Identification of ScLB, a Zn(II)$_2$Cys$_6$ transcription factor involved in sclerotium formation in Aspergillus niger

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**A B S T R A C T**

Certain *Aspergillus* species such as *A. 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)$_2$Cys$_6$ 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 indoloterpane synthesis was confirmed and also led 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...
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 some 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 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 ScIR vs EcdR determines a developmental decision towards either sclerotium formation or asexual reproduction (Brown et al., 2009). By contrast, deletion of psi genes altered the balance between conidiation and sclerotium formation in A. flavus, with ppaA and ppaC having antagonistic roles (Brown et al., 2009). In addition, deletion of ppa genes altered the balance between conidiation and sclerotium formation in A. flavus, whereas higher expression of sclR favours sclerotium formation (low EcdR/high ScIR) or asexual development (high EcdR/low ScIR) (Jin et al., 2011a, 2011b). Meanwhile, the light/dark response regulator protein VeA is required for sclerotial development in A. parasiticus and A. flavus (Calvo et al., 2004, Cary et al., 2007). Finally, a calcineurin-basal transcription factor 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; Amelem 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 aflavin-type apolar indoloterpenes. Production of aflavins 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; Pomranging 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 ScIR, 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 solid medium (MM) or complete medium (CM). MM contains 7 mM KCl, 8 mM KH2PO4, 70 mM NaNO3, 2 mM MgSO4, and 0.5% (w/v) glucose. Plates were incubated at 30 °C in continuous light or complete dark. MM supplemented with 10 mM uridine or 2.5 mg/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 for 7 days in continuous light or complete dark as indicated. SEM-pictures were taken as previously described (Jørgensen et al., 2010) and safranin staining was performed according to Xiaoke and Shuxing (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-32P-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 sclAP1-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-AmpyG split marker fragments were created by fusion PCR (see Arentshorst et al. (2015a) for details about primers and selection
marker template]. These sclB-APyrG split marker fragments were transformed into A. niger strains MA169.4 (kusA::DR-amds-DR, pyrG) and MA100.1 (kusA::amds, ∆fwnA::hygB,pyrG), resulting in strains MA336.1 (kusA::DR-amds-DR, ∆sclB::AopyrG) and strain MA337.1 (kusA::amds, ∆sclB::AopyrG, ∆fwnA::hygB) (Table 1). Deletion of the sclB gene was confirmed by Southern blot analysis (data not shown). Deletion cassettes to inactivate the noxA and noxB 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 (An03g05440), including 1000 bp upstream region and 650 bp downstream region, was PCR amplified using primers sclAP1f-NotI and ligated into the pJet1.2 vector. The resulting PCR product (3346 bp) was ligated into PCR cloning vector pJet1.2 (Thermo Scientific) and sequenced. The whole complementing sclB fragment was isolated with NotI and ligated into the NotI Open reading frame (ORF) targeting vector pMA334 (Arentshorst et al., 2015b). The resulting plasmid, pMA363, was digested with Ascl 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. The resulting PCR product (3346 bp) was ligated into PCR cloning vector pJet1.2 (Thermo Scientific) and sequenced. The whole complementing sclB fragment was isolated with NotI and ligated into the NotI Open reading frame (ORF) targeting vector pMA334 (Arentshorst et al., 2015b). The resulting plasmid, pMA363, was digested with Ascl 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).

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 PorosHil 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).

Table 1

| Strain   | Genotype                                    | Description                                                        | Reference          |
|----------|---------------------------------------------|--------------------------------------------------------------------|--------------------|
| N402     | cpA1                                        | 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                             | kusA70 derivative of AB4.1                                       | Meyer et al. (2007) |
| MA169.4  | kusA::amds, fwnA::hygB,pyrG                 | curable kusA70 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, pLAB4.1 (A. niger pyrG containing plasmid) | TJ1.2 complemented with A. niger pyrG                        | Jørgensen et al. (2011b) |
| MA192.3  | scl-2, pLAB4.1 (A. niger pyrG containing plasmid) | TJ1.2 complemented with A. niger pyrG and fwnA                   | This study         |
| MA145.1  | scl-2, noxA::pyrG                           | ∆noxA in TJ1.2                                                     | This study         |
| MA144.1  | scl-2, noxB::pyrG                           | ∆noxB in TJ1.2                                                     | This study         |
| N6.2     | capA1, olvA::ApypG, nicB::hygB              | ∆nicB in MA169.4                                                   | Niu et al. (2016)  |
| AR206    | capA1/capA1, fwnA::fwnA::hygB,ohvA/ ohvA::AopyrG, pyrG/yc37, nicB::hygB | diploid TJ1.2 × N6.2                                               | This study         |
| AM336.1  | ∆sclB::pyrG                                 | ∆sclB in MA169.4                                                   | This study         |
| AM337.1  | ∆fwnA::hygB, ∆sclA::pyrG                    | ∆sclA in MA169.4                                                   | This study         |
| AM338.1  | scl-2, pLAB4.1 (sclA)                       | ∆sclB in MA169.4                                                   | This study         |
| TS51.5, TS51.9 | An03g05440::AopyrG           | ∆An03g05440 in TJ1.2                                              | This study         |
| TS58.4, TS58.5 | An03g05440::AopyrG              | ∆An03g05440 in MA100.1                                            | This study         |

2.4. Bulk segregant analysis and whole genome sequencing

Parasexual crossing of haploid strains TJ1.2 (scl-2, ∆fwnA::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) 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/CA-SAVA v1.8.2. Based on the mapped reads, variants in the sample data sets were identified and between the samples using an in-house SNP pipeline v3.2 (ServiceXS). Validated variants had to be found in one organism=Aspni5) and between the samples using an in-house SNP pipeline v3.2 (ServiceXS). Validated variants had to be found in one
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 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 Bioanalyzer. The spectrum generated by the Agilent Bioanalyzer 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 ΔfwnA 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 continuous 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[1,8-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 conidiophores 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 became 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,
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 ΔfwnA 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-aflavilin, hydroxyaflavilin, and 10,23-dihydro-24,25-dehydro-21-oxo-aflavilin, 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 ΔfwnA scl-2 strain. This was surprising, as we and others had previously shown that production of naphtho-γ-pyrones 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 fumonisins B2/B4, kotanin (and its precursors demethylkotanin and orlandin), fungitoxin A, pyranonigrin A, nigerazine and funalemone, 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 was obtained 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.
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 ZnII2Cys6 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 \( \Delta fwnA \) 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. 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 ZnII(Cys6 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)$_2$Cys$_6$ 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 mycelia

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 606 genes encompassing 32 biological processes including 9 terminal nodes (Table 3 and Supplementary Table 5). Six of these terminal nodes contain genes encoding glycosylhydrodrolases.

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 (Frivad 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 indoloterpene paspaline in P. pastoris, 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 piimaradiene 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).
Regardino, 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 naphth-o-gly-pyrone class of secondary metabolites. Previous studies in A. niger have shown that the production of naphtho-gly-pyrone was dependent on the PKS gene fwmA (Jørgensen et al., 2011b; Chiang et al., 2011). Given that An03g05440 exhibited similarity to fwmA (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 (indoloterpines and fumonisin) were still found (Supplemental Fig. 5). Two genes (An01g06890 (NRPS) and An01g06930 (PKS)) involved in fumonisin biosynthesis (Pel et al., 2007) were significantly down-regulated 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 adjacent genes (Table 5). Upregulation of An14g02540, which encodes a TF of the basic helix-loop-helix family of TFs, was also observed down-regulation of many extracellular enzymes (Table 3). 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).

3.8. 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)Cys6 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 ScrA, 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)Cys6 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 down-regulation 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 (agaD) and down-regulation of an alpha-1,3-glucan processing enzyme (agaC) 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-glucanosiyltransferases which were expressed upregulation, and there was even downregulation of An04g07400, a Zn(II)Cys6 transcription factor homologous to Pro1 essential for sexual development in Sordaria macrospora (Masloff et al., 1999) (Supplemental Table 8).

Table 2

| Gene ID               | Ortholog(s) | Description                                      | Myc | Scl | FC   | FDR  |
|-----------------------|-------------|--------------------------------------------------|-----|-----|------|------|
| An04g05950            | Ortholog(s) | have inositol hexakisphosphate kinase activity   |     |     |      |      |
| An08g05260            | Ortholog(s) | have phospholipase C activity (ScPLCI)           |     |     |      |      |
| An09g05250            | Ortholog(s) | have inositol hexakisphosphate 3-kinase activity |     |     |      |      |
| An10g07260            | Ortholog(s) | have anthranilate phosphoribosyl transferase activity |  | | | |
| An08g06080            | Ortholog(s) | have anthranilate synthase activity              |     |     |      |      |
| An10g06010            | Ortholog(s) | have chorismate mutase activity                  |     |     |      |      |
| An14g01820            | Ortholog(s) | strong similarity to hypothetical cell wall protein bina |  | | | |
| An04g05880            | Ortholog(s) | Putative dioxygenase                             |     |     |      |      |
| An01g01550            | Ortholog(s) | BrA transcription factor                         |     |     |      |      |
| An01g02970            | Ortholog(s) | have protein histidine kinase activity           |     |     |      |      |
| An08g06080            | Ortholog(s) | have anthranilate synthase activity              |     |     |      |      |
| An02g06820            | Ortholog(s) | putative hydantoin transaminase                  |     |     |      |      |
| An01g07260            | Ortholog(s) | have anthranilate synthase activity              |     |     |      |      |

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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 *An15g07550* showed strong similarity to neutral amino acid permease *Mtr*.

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

| Gene ID* | Description | Mean Hyphae | Mean Sclerotia | FC S vs H qValue |
|----------|-------------|-------------|----------------|------------------|
| An03g05440 | similarity to polyketide synthase ORF13 | 12.8 | 585.9 | 45.7 | 1.6E–07 |
| An03g05450 | similarity to zeaalexin epoxide ABAA2 | 17.2 | 626.5 | 36.4 | 1.65E–07 |
| An03g05460 | strong similarity to cytochrome P450 monooxygenase avnA - Aspergillus parasticus | 21.8 | 3829.8 | 175.5 | 8.37E–08 |
| An03g05470 | strong similarity to hypothetical protein SCD49.03 - Streptomyces coelicolor | 47.6 | 7123.6 | 149.6 | 1.85E–06 |
| An03g05480 | similarity to O-methyltransferase ommA - Aspergillus parasticus | 18.7 | 7301.3 | 390.0 | 2.13E–08 |
| An03g05490 | similarity to maleylacetate reductase macA - Rhodococcus opacus | 30.2 | 1189.7 | 39.4 | 3.96E–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 |

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 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 auraspore 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.

| Gene ID* | Description | Mean Hyphae | Mean Sclerotia | FC S vs H qValue |
|----------|-------------|-------------|----------------|------------------|
| An1g02480 | Orthologs have salicylate hydroxylase activity (paxM-like) | 19.5 | 2259.1 | 116.1 | 6.02E–08 |
| An1g02490 | paxB homolog | 19.8 | 204.7 | 10.4 | 3.46E–07 |
| An1g02500 | Orthologs have geranylgeranyl pyrophosphate synthetase activity PaxG-like | 21.6 | 97.1 | 4.5 | 1.14E–04 |
| An1g02510 | similarity to cytochrome P450 monoxygenase TR04 - Myrothecium verrucaria | 17.0 | 2111.2 | 124.3 | 4.88E–08 |
| An03g0830 | weak similarity to intestinal mucin MUC2 - Homo sapiens | 20.8 | 57.7 | 2.8 | 6.73E–05 |
| An03g0840 | strong similarity to cDNA an_3113 - Aspergillus niger | 28.0 | 325.1 | 11.6 | 1.02E–05 |
| An03g0850 | Orthologs have prenyl transferase activity paxC - Penicillium parishii | 40.3 | 8306.5 | 206.2 | 6.02E–08 |
| An03g0860 | Orthologs have geranyl/geranyl pyrophosphate synthetase activity | 17.2 | 9528.1 | 554.6 | 2.13E–08 |
| An03g0870 | strong similarity to hypothetical protein An1g10520 - *A. niger* | 25.5 | 402.8 | 15.8 | 6.04E–07 |
| An03g0880 | paxB homolog | 14.7 | 369.5 | 247.7 | 5.67E–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 Velegkri, 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 sclerotal 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 pseudo-parenchymatous 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 indolopertenes, consistent with the findings of Frisvad et al. (2014) who reported that sclerotial development in *A. niger* is associated with the indolopertene 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 sclerotial 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 ZnII2Cys6 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 *scIB*, in a wild-type background resulted in a sclerotia-forming phenotype. It is therefore apparent that the ScIB 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 *scIB* was identified from *A. nidulans* using the present *A. niger* *scIB* gene sequence as a search term (Thieme et al., 2018). Deletion of the *A. nidulans scIB* 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 *scIB* is involved primarily in regulation of asexual, rather than sexual, development in *A. nidulans*. Further work is now required to examine the expression of *scIB* 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 *scIB* (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 and cleistothecia 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 down-regulated 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 term related to sporocarp development (fructifying 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 term ‘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 fwaA, the PKS required for melanin and naphto-γ-pyrone synthesis (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 aurasperone 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 (Loustert 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 (Jim et al., 2011a,b). ScIR and EcdR both encode basic helic-loop-helix (bHLH) TFs with the ScIR transcription factor being required for sclerotium formation. In the A. niger scl-2 sclerotia-forming strain, the scIR homolog (An14g02540) was found to show 3.5 fold increased expression compared to vegetative mycelium, indicating that expression of the ScIR 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 scIR 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 ScIR 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 ScIR. 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 sclerotia might be a first requisite 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 recombination and metabolite analysis; KFN: metabolite analysis; VM: project funding, data analysis and writing; GL: microscopy; JN: parasexual recombination and metabolite analysis; KFN: metabolite analysis; VM: project funding, data analysis and writing; AFJR: project funding; project design, data analysis, writing. All authors read and approved the final manuscript.

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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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