The role of melanin pathways in extremotolerance and virulence of Fonsecaea revealed by de novo assembly transcriptomics using illumina paired-end sequencing

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Abstract: Melanisation has been considered to be an important virulence factor of Fonsecaea monophora. However, the biosynthetic mechanisms of melanisation remain unknown. We therefore used next generation sequencing technology to investigate the transcriptome and digital gene expression data, which are valuable resources to better understand the molecular and biological mechanisms regulating melanisation in F. monophora. We performed de novo transcriptome assembly and digital gene expression (DGE) profiling analyses of parent (CBS 122845) and albino (CBS 125194) strains using the Illumina RNA-seq system. A total of 17352 annotated unigenes were found by BLAST search of NR, Swiss-Prot, Gene Ontology, Clusters of Orthologous Groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) (E-value <1e−5). A total of 2283 unigenes were judged to be the differentially expressed between the two genotypes. We identified most of the genes coding for key enzymes involved in melanin biosynthesis pathways, including polyketide synthase (pks), multicopper oxidase (mco), laccase, tyrosinase and homogentisate 1,2-dioxygenase (hmgA). A total of 17352 annotated unigenes were found by BLAST search of NR, Swiss-Prot, Gene Ontology, Clusters of Orthologous Groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) (E-value <1e−5). A total of 2283 unigenes were judged to be the differentially expressed between the two genotypes. We identified most of the genes coding for key enzymes involved in melanin biosynthesis pathways, including polyketide synthase (pks), multicopper oxidase (mco), laccase, tyrosinase and homogentisate 1,2-dioxygenase (hmgA). DEG analysis showed extensive down-regulation of key genes in the DHN pathway, while up-regulation was noted in the DOPA pathway of the albino mutant. The transcript levels of partial genes were confirmed by real time RT-PCR, while the crucial role of key enzymes was confirmed by either inhibitor or substrate tests in vitro. Meanwhile, numbers of genes involved in light sensing, cell wall synthesis, morphology and environmental stress were identified in the transcriptome of F. monophora. In addition, 3533 SSRs (Simple Sequence Repeats) markers were identified from 21600 consensus sequences. Blocking of the DNH pathway is the most likely reason of melanin deficiency in the albino strain, while the production of pheomelanin and pyomelanin were probably regulated by unknown transcription factors on upstream of both pathways. Most of genes involved in environmental tolerance to oxidants, irradiation and extreme temperatures were also assembled and annotated in transcriptomes of F. monophora. In addition, thousands of identified cSSR (combined SSR) markers will favour further genetic linkage studies. In conclusion, these data will contribute to understanding the regulation of melanin biosynthesis and help to improve the studies of pathogenicity of F. monophora.

Key words: Chromoblastomycosis, Fonsecaea monophora, Melanin, Paired-end sequencing, Transcriptome.

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INTRODUCTION

The genus Fonsecaea comprises etiologic agents of human chromoblastomycosis, a chronic (sub)cutaneous infection eventually leading to cauliflower-like eruptions on the skin (Kombila et al. 1995, Estere et al. 1996, Attappattu 1997, Silva et al. 1998, Queiroz-Telles et al. 2009, Xi et al. 2009a,b). The disease has mostly been reported in tropical and subtropical climate zones, and has a high incidence in endemic areas (Najafzadeh et al. 2011, Sun et al. 2012), but autochthonous infections have also been reported from temperate Europe (Pindycka-Piaszcynska et al. 2014). One of the consistent features of etiologic agents of chromoblastomycosis is their consistent melanisation, all species having an olivaceous black thallus. Upon entering human tissue, a shift is observed from hyphal to meristematic growth, leading to formation of the isodiametrically enlarging tissue phase, the muriform cell.

Published reports on melanisation of Fonsecaea showed that the pathogens are able to produce secreted as well as cell-wall-associated melanin-like components (Cunha et al. 2005, Franzen et al. 2006, Santos et al. 2007). These melanins are either immunological activators or involved in interaction with host immune cells (Farbierz et al. 1992, Nosanchuk et al. 1998), while effects on susceptibility to antifungal agents are not congruent (Polak & Dixon 1989, van de Sande et al. 2007, Sun et al. 2011).

Fonsecaea monophora, one out of four pathogenic species of Fonsecaea, is able to cause subcutaneous as well as brain infections (de Hoog et al. 2004, Surash et al. 2005). In our previous study, a morphological mutant of F. monophora showing meristematic growth in vivo and in vitro was isolated from a case of chromoblastomycosis in China (Xi et al. 2009a,b). After two years of subculturing (transferred every three months) an albino mutant was obtained (Sun et al. 2011). The parent strain showed slow-growing, black, heaped colonies, and produced cell-wall-associated secreted melanin, while the albino mutant was melanin-deficient and was sensitive to environmental stress factors of temperature, pH, UV irradiation and oxidative stress (Sun et al. 2011). Moreover, melanisation of the parent strain inhibited production of nitric oxide and Th1 cytokines of murine

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macrophages, which probably enhanced persistence of the fungus in tissue (Zhang et al. 2013). The loss of melanin production ability in albino strain perhaps was due to the mutations of key enzymes in melanin biosynthetic pathway. Differ from those resulting from random mutagenesis or recoverable mutations generated by UV light (Romero-Martinez et al. 2000, Ruiz-Diez & Martinez-Suarez 2003), these mutations of key enzymes are fixed after generated from parent strain, result in permanent loss of melanin production in albino strain. Therefore, clarification of the melanin biosynthesis pathway in the albino mutant is essential to elucidate the physiological processes involved in melanisation and will help to understand the pathogenesis of Fonsecaea. However, large-scale identification of melanin biosynthetic pathway genes at genome or transcriptome levels is still not available in *F. monophora*.

RNA deep sequencing technologies such as Solexa/Illumina RNA-seq and Digital Gene Expression (DGE) have changed approaches in functional gene identification dramatically. These technologies greatly facilitate investigation of functional complexity of transcriptomes (Anisimov 2008, Wang et al. 2009). RNA-seq refers to transcriptome shotgun sequencing wherein mRNA or cDNA is mechanically fragmented, resulting in overlapping short fragments that cover the entire transcriptome. DGE is a tag-based transcriptome sequencing approach where short raw tags are generated by endonucleases. The expression level is a tag-based transcriptome sequencing approach where short raw tags are generated by endonucleases. The expression level of virtually all genes in the sample is measured by counting the number of individual mRNA molecules produced from each gene. Compared with DGE analysis, RNA-seq is a powerful approach to unravel transcriptome complexity, and to identify genes, transcript structure, alternative splicing, non-coding RNAs, and new transcription units. In contrast, the DGE protocol is more suitable and affordable for comparative gene expression studies because it enables direct transcript profiling without compromise and potential bias, thus allowing sensitive and accurate profiling of the transcriptome that reflects the biology of the cell more closely (Morozova & Marra 2008, Mortazavi et al. 2008, Nagalakshmi et al. 2008, Sultan et al. 2008). These two technologies have increasingly been used in transcriptome profiling studies of divergent organisms (Xiang et al. 2010, Wei et al. 2011, Liu et al. 2012, Liang et al. 2013).

The present study conducts a transcriptome profiling analysis of *F. monophora* using RNA-seq and DGE to gain deep insight into melanin biosynthetic pathways, as well as in cell wall biosynthesis genes and tolerance genes responding to environmental stress (oxidants, irradiation and extreme temperatures). were scraped from the colony surface, immediately frozen in liquid nitrogen and transferred to the sequencing company with dry ice. Total RNA of each sample was isolated using a hexadecyltrimethylammonium bromide (CTAB) based protocol and further purified with the RNeasy Plus Universal Kits (Qiagen, Valencia, USA). RNA quality was verified using a 2100 Bioanalyzer RNA Nanochip (Agilent, Santa Clara, USA), and both samples had RNA Integrity Number (RIN) value more than 9.0. Then RNA was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA). A total of each 20 μg of RNA was used for cDNA library preparation.

cDNA library construction and sequencing

Illumina sequencing using the HiSeq™ 2000 platform was performed at the Beijing Genomics Institute (BGI), Shenzhen, China (www.genomics.cn/index.php) according to the manufacturer’s instructions (Illumina, San Diego, USA). Briefly, poly (A) RNA was isolated from total RNA using Sera-mag Magnetic Oligo (dT) Beads (Illumina). To avoid priming bias when synthesising cDNA, purified mRNA was first fragmented into small pieces (100–400 bp) using divalent cations at 94 °C for exactly 5 min. Then the double-stranded cDNA was synthesised using the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Camarillo, USA) with random hexamer (N6) primers (Illumina). The synthesised cDNA was subjected to end-repair and phosphorylation using T4 DNA polymerase, Klenow DNA polymerase and T4 PNK. These repaired cDNA fragments were 3’-adenylated using Klenow Fragment (3’–5’ exo−, Illumina). Illumina paired-end adapters were ligated to the ends of these 3’-adenylated cDNA fragments. To select a size range of templates for downstream enrichment, products of the ligation reaction were purified on a 2 % TAE-agarose gel (Certified Low-Range Ultra Agarose, Biorad, USA). A range of cDNA fragments (200 ± 25 bp) was excised from the gel. Fifteen rounds of PCR amplification were performed to enrich the purified cDNA template using PCR Primers PE 1.0 and PE 2.0 (Illumina) with Phusion DNA Polymerase. The cDNA library was constructed with a fragment length range of 200–400 bp. Finally, after validating on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA 1000 chip kit (Agilent, Santa Clara, USA), the cDNA library was sequenced on a PE flow cell using Illumina HiSeq™ 2000 platform, clean sequencing reads with Q20 > 97 % were used for further analysis.

Data filtering and de novo assembly

Quality requirements for de novo transcriptome sequencing are significantly higher than that for re-sequencing, because sequencing errors can create difficulties for the short-read assembly algorithm. We therefore carried out a stringent filtering process. Firstly, we removed reads that did not pass the built-in Illumina’s software Failed-Chastity filter according to the relation “failed-chastity ≤ 1”, using a chastity threshold of 0.6, on the first 25 cycles. Secondly, we discarded all reads with adapter contamination. Thirdly, we ruled out low-quality reads with ambiguous sequences “N”. Finally, the reads with more than 10 % Q < 20 bases were also removed.

De novo assembly was carried out with the Trinity short reads assembling program (http://trinityrnaseq.sourceforge.net/)

**MATERIAL AND METHODS**

**Strains and RNA extraction**

CBS 122845 was isolated from lesion of an 81-year-old male patient (Xi et al. 2008a,b). The isolate was confirmed to be a meristematic mutant of *F. monophora* by morphology and multilocus molecular data including ITS, ACT, BT2 and CDC42 genes (Sun et al. 2011). Its albino mutant CBS 125194 (the mutant was initially assigned as CBS 125149, while the accession number was changed to be CBS 125194) was generated by subculture in vitro (once per three months) (Sun et al. 2011). For the transcriptome sequencing, both strains were cultured at 25 °C for 14 days on potato dextrose agar (PDA) medium. Cells

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Gene Ontology (GO) functional annotation has three ontologies: molecular function, cellular component and biological process. With NR annotation, we used the program Blast2GO (v2.5.0) to get GO annotation of unigenes (Conesa et al. 2005). After obtaining GO annotation for every unigene, we used WEGO software (www.wego.genomics.org.cn) to perform GO functional classification for all unigenes and to understand the distribution of gene functions of the species from the macro level (Ye et al. 2006).

**Unigene expression difference analysis**

This analysis aims to predict genes with different expression levels, subsequently carrying out GO functional analysis and KEGG pathway analysis. Unigene expression calculations were aided by FPKM (Fragments Per kb per Million reads) (Audic & Claverie 1997) which were used to eliminate the influence of differences in gene lengths and sequencing level. Accordingly, verified gene expressions could be used directly for comparison of differences in gene expression between samples. The formulae of FPKM and RPKM are identical (Fig. 1A), if both pairs of reads were aligned to a gene, we treated them as a single fragment with FPKM; otherwise they were treated as two reads with RPKM.

We developed a rigorous algorithm to identify differentially expressed genes between two samples (Fig. 1B). The null hypothesis and alternative hypothesis to identify expressed genes between two samples are defined as follows: \( H_0 \) is a gene with the same expression level in two samples; \( H_1 \) is a gene with different expression levels in two samples. The probability of gene A expressed equally between two samples can then be calculated with the following formula (Fig. 1C).

With thousands of hypotheses tested, the suitable p-value for an individual test is insufficient to guarantee a low rate of false discovery. There we had to do multiple testing corrections for each individual hypothesis to guarantee a low false discovery rate in the entire dataset. FDR (False Discovery Rate) control is a statistical method used in multiple hypotheses testing to correct for p-value. In practical terms, the FDR is the expected false discovery rate

**Unigene function annotation, GO classification, pathway analysis**

For validation and annotation of assembled unigenes, sequence similarity search was conducted against NR, the Swiss-Prot protein, KEGG, COG and two closely species (F. pedrosoi, F. multiformis) genome data on Broad institute using blastx alignment with an E-value threshold of 10\(^{-5}\). The KEGG database contains a systematic analysis of intracellular metabolic pathways and functions and enhances pathway annotation of unigenes (Pathfinder: http://www.genome.jp/) (Kanehisa et al. 2008). COG is a database where orthologous genes are classified, assuming that every protein has evolved from an ancestor protein. NT is the non-redundant NCBI nucleotide database, with entries from all traditional divisions of GenBank, EMBL, and DDBJ excluding bulk divisions.
GO classification of differentially expressed unigenes and pathway analysis

GO functional analyses provide functional classification annotation and enrichment analysis for DEGs (Differentially Expressed Genes). First, mapping was done of all differentially expressed genes to each term of the Gene Ontology database (www.geneontology.org) and calculating the gene numbers of each GO term. Subsequently, hyper-geometric tests were used to find significantly enriched GO terms in DEGs comparing to the genome background. The formula calculating a p-value in this hypothesis test is as in Fig. 1D. The calculated p-value goes through Bonferroni Correction, assuming a corrected p-value < 0.05 as a threshold. GO terms fulfilling this condition were defined as significantly enriched GO terms in DEGs. This analysis was able to recognise the main biological functions that DEGs exercise. Our GO functional enrichment analysis also integrated clustering analysis of expression patterns. Thus, expression patterns of DEGs annotated to a given GO-term were obtained relatively easily.

Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing the entire genome background. The calculating formula of p-value is the same as in GO analysis (Fig. 1D). Here $N$ is the number of all genes with KEGG annotation, $n$ is the number of DEGs in $N$, $M$ is the number of all genes annotated to specific pathways, and $m$ is number of DEGs in $M$. After multiple testing corrections, we observed pathways with Q-values < 0.05 being significantly enriched in DEGs.

Development of cDNA-derived SSR markers

A Perl script known as MicroSatellite (www.pgrc.ipk-gatersleben.de/misa) was used to identify microsatellites in the unigenes. In this study, cDNA-based SSRs (Simple Sequence Repeats) were considered to contain motifs with two to six nucleotides in size and a minimum of 4 contiguous repeat units. Frequency of cSSR refers to kilobase pairs of cDNA sequences containing one SSR.

Confirmation of genes transcript using Real time RT-PCR

Thirty-six assembled genes distributed in melanin synthesis, light sensing, cell wall synthesis, morphology regulation and environmental resistant pathways (UV, temperature, oxidative stress) were selected for DEG data confirmation. The primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA), and all the primers were synthesised in Life Technology Company (Life Technology, International, Palo Alto, USA), and all the primers were synthesized using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA). Real-time PCR reactions were performed using SYBR ExScript™ RT-PCR Kit (TakaRa, Dalian, China). The cycling conditions were 95 °C for 30 s, followed by 45 repetitive cycles at 95 °C for 5 s, 60 °C for 10 s. A melting curve analysis was performed from 65 °C to 95 °C. ACT (unigene7394) was used as the internal normalise gene. The specific amplification of each gene was confirmed by melting curve analysis. Results analyses were same as our described before (Liu et al. 2007, Sun et al. 2014).

DHN inhibitor test, laccase, tyrosinase activity assays and light-sensor test

Tricyclazole was used as inhibitor of pentaketide melanin biosynthesis to evaluate the pathway of DHN melanin synthesis (Franzen et al. 2006). All strains were cultured PDA medium with 50 mg/L tricyclazole at 25 °C for 7 days.

Laccase and tyrosinase enzymes activities were tested according to Laufer et al. (2006). Both strains were cultured on PDA medium at 25 °C for 7 days. Total protein extraction and purification were carried out as described previously. 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Sigma) and syringaldazine (Sigma) were used as substrate of laccase activity test, while DOPA (Sigma) and tyrosine (Sigma) were used as substrate of tyrosinase as described by Laufer et al. (2006).

For the light effects experiment, both strains were grown on PDA medium (Chen et al. 2014). Cultures were exposure to a light and incubated at 25 °C for 7 days.

RESULTS AND DISCUSSION

Sequencing and transcriptome assembly

To obtain a global overview of the transcriptome and gene activity at nucleotide resolution of parent (CBS 122845) and albino mutant strain (CBS 125194), each three independent cDNA libraries were constructed and sequenced separately using an Illumina HiSeq2000 genome analyzer. After stringent quality check and data cleaning, more than 143 million clean reads were obtained from each sample (Q20 bases > 97 %, G+C about 53 %) (Table 1). The total length in nucleotides was

| Table 1. Summary of reads in melanised strain and albino strain of F. monophora, and statistics of assembly quality. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| CBS 122845 | CBS 125194 | CBS 125194 |
| Total reads | 15 36 78 424 | 14 35 01 576 |
| Total clean reads | 15 05 29 368 | 14 04 47 326 |
| Total clean nucleotides (nt) | 15 05 29 36 800 | 14 04 47 32 600 |
| Q20 | 97.42 % | 97.31 % |
| N | 0.00 % | 0.00 % |
| GC | 53.60 % | 53.36 % |
| Contig total number | 26 939 | 27 267 |
| Contig total length (nt) | 2 54 82 551 | 2 60 50 225 |
| Contig mean length (nt) | 945 | 955 |
| N50 | 2 273 | 2 370 |

Total reads and total nucleotides are clean reads and clean nucleotides. Total nucleotides should be more than contract provision. Q20 percentage is proportion of nucleotides with quality value larger than 20. N percentage is proportion of unknown nucleotides in clean reads. GC percentage is proportion of guanine and cytosine nucleotides among total nucleotides. Total clean nucleotides = total clean reads x read1 size + total clean reads x read2 size.
We used the Blast2GO program to get GO annotation of unigenes and WEGO software to do GO functional classification for all unigenes. Based on the NR annotation, 17 138 unigenes were assigned gene ontology terms. GO-annotated unigenes belonged to the biological processes, cellular components, and molecular functions clusters were distributed in 46 categories (Fig. 3). Among the biological processes category, metabolic processes (3 475, 20.28 %) composed the most dominant group, followed by cellular processes (3 063, 17.72 %), single-organism processes (1 602, 9.35 %) and localisation (1 282, 7.48 %) (Fig. 3). Regarding molecular functions, 23.51 % (4 030) of the unigenes were assigned to catalytic activity, followed by binding (2 682, 15.65 %), transporter activity (694, 4.05 %) and structural molecule activity (99, 0.58 %) (Fig. 3). Among the cellular components category, cell (1 404, 8.19 %) and cell part (1 404, 8.19 %) were the dominant groups, followed by organelles membrane (1 376, 8.03 %) and (946, 5.52 %) (Fig. 3). In addition, all unigenes were subjected to a search against the COG database for functional prediction and classification. Similarly, all unigenes were assigned to COG classifications (Fig. 4). COG-annotated putative proteins were functionally classified into 25 molecular families such as cellular structure, biochemistry metabolism, molecular processing, and signal transduction (Fig. 4). The cluster for general function prediction represented the largest group (4 413, 25.76 %), followed by carbohydrate transport (2 303, 13.44 %), amino acid transport (2 033, 11.68 %), inorganic ion transport (1 710, 9.98 %) and secondary metabolites catalysis (1 401, 8.17 %). Only a few unigenes were assigned to nuclear structure and extracellular structure (14 and 40 unigenes, respectively) (Fig. 4).

The KEGG database was used to analyse potential involvement of the consensus sequences in cellular metabolic pathways. Among the 17 138 annotated genes, 12 360 could be grouped into according to the KEGG database. The unigenes were grouped into 108 cellular metabolic or signalling pathways. For example, among the 12 360 annotated genes, 1 401 unigenes were classified into 46 categories (Fig. 3). Among the biological processes category, metabolic processes (3 475, 20.28 %) composed the most dominant group, followed by cellular processes (3 063, 17.72 %), single-organism processes (1 602, 9.35 %) and localisation (1 282, 7.48 %) (Fig. 3). Regarding molecular functions, 23.51 % (4 030) of the unigenes were assigned to catalytic activity, followed by binding (2 682, 15.65 %), transporter activity (694, 4.05 %) and structural molecule activity (99, 0.58 %) (Fig. 3). Among the cellular components category, cell (1 404, 8.19 %) and cell part (1 404, 8.19 %) were the dominant groups, followed by organelles membrane (1 376, 8.03 %) and (946, 5.52 %) (Fig. 3). In addition, all unigenes were subjected to a search against the COG database for functional prediction and classification. Similarly, all unigenes were assigned to COG classifications (Fig. 4). COG-annotated putative proteins were functionally classified into 25 molecular families such as cellular structure, biochemistry metabolism, molecular processing, and signal transduction (Fig. 4). The cluster for general function prediction represented the largest group (4 413, 25.76 %), followed by carbohydrate transport (2 303, 13.44 %), amino acid transport (2 033, 11.68 %), inorganic ion transport (1 710, 9.98 %) and secondary metabolites catalysis (1 401, 8.17 %). Only a few unigenes were assigned to nuclear structure and extracellular structure (14 and 40 unigenes, respectively) (Fig. 4).

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Differentially expressed unigenes

Compared with the parent strain, we obtained 2 283 significantly differentially expressed genes between the two samples judged by using the threshold of false discovery rate (FDR < 0.001) and the absolute value of log2 ratio ≥1, including 1 316 up-regulated and 967 down-regulated genes (Fig. 5).

GO functional analysis showed that these unigenes belonged to three functional clusters and were distributed in 41 categories based on the NR annotation (data not shown). To explore the biological function of the significantly differentially expressed genes, all 2 283 genes were mapped to the KEGG database, and then the hit genes were enriched to important pathways such as metabolism and signal transduction. There were 907 unigenes mapped to 96 pathways. Specific pathways were observed that are involved in metabolic pathways, biosynthesis of secondary metabolites, RNA transport, TCA cycle, and cell cycle. Interestingly, the majority of genes mapping to the above pathways were dramatically up-regulated in the albino strain which indicated more biological relevance to growth and reproduction. In particular, genes involved in phenylpropanoid biosynthesis pathways which related to melanin production were also
substantially up-regulated. These results seem inconsistent with melanin deficiency in the albino mutant which drives us for further analysis.

Melanin biosynthesis pathway and regulation

Fungal melanins are negatively charged, hydrophobic, high molecular weight polymers which arise by oxidative polymerisation of phenolic and/or indolic precursors (Nosanchuk & Casadevall 2003). The polymers help to defend against diverse environmental stresses such as UV radiation, oxidising agents, and extreme temperatures (Jacobson 2000, Langfelder et al. 2003). Melanins are consistently present in the fungal agents of chromoblastomycosis (Schnitzler et al. 1999, Kogej et al. 2004, Santos et al. 2007, Thornton et al. 2015), as well as in fungi that otherwise have hyaline cell walls, e.g. Candida albicans (Morris-Jones et al. 2005), Cryptococcus neoformans (Chaskes et al. 2014), Paracoccidioides brasiliensis (Gomez et al. 2001), Histoplasma capsulatum (Nosanchuk et al. 2002), Blastomyces dermatitidis (Nosanchuk et al. 2004), and Aspergillus fumigatus (Tsai et al. 2001). Three different pathways contribute to melanin production: the DHN-melanin pathway (Fig. 6), the DOPA-melanin pathway (Fig. 7), and the 3,4-tirosine degradation pathway (Fig. 8) (Langfelder et al. 2003). The DHN-melanin biosynthetic pathway is the best characterised pathway for producing fungal eumelanin, a black pigment localised in cell walls (Wheeler et al. 2008). All the enzymes of the DHN-melanin pathway were found in the transcriptome of both strains (Table 3), including polyketide synthase (pks), αβ hydrolase (ag1), scytalone dehydratase (arp1), 1,3,6,8-tetrahydroxynaphthalene reductase (arp2), multicopper oxidases (abr1), and ferroxidase (abr1) (Table 3). Polyketide synthase and the αβ hydrolase are essential for melanin production in many black fungi (Wheeler et al. 2008). In total, three pks and two ag1 gene homologues were found in both samples. Most of the genes in the DHN-melanin pathway were down-regulated in the albino mutant, which suggests that the DHN-melanin pathway might have been blocked, resulting in loss of melanin production (Fig. 6A). The down-regulated transcription level of partial genes was confirmed by real time RT-PCR (Fig. 6B). The inhibition test using tricyclazol showed significant decrease of DHN melanin in the parent strain, while no effects were noted in
Fig. 3. GO annotations of non-redundant consensus sequences. Best hits were aligned to the GO database, and 6,876 transcripts were assigned to at least one GO term. Most consensus sequences were grouped into three major functional categories, namely biological process, cellular component, and molecular function.
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Fig. 4. Histogram presentation of clusters of orthologous groups (COG) classification. All unigenes were aligned to COG database to predict and classify possible functions.
Fig. 5. Algorithm to identify differentially expressed genes between two samples (A). In the follow figure, CBS 122845 is control and CBS 125194 is treated. The genes were classified into three classes. Red genes are up-regulated that gene expression of right sample is larger than left sample. Green genes are down-regulated that gene expression of left sample is larger than right sample. Blue genes are not differentially expressed genes. The horizontal coordinate is the expression level of right and the vertical coordinate is the expression level of left sample. The distribution of Differentially Expressed Genes (DEGs) (B). The X-axis is the treat/control, and the Y-axis is the number of the DEGs. The red bar is up-regulated genes, and the green bar is down-regulated genes.

Fig. 6. The DHN-melanin pathway. The flowchart of the DHN-melanin pathway and transcription level of particular genes (blue) detected in this study (A). Red colour arrow marked up-regulated genes, while green colour arrow marked down-regulated genes. The differential expression genes supported by statistical analysis were marked as star. Transcription levels of partial genes between two strains were confirmed by Real time RT-PCR (B). The X-axis is the selected unigenes, Y-axis is the value of log2 ratio; positive value means up-regulation in albino strain compare with parent strain. DHN pathway inhibition test (C). Tricyclazole was used as inhibitors to study the pathway of melanin synthesis both strains were culture in PDA medium supplied with 50 mg/L tricyclazole. The type strain of *F. monophora* (CBS 269.37) was used as the control.
the albino mutant (Fig. 6C). However, the tricyclazol could not completely inhibit the melanin production in parent strain or control strain (*F. monophora* CBS 269.37), alternatively, others melanin pathways were probably active simultaneously in parent and control strain (Fig. 6C). Both *arp1* and *arp2* are single copy in *F. monophora*. In contrast, oxidases *abr1* and *abr2* each have multiple homologous genes in the transcriptome. Multicopper oxidases (*mco*) are over-represented in *F. monophora*, including ABR-like pigment-related oxidases, fungal ferroxidase and laccases. A total of 14 multicopper oxidase genes were annotated in the two transcriptomes of *F. monophora* (Table 3). These *mco* genes were classified into fungal pigment multi copper oxidase...
of the 14 genes, six were up-regulated in the albino mutant strain, suggesting that most of these copies could be functionally active. Indeed, the enzyme test showed significant transcription of laccase in the albino mutant compared with the parent strain (Fig. 9A) \( (p < 0.05) \).

In addition to DHN melanin, many fungi produce pheomelanin through the DOPA melanin pathway (Liu et al. 2014, Pal et al. 2014), in which tyrosinases or laccases generate dopaquinone either through hydroxylation of L-tyrosine, or through oxidation of DOPA. Dopaquinone auto-oxidises and polymerises to form melanins (Fig. 7A). *Fonsecaea monophora* contains 8 laccases and 2 tyrosinases coding genes (Table 3). All laccases were up-regulated in the albino mutant except for two unigenes \( (p < 0.05) \), as well as tyrosinases compared to the parent strain \( (p > 0.05) \), which matches with the following enzyme activity tests (Fig. 9A, B) and real time RT-PCR results (Fig. 9C). However, when DOPA and L-tyrosine as separate substrates were added to the medium, DOPA melanin were detected in the PDA medium with DOPA, but not L-tyrosine in both strains (Fig. 9D). These data suggested that DOPA melanin biosynthesis pathway were affected by substrate in culture medium, as well as the genes in DOPA melanin biosynthesis pathway. Indeed, in C. neoformans, the melanin production was regulated by specific transcript factors only in the presence of specific substrates, mainly diphenolic compounds (Chaskes & Tyndall 1978).

The pathway to produce a third type of melanin, pyomelanin (allomelanin), viz. the L-tyrosine degradation pathway (Keller et al. 2011), is also conserved in *F. monophora*. The two enzymes involved in this pathway are 4-hydroxyphenylpyruvate dioxygenase (hppD) and homogentisate dioxygenase (hmgA). HppD converts 4-hydroxyphenylpyruvate to homogentisate, which can be converted to pyomelanin through oxidation and polymerisation. Homogentisate can alternatively be degraded to other compounds by hmgA and two downstream enzymes, maleylacetacetate isomerase (maiA) and fumarylacetacetate hydrodrolase (fahA) (Fig. 8A). All these enzymes were found in both strains (Table 3). Interestingly, we found that hppD was up-regulated (3.528 fold, log2 ratio = 1.819, \( p < 0.05 \)), while hmgA was down-regulated (2.156 fold, log2 ratio = −1.108, \( p < 0.05 \)) in the albino mutant, and no significant variation in transcription levels were detected in downstream maiA and fahA, suggesting that the down-regulation of hmgA blocks the degradation of homogentisate, resulting in accumulation of pyomelanin. Although the real time RT-PCR results confirmed these results (Fig. 8B), the following enzyme activity test using L-tyrosine as substrate showed that pyomelanin was not detected in both strains (Fig. 9D). The possible explanation is that down-regulation of tyrosine aminotransferase (tat) (3.575 fold, log2 ratio = −1.910, \( p < 0.05 \); 3.573 fold, log2 ratio = −1.837, \( p < 0.05 \)) attributed to the inhibition of 4-hydroxyphenylpyruvate production, result in blocking of pyomelanin biosynthesis pathway.

![Diagram](https://www.studiesinmycology.org/fig8.png)

**Fig. 8.** The L-tyrosine degradation pathway. The flowchart of the DOPA-melanin pathway and transcription level of particular genes (blue) detected in this study (A). Red colour arrow marked up-regulated genes, while green colour arrow marked down-regulated genes. The differential expression genes supported by statistical analysis were marked as star. Transcription levels of partial genes between two strains detected by Real time RT-PCR (B). The X-axis is the selected unigenes, Y-axis is the value of log2 ratio; positive value means up-regulation in albino strain compare with parent strain.
# Table 3. Melanin biosynthesis pathway genes.

| Gene                                      | F. monophora | Homologues in other fungal genomes | p-Value (*<0.05) |
|-------------------------------------------|--------------|-----------------------------------|-----------------|
| Homologues in other fungal genomes        | W. dermatitidis | C. carrioni | A. fumigatus | A. niger |
| Assemble gene code | Transcript level (log2 ratio) (CBS125194/ CBS122845) | | | |
| **DHN—melanin pathway**                   | | | | |
| Polyketide synthase (Pks1)                | unigene4246 | -0.948 | 0.037* | HMPREF1120-03173 G647-03926 | Afu2g17600 (Pk1) An03g05440 |
|                                           | unigene4245 | -0.266 | 0.025* | G647-03240 | Afu4g00210 (EncA) An04g9530 |
|                                           | unigene10003 | -0.392 | 0.032* | G647-03914 | Afu4g14560 Afu7g0160 | An09g05730 (FamA) An11g07310 |
| Abhydrolase (Ayg1)                        | unigene1384 | 0.250 | 0.309 | HMPREF1120-00377 G647-03240 | Afu4g00210 (EncA) An04g9530 |
|                                           | unigene1176 | 0.301 | 0.244 | G647-03620 | Afu4g14560 Afu7g0160 | An09g05730 (FamA) An11g07310 |
| 1,3,6,8-Tetrahydroxynaphthalene reductase (Arp2) | unigene2337 | -0.126 | 0.142 | HMPREF1120-05939 G647-01180 | Afu2g17560 (Arp2) An02g00220 |
|                                           | unigene1389 | -0.793 | 0.04* | HMPREF1120-07724 G647-03339 | Afu2g17560 (Arp2) An02g00220 |
|                                           | unigene9376 | -1.874 | 0.027* | HMPREF1120-01590 G647-00601 | An15g05520 (McoK) |
|                                           | unigene9070 | -1.710 | 0.004* | HMPREF1120-03706 G647-00377 | An15g05520 (McoK) |
| **DOPA—melanin pathway**                  | | | | |
| Tyrosinase (MelC2)                        | unigene1125 | 0.472 | 0.444 | HMPREF1120-05316 G647-08944 | Afu3g01070 | An01g09220 (MelC2) |
|                                           | unigene6269 | 0.641 | 0.058 | HMPREF1120-03345 G647-02663 | An03g00280 |
|                                           | | | | | An09g02980 G647-09055 |
| Laccase (Lac)                             | cl2942.contig1 | 1.690 | 0.008* | HMPREF1120-05865 G647-00377 | Afu4g14490 | An12g05810 (McoJ) |
|                                           | cl2942.contig2 | 1.027 | 0.014* | HMPREF1120-00199 G647-03384 | Afu4g14490 | An16g02020 (McoM) |
|                                           | cl422.contig2 | 1.336 | 0.001* | HMPREF1120-08116 G647-09390 | An11g03580 (McoD) |
|                                           | cl31.contig1-23 | -1.008 | 0.334 | HMPREF1120-08564 G647-05054 | An08g08450 (McoG) |
|                                           | unigene8445 | 3.902 | 0.002* | HMPREF1120-04578 G647-09922 | An05g02340 (McoF) |
|                                           | unigene9375 | 1.717 | 0.016* | HMPREF1120-02754 G647-06061 | An01g00860 (McoN) |
|                                           | unigene3099 | -0.073 | 0.779 | G647-00947 | An18g02690 (McoN) |
|                                           | unigene1837 | 4.113 | 0.026* | G647-00947 | An18g02690 (McoN) |
| **L-tyrosine degradation pathway**         | | | | |
| Tyrosine aminotransferase (Tat)           | cl1774.contig1 | -1.910 | 0.003* | HMPREF1120-02164 G647-02757 | Afu2g13630 | An02g05540 |
|                                           | cl1774.contig2 | -1.837 | 0.006* | G647-07845 | Afu2g13630 |
| 4-Hydroxyphenylpyruvate dioxygenase (hppD) | cl24.contig4 | 1.819 | 0.012* | HMPREF1120-05584 G647-06922 | Afu2g04200 | An11g02200 |
| Homogentisate dioxygenase (hmgA)          | unigene1219 | -1.108 | 0.017* | HMPREF1120-03827 G647-06522 | Afu2g04200 | An11g02180 |
| Fumarylacetoacetate hydrolase (fahA)      | unigene2130 | -0.313 | 0.043* | HMPREF1120-03825 G647-06520 | Afu2g04200 | An11g02170 |
| Maleylacetoacetate isomerase (maiA)       | unigene4460 | -0.557 | 0.155 | HMPREF1120-03438 G647-03643 | Afu2g04200 | An11g02160 |

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To explore potential mechanism of significant down-regulation of most genes in the DHN pathway, the unique transcribed genes of each strain were collected, and the genes associated with melanin synthesis were evaluated according to archived literature. A total of 17 and 25 unigenes were uniquely transcribed in parent and albino strains, respectively (Table S2). After removing the unigenes which contained homologues in both strains, no novel genes directly linked to melanin production was uniquely transcribed in parent or albino strain. DHN-melanin defect in the albino strain may not be due to a sudden gain/loss of particular genes in melanin biosynthesis pathways. Melanin biosynthesis, including DHN-melanin, perhaps is regulated by multiple transcription factors. For example, melanin production of *C. neoformans* is regulated by a number of genes including cAMP signal pathway, iron regulated gene *SIT1* and chitin synthase (*Chs3*) (Zhu et al. 2001, Waterman et al. 2007). They found the key enzyme of DOPA melanin production, laccase, is synthesised in the cytoplasm, transported in vesicles, and tightly linked to the cell wall through disulfide or thioester bonds. If the strains have cell wall defects, the laccase will miss localisation of chitin-like structures, and consequently result in melanin deficiency (Walton et al. 2005). Our previously studied in *F. monophora* already identified the cell wall defects of the albino strain by transmission electron microscopy (Zhang et al. 2013). Along with the up-regulation of several chitin degradation coding genes in the albino strain (Table S3), all these suggested the absence of laccase localisation is the possible mechanism of DOPA melanin deficiency in albino strains, although this needs further verification. Recently, Sapmak et al. (2015) found that *pbrB* gene encodes a laccase required for DHN-melanin synthesis in conidia of *Talaromyces marneffei*. Cary et al. (2014) found that *veA*, a sclerotium-specific pigment melanin synthesis gene, regulates asparasone in *Aspergillus flavus*. We also verified *veA* regulates asparasone synthesis in *F. monophora*, while it seems to have no effect on DHN melanin synthesis (Fig. 10). Pal et al. (2014) concluded that the amount and type of melanin in aspergilli largely differ from species to species. Therefore, which transcription factors are involved in regulation of DHN melanin production in *F. monophora* still needs further investigation.

**Cell wall synthesis and morphogenesis**

Numerous key enzymes involved in cell wall biosynthesis were identified in the transcriptome of *F. monophora* (Table S3). Chitin synthase genes encompass several subfamilies. We identified six out of seven chitin synthase genes previously described in fungi. Proteins involved in the regulation of chitin synthase activity, export of chitin synthases, and chitin degradation (chitinases) were all present in both strains. The glycosyl transferase family was also identified, while the copy number was significantly lower than in other fungi, such as *E. dermatitidis* or...
**Aspergillus** (Chen et al. 2014). Glucanases (β-1,3-glucanase, β-1,4-glucanase, endo and exo-glucanases) and GlcNAc-mannosyltransferase genes, which are important enzymes in glucan synthesis, were also observed in both transcriptomes (Table S3). Although transcript levels of partial chitin synthase coding genes were up-regulated in albino strain, some chitin degradation coding genes, e.g. chitinase, were also up-regulated, as well as numbers of β-1,3-glucan synthesis and regulation genes including β-1,3-transglucosylases and transglycosidases (Table S3, Fig. S2), which probably suggested a change of cell wall components and increasing cross linking beta-glucan to chitin in albino strain.

Alpha-1,3-glucanases play a key role in biosynthesis of α-1,3-glucan which normally transcript in yeast form of pathogenic fungi, such as *Histoplasma capsulatum* and *Aspergillus* species. The archived studies showed that α-1,3-glucan helps to mask β-glucans from detection by the host receptor dectin-1, and results in immunological escape (Aimanianda et al. 2009, Fujikawa et al. 2012). In contrast, α-1,3-glucanase is able to remove this α-glucan mask by catalysing the degradation of α-1,3-glucan. Both α-1,3-glucan synthases (Ags family) and α-1,3-glucanases (Agn family) was identified in *F. monophora*, as well as *F. pedrosoi* and *F. multiformis* genomes, while not detected in *E. dermatitidis* (Chen et al. 2014). Interestingly, both Ags and

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**Fig. 10.** Effects of light on pigment and carotenoid biosynthesis. Both strains were grown on PDA agar medium exposing to light or keep in dark, and both cultures were incubated at 25 °C for 7 days.
Agn families are present as multiple copies in *Histoplasma capsulatum*, *Magnaporthe oryzae*, and *Aspergillus* species (Rappleye et al. 2007, Amanianda et al. 2009, Fujikawa et al. 2012). This variation in α-1,3-glucan biosynthesis of *F. monophora* might affect the interactions potentially between this pathogen and the host's immune system. That is because the knock-out of Ags significantly up-regulated the recognition of host cell, and results in the attenuate of pathogenesis of *A. fumigatus* and *M. oryzae*. Therefore, we speculated the deficiency of melanin, up-regulated chitin degradation coding genes and β-1,3-glucan synthesis genes in albino strain perhaps promote the recognised by host immune cell (Fig. 11), and results in sufficient killing factor secretion of host cells, consequently, leading to clear of infection. In contrast, insufficient exposing of antigens (β-1,3-glucan) due to shield effects of α-1,3-glucan and melanin probably caused a chronic infection in parent strain (Fig. 11). Our previously studies in a marine macrophage infection model (Zhang et al. 2013), as well as animal infection model (unpublished data) confirmed this speculation.

Another class of enzymes, α-amylases, performing transglycosylation or hydrolysis of glycosidic linkages, is present in *F. monophora*. Moreover, both intracellular and secreted α-amylases were identified simultaneously, as well as in many other fungi (van der Kaaij et al. 2007). In addition, similar to...
E. dermatitidis and Schizosaccharomyces pombe. F. monophora lacks copies of conserved chitosinases, whereas they exist ordinarily in genome of other filamentous fungi, including A. fumigatus, A. niger, A. flavus, Coccidioides immitis, Trichophyton rubrum and Neurospora crassa (Shimono et al. 2002, Martinez et al. 2012, Chen et al. 2014).

The genetic mechanisms of activating developmental dimorphic switches in other pathogenic fungi have been clarified, e.g. Candida albicans (Biswas et al. 2007). Several gene homologues coding for morphology regulatory enzymes were identified in both transcriptomes of F. monophora, including mitogen-activated protein kinase family, cell division cycle protein family (CDC5, CDC42) and Ras family [a protein superfamily of small GTPases which function independently as hydrolase enzymes to bind to and hydrolyse a guanosine triphosphate (GTP) to form guanosine diphosphate (GDP)] (Table S4). Some key genes encoding important transcription factors which regulate the developmental switches from yeast to hyphae were present in the transcriptome of F. monophora, including APSES transcription factors (Wang & Szanielszlo 2007, Qi et al. 2012, Connolly et al. 2013) and STE transcription factor (Biswas et al. 2007). Since dimorphic switches between the muriform cell and hyphae were never detected in any of the strains, it seems that this process was regulated by an unidentified transcription factor; thus further genome wide extension study are needed.

Light-sensing and pigment production

Fungi have multiple sensors that respond to light of different wavelengths. Genes associated with light sensing were also identified in both F. monophora strains, including the velvet family of regulatory genes (veA, veB, veC and vosA) which play a key role in coordinating development and secondary metabolism responding to light. Opsin-like protein is one of the members of Opsin-like protein, including T. rubrum and C. immitis (Idnurm et al. 2010), while F. monophora contains this class of light sensing proteins (Table S5). Colonies of the albino strain showed increased pink colour when incubated under light, while it maintained its white colour when incubated in the dark, indicating production of carotenoids (Fig. 10). The coordination of these responses would enable protection against and response to light stimuli as observed in Exophiala dermatitidis (Chen et al. 2014).

Environmental stress

In pathogenic fungi, several genes were proven to be involved in the resistance to the environmental stress from the host cells, including superoxide dismutase (SOD), catalase, NADH dehydrogenase, members of Hog1 family and the transcriptional regulator Skn7p (Abad et al. 2010). Most of those genes were identified in the transcriptomes of F. monophora (Table S6, Fig. S2). Numerous transcripts were homologues of genes encoding enzymes in resistance to oxidants, irradiation and extreme temperatures. In addition, heat shock proteins (HSPs) and heat shock factors (HSFs), which serve as chaperones to couple with target proteins and activators of HSPs, respectively, were identified in both transcriptomes. However, the transcription levels of those genes were comparable because the same medium and culture conditions were used for both strains. Only the c1296.contig4 and unigene8441, which coding superoxide dismutase and deoxyribopyrimidine photo-lyase resistant to oxidants and UV-A damage, respectively, were significantly down-regulated in the albino mutant (16.167 fold, log2 ratio = −4.015, p < 0.05; 4.95 fold, log2 ratio = −2.307, p < 0.05) (Tables S5 and S6). These results were concordant with our previous finding that the albino strain was sensitive to oxidants and UV light (Sun et al. 2011). However, since no pairwise experiments were done under different conditions of oxidants, temperature and pH were done, stress tolerance were not detected, thus no significant transcription difference was to be expected between the strains.

![Fig. 12. Frequency distribution of cSSRs based on motif sequence types. The X-axis is the repeated nucleotides types, and Y-axis is the SSR motif numbers in total.](image-url)
Frequency and distribution of EST-SSRs in the same transcriptome

In total, 2,849 sequences containing 3,353 SSRs were identified from 21,600 consensus sequences, and 425 of the sequences contained more than one SSR. The SSR frequency in the consensus transcriptome was 13.18 %. The distribution density was 67.33 per Mb. The most abundant type of repeat motif was trinucleotide (36.69 %), followed by dinucleotide (30.25 %), quadri-nucleotide (9.81 %), mononucleotide (9.08 %), pentanucleotide (7.75 %) and hexanucleotide repeat units (6.62 %) (Table S7). SRRs with five tandem repeats (27.39 %) were the most common, followed by six tandem repeats (23.41 %), 10 tandem repeats (13.50 %) and seven tandem repeats (12.29 %) (Table S7). The dominant repeat motif in SSRs was AG/CT, followed by AC/CT, ACC/GGT, AGC/CTG, and ACG/CGT (Fig. 12).

CONCLUSIONS

This study investigated the transcriptome profiles of melanised and albino strains of *F. monophora* using illumina RNA-seq and DGE deep sequencing technologies. The substantial amount of transcripts obtained revealed the genes involved in melanin biosynthesis in *F. monophora*, and suggested the loss of melanin in albino mutant is most likely due to blocking of the DNH pathway. Moreover, most of the genes involved in light sensing, cell wall synthesis, morphology regulation and tolerance to environmental stress (UV, pH, temperature, oxidants, and osmotic condition) were assembled and annotated in transcriptomes of *F. monophora*. The thousands of cSSR markers produced in this study will enable genetic linkage mapping construction and gene-based association studies.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.simyco.2016.02.001.

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