of formation of sclerotia in the scl-2 strain

Analysis by microscopy revealed that the putative sclerotia formed by the *scl-2* strain showed the characteristic morphology of sclerotia, exhibiting outer layers of thick walled hyphal cells and inner pseudoparenchymatous hyphae. They resembled in colour and appearance the sclerotia reported by Frisvad et al. (2014) from *A. niger*. The putative sclerotia formed by the *scl-2* strain were also shown to contain at least three different indoloterpenes, consistent with the findings of Frisvad et al. (2014) who reported that sclerotial development in *A. niger* is associated with the indoloterpene production. The associations between fungal secondary metabolism and sclerotial formation in *Aspergillus* species has been reviewed by Calvo and Cary (2015). In addition, aurasperones were detected in sclerotia of the *scl-2* strain. This was surprising, as we and others had previously shown that production of naphtho-γ-pyrone class compounds requires the *fwnA* polyketide synthase (Jørgensen et al, 2011b; Chiang et al., 2011), which is disrupted in the *scl-2* background. It was observed that one of the highest induced PKS genes was An03g05440. Furthermore, An03g05440 shows relative high sequence similarity to *fwnA*, suggesting that An03g05440 might be involved in the production of naphtho-γ-pyrone class compounds, such as aurasperones. Deletion of An03g05440 in the *scl-2* mutant indeed abolished the production aurasperones suggesting that aurasperones and the genes clustered and co-regulated with An03g05440 are responsible for aurasperone production in sclerotia of *A. niger*.

It was also noted that sclerotia were only formed in the dark, which is typical for sexual development in the aspergilli (Dyer and O'Gorman, 2012), suggesting a shared evolutionary origin of scleriotial and cleistothecial development as regards regulation of developmental choices toward sexual or asexual development (e.g. Bayram and Braus, 2012; Amaike and Keller, 2011).

Further confirmatory evidence that the sclerotia-like structures formed by the *scl-2* strain were indeed sclerotia of *A. niger* came from gene disruption work. Previous work by Kim et al. (2011) and Segmüller et al. (2008) has shown that a functional NADPH-complex was required for sclerotia development in *S. sclerotiorum* and *B. cinerea* respectively. Consistent with these reports, it was here found that deletion of the *noxA* or *noxR* genes in the *scl-2* strain resulted in the loss of production of sclerotia (Fig. 3).

4.2. Genetic basis of formation of sclerotia in the scl-2 strain

A combination of parasexual crossing, bulk segregant analysis (BSA), and high-throughput next generation sequencing (NGS) as first employed by Niu et al. (2016) was used to identify the genetic basis of sclerotial formation in the *scl-2* strain. This approach was facilitated by the fact that the JN6.2 crossing partner used for parasexual crossing was derived from the same ultimate parental strain as *scl-2*, meaning that a fairly uniform genetic background was present, reducing the number of SNPs to be analysed. It was observed that the diploid strain did not produce sclerotia, indicating that the *scl-2* mutation(s) could be complemented by the presence of a functional gene copy. The BSA/NGS analyses then revealed that the *scl-2* phenotype most likely arose from a mutation in a gene (An08g07710) encoding a novel putative $ZnII_2Cys_6$ transcription factor, with a stop codon located in the Zn-finger domain of the gene in the *scl-2* strain. This was confirmed by the fact that introduction of a functional copy of the gene complemented the *scl-2* mutant, and not only led to loss of sclerotial production but also restored the growth and asexual sporulation phenotype of the wild-type strain. Further evidence that the An08g07710 gene is involved in sclerotial development came from the finding that deletion of the gene, here named *sclB*, in a wild-type background resulted in a sclerotia-forming phenotype. It is therefore apparent that the SclB transcription factor has a role in promoting asexual growth and sporulation whilst acting as a negative regulator of sclerotial development in *A. niger* under standard growth conditions. Indeed, during the course of the present study a homologue of *sclB* was identified from *A. nidulans* using the present *A. niger sclB* gene sequence as a search term (Thieme et al., 2018). Deletion of the *A. nidulans sclB* gene led to a similar phenotype of reduced asexual sporulation and also impacted secondary metabolism. However, there was no clear effect on cleistothecial development, suggesting that *sclB* is involved primarily in regulation of asexual, rather than sexual, development in *A. nidulans*. Further work is now required to examine the expression of *sclB* during different growth states of *A. niger*.

The same phenotype of reduced conidiation and increased formation of sclerotia in the *scl-2* strain was also observed in an *A. oryzae* strain overexpressing *sclR* (Jin et al., 2011a). This suggests a similar mechanism(s) across the aspergilli in which the fungus switches between two primary developmental programs, and that choosing one will repress the other pathway. Assuming that sclerotium formation and cleistothecium formation are evolutionary connected and triggered by the same environmental signals, it seems that in *A. niger* the pathway leading to asexual sporulation is strongly preferred over the sclerotium/cleistothecium developmental pathway under standard growth conditions. Conditions to activate the sexual/sclerotial differentiation pathway have been identified in several *Aspergillus* species, including species once thought to be asexual such as *A. fumigatus, A. flavus*, *A. parasiticus* and *A. clavatus* (O'Gorman et al., 2009; Dyer and O'Gorman, 2012; Horn et al., 2009, 2014; Ojeda-López et al., 2018), but conditions leading to sclerotia formation in *A. niger* have only recently been established (Frisvad et al., 2014).

4.3. Transcriptome expression analysis of sclerotia compared to vegetative hyphae

Finally, transcriptomic analyses were undertaken to assess changes in gene expression linked to production of sclerotia. This involved data collection in the present study to directly compare gene expression of *scl-2* mycelial or sclerotial samples on the same plate, and also a broader comparison to publicly available microarray data for *A. niger* to identify sclerotia-specific gene expression. This revealed several interesting insights into sclerotia as unique differentiated structures.

The GO analysis revealed that the production of extracellular enzymes such as glucoamylases and many proteases is strongly downregulated in sclerotia. Terminal nodes included those related to dipeptide transport and neutral amino acid uptake, and for dicarboxylic acid metabolic processes. This might indicate a reshuffling of C-metabolism during sclerotia formation possibly for the production of secondary metabolites. The presence of the GO terḿ related to sporocarp developmenť (fruiting body development) was consistent with the hypothesis that sclerotia are an initial stage of sexual development in *A. niger*, although relatively few genes related to the sexual cycle were upregulated. The GO terḿ tryptophan metabolic process' suggested upregulation of tryptophan biosynthesis. possibly as a consequence of indoloterpene production. Indoloterpenes have a common core structure comprising of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGDP) and an indole group that is most likely derived from indole-3-glycerol phosphate, a precursor of tryptophan.

The sclerotium upregulated or specific genes included a secondary metabolite gene cluster (An03g05420 to An03g05510) which was found to be uniquely expressed in sclerotia. Blast analysis showed that the polyketide synthase (An03g05440) in the cluster is highly similar to *fwnA,* the PKS required for melanin and naphtho-γ-pyrones synthesis in (Jørgensen et al., 2011b; Chiang et al., 2011). Deletion of An03g05440 in the *scl-2* mutant resulted in the absence of aurasperone indicating that this gene cluster is indeed responsible for aurasporone production in sclerotia of *A. niger*.

Sclerotia are differentiated structures in which hyphae are densely packed and intertwined, with the outer layers being hardened to give robustness to the sclerotium. The cell wall composition of sclerotia of *A. niger*, and whether the composition differs from vegetative hyphae, has not yet been analysed. In the present study, genes possibly involved in galactosaminogalactan (GAG) synthesis were found to be strongly induced in sclerotia. The expression of genes involved in GAG-biosynthesis suggests that this cell wall polymer is present in the cell wall of sclerotia. GAG has been found together with α-1,3-glucan as an important cell wall component in biofilms of *A. fumigatus* in which hyphae are also densely packed (Loussert et al., 2010). Imaging by electron microscopy of sclerotia indicated the presence of an extracellular matrix covering the sclerotia of the *scl-2* strain, and it will be of interest to determine whether GAG and α-glucan are important components of this matrix. It is also noted that expression of the α-glucan synthase gene AgsD (An02g03260) in *A. niger* is also very specific for sclerotia (the only other detected expression being during starvation conditions after growth on fructose and sorbitol). Thus, the overall data suggests that the cell wall composition of hyphae in some parts of sclerotia might be different from that of vegetative hyphae, being rich in galactosaminogalactan and containing an altered alpha-glucan content and possibly other cell wall proteins. In a recent RNA-seq study, Luo et al. (2019) found evidence for abundant expression of enzymes related to cell-wall remodelling, together with upregulation of genes linked to melanin biosynthesis, during the development of microsclerotia of *Verticillium dahliae*.

Previous studies in *A. oryzae* have resulted in the identification of TFs involved in sclerotium formation (Jin et al., 2011a,b). SclR and EcdR both encode basic helic-loop-helix (bHLH) TFs with the SclR transcription factor being required for sclerotium formation. In the *A. niger scl-2* sclerotia-forming strain, the *sclR* homolog (An14g02540) was found to show 3.5 fold increased expression compared to vegetative mycelium, indicating that expression of the SclR homolog also contributes to sclerotium formation in *A. niger*. Transcriptomic analysis of the EcdR homolog in *A. niger* (An04g01250) showed no differential expression. Further genetic manipulation of the *sclR* and *ecdR* homologues in *A. niger* should give insights into whether and how these TFs regulate sclerotium formation in *A. niger*.

4.4. Concluding remarks

The present study is the first to investigate the molecular-genetic basis of formation of sclerotia in *A. niger*. Results indicate that the SclB transcription factor promotes asexual reproduction and represses sclerotium formation under standard growth conditions i.e. *A. niger* has developed a default strategy towards asexual development due to activity of SclB. In the *scl-2/ΔsclB* mutant this default strategy is broken, allowing the fungus to respond to environmental signals (such as darkness) that promote sclerotium development.

Many genetic tools have been developed and employed to optimise *A. niger* as a cell factory for protein and metabolite production given the importance of the species in the biotechnology industry (de Vries et al., 2017; Cairns et al., 2018, 2019). It would be a great advance if the possibility of sexual crossing could be added to the *A. niger* genetic tools box (Ashton and Dyer, 2016). Genetic manipulation of *sclB* to enhance formation of sclerotium might be a first prerequisite step towards engineering *A. niger* strains capable of sexual reproduction. This process would still require identification of sexually compatible *MAT1-1* and *MAT1-2* strains of *A. niger*, which is not straightforward given a strong bias towards the *MAT1-1* genotype in populations of *A. niger* (Pel et al., 2007; Darbyshir, Houbraken, Samson, Baker, Susca, van de Vondervoort and Dyer, unpublished results). Alternatively, complementary mating-type genes could be expressed in the same strain, as is the case in homothallic species such as *A. nidulans* (Paoletti et al., 2007). The *sclB* mutant could act as an important host for such experiments as the pathway towards sclerotium and possibly cleistothecium development is already activated. Results from such studies could also be pivotal for induction of sexual reproduction in other asexual species of importance in biotechnology such as *A. oryzae,* which can be induced to form sclerotia and has proven *MAT1-1* and *MAT1-2* genotypes but for which no sexual stage has yet been identified (Wada et al., 2012).

5. Contributions of the authors

TRJ: project design, isolation of mutants, identified sclerotia, transcriptomics data analysis, writing; ABW: transcriptomics data analysis, MA: strain construction and genome sequencing; TS: strain construction and metabolite analysis, writing; GL:microscopy: JN: parasexual crossing; MJK: strain construction; JP: strain construction; JF: metabolite analysis; KFN: metabolite analysis; VM: project funding, data analysis and writing; CAMH: project funding, project design, data analysis, writing; PD: data analysis and writing. AFJR: project funding; project design, data analysis, writing. All authors read and approved the final manuscript.

Funding

This work was partly funded by the Kluyver Centre for Genomics of Industrial Fermentation (NGI/NWO) and the Marie Curie International Training Network QuantFung FP7-People-2013-ITN, Grant 607332.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2020.103377.

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