Gene transcription profiling of *Aspergillus oryzae* 3.042 treated with ergosterol biosynthesis inhibitors

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Received: 26 March 2018 / Accepted: 4 October 2018 / Published online: 3 December 2018
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Abstract

Ergosterol, a unique component of fungal cells, is not only important for fungal growth and stress responses but also holds great economic value. Limited studies have been performed on ergosterol biosynthesis in *Aspergillus oryzae*, a safe filamentous fungus that has been used for the manufacture of oriental fermented foods. This study revealed that the ergosterol biosynthesis pathway is conserved between *Saccharomyces cerevisiae* and *A. oryzae* 3.042 by treatment with ergosterol biosynthesis inhibitors and bioinformatics analysis. However, the ergosterol biosynthesis pathway in *A. oryzae* 3.042 is more complicated than that in *S. cerevisiae* as there are multiple paralogs encoding the same biosynthetic enzymes. Using RNA-seq, this study identified 138 and 104 differentially expressed genes (DEG) in response to the ergosterol biosynthesis inhibitors tebuconazole and terbinafine, respectively. The results showed that the most common DEGs were transport- and metabolism-related genes. There were only 17 DEGs regulated by both tebuconazole and terbinafine treatments and there were 256 DEGs between tebuconazole and terbinafine treatments. These results provide new information on *A. oryzae* ergosterol biosynthesis and regulation mechanisms, which may lay the foundation for genetic modification of the ergosterol biosynthesis pathway in *A. oryzae*.

Keywords *Aspergillus oryzae* · Ergosterol biosynthesis · Inhibitors · Transcriptome

Introduction

Sterols, a large group of natural macromolecule alcohol compounds, are key components of the cell membrane of animals, plants, and fungi [1, 2]. Ergosterol is specific for fungal cell membranes and has been widely used as a marker to assess fungal biomass [3]. As ergosterol is an important cell membrane

component that affects fluidity and permeability, and it plays important roles in fungal growth, reproduction, and stress tolerance [4]. Furthermore, ergosterol is also a very important pharmaceutical raw material, which can be used for cortisone and progesterone production, and can be used as a precursor of vitamin D2 synthesis [5]. Recently, new functions of steroid drugs have been revealed, for example tetrahydroxysterol has a significant anti-tumor activity, and some anti-HIV compounds have similar structures to ergosterol [6]. Steroids have important physiological activities, have been widely used in clinical practice, and are the second most widely used drugs after antibiotics [5].

Research on yeast and other fungi has provided great progress in understanding the ergosterol biosynthesis pathway. This pathway is a complex process, which involves about 18 reactions and 24 enzymes in *Saccharomyces cerevisiae*, and the detailed process of ergosterol biosynthesis pathway has been researched and reviewed in detail [7–10]. As ergosterol is a unique component of fungal cells, several studies have reported a range of ergosterol biosynthesis inhibitors. On one hand, these inhibitors can be used as fungicides in agriculture and medicine [11]. On the other hand, these inhibitors provide a very important tool for the study of ergosterol synthesis, metabolism, and

Zhihong Hu and Ganghua Li contributed equally to this work.

Responsible Editor: Rosana Puccia

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42770-018-0026-1>) contains supplementary material, which is available to authorized users.

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regulation. Currently, most antifungal agents are inhibitors of essential enzymes in the ergosterol biosynthesis pathway, which can inhibit the biosynthesis of ergosterol by competitively binding to key enzymes, resulting in destruction of the cell membrane and inhibition of fungal growth and reproduction [12]. Ergosterol biosynthesis inhibitors are of four main types. The first type is C-14 α -demethylase inhibitors, most commonly used in fungicides and these compounds are mainly imidazoles (azoles) that inhibit the activity of ERG11 (CYP51), blocking ergosterol biosynthesis [13]. The second type includes $\Delta^8 \rightarrow \Delta^7$ isomerase or Δ [14] reductase inhibitors, including morpholines and fenpropimorphs, which can competitively inhibit the activities of these two enzymes and thereby inhibit ergosterol synthesis. The main target of these drugs is ERG24 [13]. The third type includes squalene cyclooxygenase (ERG1) inhibitors, including acrylamide compounds [14]. On one hand, these inhibitors can cause fungal ergosterol deficiency, but on the other hand, they can cause squalene accumulation in the plasma membrane, leading to increased fragility of the plasma membrane and resulting in the destruction of cell membrane structure and function [15]. The last type includes $\Delta^{5(6)}$ desaturase inhibitors, which can competitively bind to oxidized sterol intermediates to inhibit ergosterol biosynthesis [14]. This inhibition can cause accumulation of $\Delta^{7,22E}$ -ergosterol precursors, resulting in a decrease in cell membrane stability. A lack of ergosterol can cause cells to synthesize ergosterol de novo by a negative feedback mechanism, generating large amounts of $\Delta^{7,22E}$ -ergosterol precursors that further inhibit cell growth [16].

Aspergillus oryzae (koji mold) is a safe filamentous fungus that has been used in the manufacture of oriental fermented foods such as sauce, miso, and sake for thousands of years. It is also commercially used for valuable enzyme production. The first sequenced genome of *A. oryzae* is of the starin RIB40, which is mainly used in Japan [17], and the sequencing of the *A. oryzae* 3.042 (mainly used in China) genome has also been completed [18]. Although the genome sequence of *A. oryzae* has been completed, functional genomics research has progressed slowly, due to its multinucleate conidia and its lack of a sexual life cycle. Until now, there have been on a few studies on ergosterol biosynthesis in *A. oryzae*. In this study, we used bioinformatics to analyze ergosterol biosynthesis in *A. oryzae* 3.042. Moreover, we also used RNA-seq to analyze the gene transcription profile of *A. oryzae* 3.042 treated with different ergosterol biosynthesis inhibitors, aiming to identify genes that are regulated after treatment with drugs that interfere with the ergosterol biosynthetic pathway. Previous studies have shown that the modification of ergosterol biosynthesis genes alters doubling time, response to stress agents, and drug susceptibility in *S. cerevisiae* [19]. Therefore, identification of these genes may provide some information about the usefulness of modifying the ergosterol biosynthetic pathway to improve the growth and stress tolerance, as well as for ergosterol production in *A. oryzae*.

Method and materials

Strain and culture conditions

The *A. oryzae* 3.042 (CICC 40092) was used in this study. To prepare the *A. oryzae* spores, wild-type *A. oryzae* 3.042 was grown on potato dextrose agar (PDA, Solarbio, China) medium at 30 °C for 3 days. Then, spores were harvested by scraping the agar surface with a sterile glass spreader under a laminar flow hood and suspending the spores in sterile water with 0.05% Tween-80. The concentration of spores was determined using a hemocytometer.

Bioinformatics analysis of ergosterol biosynthesis genes in *A. oryzae* 3.042

The ergosterol biosynthesis pathway in *S. cerevisiae* was obtained from a review article [7]. The protein sequences of the enzymes involved in ergosterol biosynthesis in *S. cerevisiae* were set as the reference sequences to identify homologous proteins in *A. oryzae* 3.042 using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). As the genomic sequence of *A. oryzae* 3.042 was not completely assembled, we also used the protein sequences of *A. oryzae* RIB40 to identify ergosterol biosynthesis enzymes in *A. oryzae* 3.042.

Tebuconazole and terbinafine treatment of *A. oryzae*

A fresh *A. oryzae* 3.042 spore suspension (about 1×10^7 spores/ml) was cultured for 2 days on PDA medium. A mycelial plug (5 mm in diameter) was then taken from the medium and placed on PDA plates with different concentrations of inhibitors and without any inhibitor as a control. After incubation at 30 °C for 3 days, the diameter of each colony was measured in two perpendicular directions. For each plate, the average of the colony diameters was used to calculate the inhibition of mycelial growth (IMG). The IMG was calculated by the following formula: $IMG = 100 \times (C - N)/(C - 0.5)$, where C is the diameter of the control colony and N is the diameter of inhibitor-treated colonies. The experiment was performed three times. For RNA-seq, a fresh *A. oryzae* 3.042 spore suspension (about 1×10^7 spores/ml) was cultured for 2 days on PDA medium covered by cellophane (Solarbio, Beijing, China), and the cellophane with mycelia was transferred onto another plate with tebuconazole (Shanghai Agricultural Chemical Co. Ltd., Shanghai, China) or terbinafine (Sigma) for 8 h. Then, the mycelia were collected for RNA-seq. Two biological replicates were performed for each treatment and the control.

Table 1 The Identification of ergosterol biosynthesis genes in *A. oryzae* 3.042

Gene	<i>S. cerevisiae</i>	<i>A. oryzae</i> 3.042			Function
		Paralogous	Protein ID	Identify	
<i>AoERG10</i>	NP_015297.1	6	EIT73661.1 EIT73496.1 EIT79671.1 EIT78121.1 EIT78942.1 EIT80840.1	60% 48% 34% 36% 36% 35%	Acetyl-CoA acetyltransferase
<i>AoERG13</i>	NP_013580.1	2	EIT76569.1 EIT75784.1	61% 56%	Hydroxymethylglutaryl-CoA synthase
<i>AoHMG1/2</i>	NP_013636.1/ NP_013555.1	5	EIT73568.1 EIT78580.1 EIT81748.1 EIT83470.1 EIT73603.1	40% 61% 34% 31% 32%	Hydroxymethylglutaryl-CoA reductase
<i>AoERG12</i>	NP_013935.1	1	EIT78374.1	40%	Mevalonate kinase
<i>AoERG8</i>	NP_013947.1	1	EIT75656.1	33%	Phosphomevalonate kinase
<i>AoERG19</i>	NP_014441.1	1	EIT78501.1	58%	Diphosphomevalonate decarboxylase
<i>AoIDI1</i>	NP_015208.1	2	EIT75013.1 EIT75157.1	52% 40%	Isopentenyl-diphosphate delta-isomerase
<i>AoERG20</i>	NP_012368.1	5	EIT77142.1 EIT82866.1 EIT78828.1 EIT74365.1 EIT77275.1	59% 27% 26% 24% 23%	Bifunctional (2E,6E)-farnesyl diphosphate synthase/dimethylallyltranstransferase
<i>AoEGR9</i>	NP_012060.1	1	EIT75610.1	45%	Squalene synthetase
<i>AoERG1</i>	NP_011691.1	1	EIT81755.1	38%	Squalene epoxidase
<i>AoERG7</i>	NP_011939.2	3	EIT83324.1 EIT77904.1 EIT79966.1	45% 42% 37%	Lanosterol synthase
<i>AoERG11</i>	NP_011871.1	3	EIT83124.1 EIT73378.1 EIT72345.1	51% 52% 49%	Sterol 14-demethylase
<i>AoERG24</i>	NP_014119.1	2	EIT78405.1 EIT72491.1	39% 38%	Sterol C-14 reductase
<i>AoERG25</i>	NP_011574.3	2	EIT77737.1 EIT75499.1	57% 52%	Sterol C-4 methyloxidases
<i>AoERG26</i>	NP_011514.1	2	EIT79126.1 EIT82518.1	27% 32%	Sterol C-4 decarboxylases
<i>AoERG27</i>	NP_013201.1	1	EIT80831.1	28%	Sterol 3-keto reductases
<i>AoERG6</i>	NP_013706.1	2	EIT83284.1 EIT80311.1	54% 51%	Sterol C-24 methyltransferases
<i>AoERG2</i>	NP_013929.1	1	EIT81783.1	52%	Sterol C-8 isomerases
<i>AoERG3</i>	NP_013157.1	3	EIT79919.1 EIT80397.1 EIT73679.1	48% 46% 44%	Sterol C-5 desaturases
<i>AoERG5</i>	NP_013728.1	2	EIT82696.1 EIT73398.1	51% 50%	Sterol C-22 desaturases
<i>AoERG4</i>	NP_011503.1	3	EIT80299.1 EIT77041.1 EIT74004.1	55% 50% 38%	Sterol C-24 reductases

Preparation of cDNA libraries and RNA sequencing

Total RNA was extracted from 0.5 g mycelia using a fungal RNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions, with the addition of a RNase-free DNase I treatment (Omega). RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA integrity was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). For cDNA library construction, each pooled

RNA sample was prepared from an equal quantity of RNA from three individual cultures to ensure reliability and reproducibility. After enrichment from pooled total RNA using oligo (dT) magnetic beads, the mRNA was then digested into short pieces in fragmentation buffer at 94 °C for 5 min. First-strand cDNA was synthesized using random hexamer-primers with the mRNA fragments as templates. Second-strand cDNA was synthesized by DNA Polymerase I and RNase H. Following end-repair and adaptor ligation, the products were amplified by PCR and purified with a QIAquick PCR purification kit (Qiagen) to create a

Fig. 1 The effects of different concentrations tebuconazole and terbinafine on the growth of *A. oryzae* 3.042. **a** Growth inhibition of *A. oryzae* 3.042 by tebuconazole (top figure) and terbinafine treatment (bottom figure). **b** and **c** The inhibition rate of different concentrations tebuconazole (**b**) and terbinafine (**c**) treatment. (Bars = 1 cm)

cDNA library. The constructed libraries were then sequenced on an Illumina HiSeq 2500 platform (Illumina, USA) at Biomarker Technologies Co., generating 125-bp paired-end reads. Sequencing data were deposited in the NCBI/SRA database (BioProject: PRJNA407274; BioSample: SAMN07637347).

Mapping reads to the reference genome and normalized gene expressions

Clean-read datasets of high quality were obtained from the raw data by removing low-quality reads, adaptor sequences, and reads with N's (uncertain bases) exceeding 10%. The clean-reads were aligned to the *A. oryzae* genome (https://www.ncbi.nlm.nih.gov/genome/526?genome_assembly_id=29881) using Tophat (v2.0.7) software. As the genome of *A. oryzae* 3.042 was not completely assembled, some reads were mapped to *A. oryzae* RIB40. Meanwhile, the Q20, Q30, GC content, and sequence duplication rate of the clean data were also calculated.

To identify differentially expressed genes between samples, transcript abundances were normalized by the RPKM metric (reads per kilobase per million mapped reads). The false discovery rate (FDR) within 0.05 and $\log_2$ (fold change) over 1 was set as the threshold for differentially expressed genes (DEGs) [20, 21]. Furthermore, all DEGs were annotated by Gene Ontology (GO) classification and KEGG pathway enrichment analyses.

Results

Identification of ergosterol biosynthesis genes in *A. oryzae* 3.042

In *S. cerevisiae*, the genes involved in ergosterol biosynthesis have been identified [7]. In order to investigate ergosterol biosynthesis in *A. oryzae* 3.042, we used a bioinformatics approach

Table 2 Summary of RNA-seq results

Tag classification	WT		Tebuconazole treatment		Terbinafine treatment	
	1	2	1	2	1	2
Total reads	43,268,556	43,621,230	43,762,690	43,265,164	43,984,152	44,658,384
Percentage of identified reads	71.02%	71.12%	73.86%	73.59%	73.77%	73.51%
Percentage of unique reads	70.88%	70.97%	73.68%	73.40%	73.60%	73.35%
% ≥ Q30	86.12%	86.41%	86.87%	86.84%	87.19%	87.09%
GC content	52.51%	52.51%	52.71%	52.63%	52.66%	52.67%
Pearson's correlation coefficient		0.843		0.925		0.827

Table 3 Summary of the DEG genes by different inhibitor treatments

DEG set	DEG number	Upregulated	Downregulated
Wt vs tebuconazole	138	22	82
Wt vs terbinafine	104	74	64
Tebuconazole vs terbinafine	256	215	41

to identify genes involved in ergosterol biosynthesis in *A. oryzae* 3.042. Therefore, we used the protein sequences of ergosterol biosynthesis enzymes from *S. cerevisiae* as the reference sequences to identify homologous proteins in *A. oryzae* 3.042. The results showed that the complete ergosterol biosynthesis pathway in *A. oryzae* 3.042 encodes homologs of all *S. cerevisiae* ergosterol biosynthesis genes (Table 1). However, these genes are very different from those in *S. cerevisiae*. Firstly, most of the ergosterol biosynthesis genes in *S. cerevisiae* appear as a single copy in the genome (except HMG1/2), while *A. oryzae* 3.042 contains multiple paralogous genes. For example, six paralogues of *ERG10* are present in the *A. oryzae* 3.042 genome, which we named as *AoERG10-A* to *AoERG10-F*, respectively. Secondly, although all of the genes homologous to those in *S. cerevisiae* can be found in *A. oryzae* 3.042, the identity ranged from 23 to 60% (Table 1). Therefore, bioinformatics analysis showed that the ergosterol biosynthesis pathway is conserved between *S. cerevisiae* and *A. oryzae* 3.042, but the ergosterol biosynthesis in *A. oryzae* 3.042 appears to be more complex than in *S. cerevisiae*.

The effect of tebuconazole or terbinafine treatment on the growth of *A. oryzae* 3.042

Through research on the ergosterol biosynthesis pathway in yeast and other fungi, many ergosterol biosynthesis inhibitors have been found [11]. We selected triazolone, tebuconazole, and terbinafine (the target site of triazolone and tebuconazole is *ERG11*, and the target site of terbinafine is *ERG1*) to treat *A. oryzae* 3.042. As shown in Fig. S1, all three drugs inhibited the growth of *S. cerevisiae*; however, only tebuconazole and terbinafine inhibited the germination

and growth of *A. oryzae* 3.042, while *A. oryzae* 3.042 appears to be resistant to triazolone. Tebuconazole and terbinafine were used to calculate the IMG (Fig. 1a). Different concentrations of both inhibitors were added into PDA media. By measuring the diameter of colonies treated with inhibitors, we found that terbinafine is more active than tebuconazole as the IMG 50% concentration of tebuconazole was 0.7 µg/L, and that of terbinafine was 0.35 µg/L. Therefore, treatments with the inhibitors further proved that ergosterol biosynthesis in *A. oryzae* 3.042 was conserved but more complex than in *S. cerevisiae*.

Transcriptome overview

In order to identify target genes regulated by ergosterol in *A. oryzae* 3.042 and investigate the function of ergosterol in *A. oryzae* 3.042, we took advantage of RNA-seq to analyze gene expression in *A. oryzae* 3.042 following treatment with the ergosterol biosynthesis inhibitors tebuconazole or terbinafine. The IMG 50% concentrations of each inhibitor listed above were chosen for the treatment for RNA-seq. Two biological replicates were performed for each treatment and control. The numbers of total reads for non-treated, tebuconazole or terbinafine treatments were greater than 43,200,200. Among those reads, the mapped reads ranged from 71.02 to 73.86%, and more than 70% were unique mapped reads. The % ≥ Q30 was greater than 86% and the GC content for all treatments was about 52%. The Pearson correlation coefficient between two repeats of each treatment was greater than 0.827, which indicates that the RNA-seq results of each treatment were repeatable and reliable. A summary of the RNA-seq results is shown in Table 2.

Table 4 COG function classification of genes regulated by inhibitors treatment

Function classes	Tebuconazole treatment			Terbinafine treatment		
	Total	Up	Down	Total	Up	Down
Energy production and conversion	5	2	3	9	9	0
Transport and metabolism	45	4	41	32	19	13
Cell wall/membrane/envelope biogenesis	1	0	1	4	3	1
General function prediction only	15	2	13	16	13	3
Function unknown	2	0	2	1	0	1
Defense mechanisms	4	0	4	3	2	1

Fig. 2 The classification of transport- and metabolism-related DEGs. **a** and **b** The classification of transport- and metabolism-related DEGs for tebuconazole and terbinafine treatments

Differentially expressed genes in response to treatment with inhibitors

To identify genes that had their expression levels altered by treatment with ergosterol biosynthesis inhibitors, we quantified the overall transcription levels of genes using the RPKM method [22]. The number of differentially expressed genes (DEGs) for non-treated vs tebuconazole, non-treated vs terbinafine, and tebuconazole vs terbinafine were 138, 104, and 256, respectively. The summary of the DEGs from different inhibitor treatments is shown in Table 3. The 20 most upregulated and downregulated

genes for non-treated vs tebuconazole, non-treated vs terbinafine, and tebuconazole vs terbinafine are shown in Supplementary Tables 1 to 4. To obtain greater information on the functional categories of the DEGs, the DEGs were analyzed by COG (cluster of orthologous groups of proteins) functional classification. As shown in Table 4, the COGs mapped to DEG gene function can be divided into six classes, including energy production and conversion, transport and metabolism, cell wall/membrane/envelope biogenesis, general function prediction only, function unknown, and defense mechanisms. The most common were transport- and metabolism-related genes, which accounted for

Table 5 Effects of inhibitors treatment on the genes involved lipid biosynthesis and transportation

Accession number	Putative production encoded by the gene	Fold changes in gene expression
Tebuconazole treatment		
EIT74279.1	Hypothetical protein with fatty acid elongase domain	− 4.1
EIT77602.1	Ergosterol biosynthesis ERG4/ERG24 family protein	− 3.0
EIT74308.1	Acetylcholinesterase/butyrylcholinesterase	− 2.8
EIT81567.1	Leucine rich repeat protein with phospholipid binding domain	− 2.8
EIT75089.1	Long chain fatty acyl-CoA synthetase	− 2.1
EIT72491.1	Delta(14)-sterol reductase (AoERG24-B)	1.6
EIT75885.1	Putative dienelactone hydrolase	1.9
EIT79617.1	Gluconate 5-dehydrogenase	2.2
EIT81441.1	Fatty acid synthetase	2.4
EIT80791.1	Esterase/lipase	2.8
EIT74373.1	Carboxylesterase family protein	3.8
EIT77624.1	Acyl-CoA synthetase	Inf
Terbinafine treatment		
EIT80216.1	Acyl-CoA synthetase	−Inf
CEJ94122.1	Hypothetical protein with cytochrome P450 domain	− 5.1
EIT76912.1	Flavin oxidoreductase/12-oxophytodienoate reductase	− 4.8
EIT78275.1	Hypothetical protein which may involves in alcohol sensor	− 3.2
EIT80997.1	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	− 2.4
EIT77697.1	Lipase	− 2.4
EIT76480.1	Benzoate 4-monooxygenase cytochrome P450	− 2.3
EIT80068.1	1-acyldihydroxyacetone-phosphate reductase	− 2.3
EIT80560.1	1-aminocyclopropane-1-carboxylate deaminase	− 1.9
EIT75499.1	C-4 methylsterol oxidase (AoERG25-B)	− 1.8
EIT78007.1	3-hydroxyacyl-CoA dehydrogenase	1.7

Inf means infinite and −inf means minus infinity

Table 6 The DEG genes involved in steroid biosynthesis

Function classes	Fold changes in gene expression		
	Wt vs tebuconazole	Wt vs terbinafine	Tebuconazole vs terbinafine
<i>ERG4/ERG24</i> family protein (EIT77602.1)	− 3.0	ND	ND
<i>AoERG24</i> (EIT72491.1)	ND	1.59	1.71
<i>AoERG25</i> (EIT75499.1)	− 1.75	ND	2.75
<i>AoERG6</i> (BAE59581.1)	ND	ND	2.39
<i>AoERG3A</i> (EIT79919.1)	ND	ND	1.59
<i>AoERG3B</i> (EIT73679.1)	ND	ND	− 1.92

ND represents not detected

61.6 and 66% of the DEGs for tebuconazole and terbinafine treatment, respectively. These transport- and metabolism-related genes can be divided into seven classes (Fig. 2). Among these seven classes, the most common for tebuconazole treated cells were amino acid, carbohydrate, and inorganic ion transporters (Fig. 2a), and the most common for terbinafine treated cells were lipid, amino acid, and carbohydrate transporters (Fig. 2b). The 10 most upregulated and downregulated transport- and metabolism-related genes for non-treated vs tebuconazole, non-treated vs terbinafine, and tebuconazole vs terbinafine are shown in Supplementary Tables 5 and 6.

Differentially expressed genes involved in lipid biosynthesis and transportation

Since ergosterol belongs to lipid, we investigated the DEGs related to lipid biosynthesis and transportation. All DEGs for tebuconazole and terbinafine treatment are shown in Table 5. For tebuconazole treatment, there were 12 DEGs related to lipid biosynthesis and transportation, including five downregulated and seven upregulated genes. Among these genes, the most upregulated gene was acyl-CoA synthetase (EIT77624.1), and the most downregulated gene was hypothetical protein with fatty acid elongase domain (EIT74279.1). For terbinafine treatment, there were 11 DEGs related to lipid biosynthesis and transportation, including 10 downregulated genes and only one upregulated gene. However, the most downregulated gene was an acyl-CoA synthetase (EIT80216.1). As the inhibitors block ergosterol biosynthesis, we also analyzed the ergosterol

biosynthesis genes in detail (Table 6). For tebuconazole treatment, two DEGs involved in ergosterol biosynthesis were identified, the one is ergosterol biosynthesis *ERG4/ERG24* family protein (EIT77602.1), the other is delta(14)-sterol reductase (*AoERG24-B*, EIT72491.1). For the terbinafine treatment, only C-4 methylsterol oxidase (*AoERG25-B*, EIT75499.1) was identified.

Comparison of gene expression profiles following tebuconazole or terbinafine treatments

In order to investigate the target genes regulated by ergosterol, the genes regulated by both tebuconazole and terbinafine treatment were identified. There were 17 genes identified as DEGs and the most common genes were classified as amino acid and carbohydrate transport, metabolism and secondary metabolite biosynthesis, and transport- and catabolism-related genes (Fig. 3). A detailed list of genes is shown in Table 7. Among the 17 DEGs, only one gene was upregulated and the other genes were all downregulated. Most of the DEGs encode hypothetical proteins with unknown functions. The only upregulated gene encodes a hypothetical protein located in the extracellular region. The remaining DEGs encode cell membrane and cell wall-related proteins, including glycosyl transferase, glucan synthases, cellulase, and cell membrane-anchored family protein. We also analyzed DEGs between tebuconazole and terbinafine treatment, and the 20 most upregulated and downregulated genes are shown in Supplementary Tables 7 and 8. As the identified DEGs involved in ergosterol biosynthesis are very different between the

Fig. 3 Analysis of DEGs between tebuconazole treatment and terbinafine treatment. **a** Venn diagram of genes regulated by both tebuconazole (right) treatment and terbinafine (left) treatment. **b** The classification of DEGs regulated by both tebuconazole treatment and terbinafine treatment

Table 7 Genes regulated by both tebuconazole and terbinafine treatment

Accession number	Putative production encoded by the gene	Fold changes in gene expression	
		Tebuconazole treatment	Terbinafine treatment
EIT80584.1	Glycosyl transferase	– 2.57	– 3.22
EIT78077.1	Alpha-1,3 glucan synthases	– 2.74	– 3.19
EIT80342.1	GPI anchored serine-threonine rich protein	– 2.79	– 2.75
EIT83128.1	Carboxylic ester hydrolase activity	– 2.91	– 3.31
EIT80508.1	Conserved hypothetical protein related to polysaccharide catabolic process with cellulase activity	– 2.43	– 3.21
EIT73411.1	Conserved threonine rich protein, Ser-Thr-rich glycosyl-phosphatidyl-inositol-anchored membrane family	– 2.30	– 2.34
EIT81688.1	Methylmalonyl-CoA decarboxylase	– 2.05	– 2.12
XP_002372960.1	Hypothetical protein AFLA_080430	– 2.70	– 3.44
XP_002382615.1	Hypothetical protein AFLA_004140	– 1.92	– 2.78
XP_002383654.1	Hypothetical protein AFLA_097810	– 3.28	– 3.40
EIT76225.1	Hypothetical protein with monooxygenase activity and oxidation-reduction process	– 2.71	– 1.76
EIT82292.1	Hypothetical protein with hydrolase activity, acting on glycosyl bonds	– 3.60	– 2.91
EIT83050.1	Hypothetical protein with DNA binding located in nucleus	– 2.51	– 3.43
XP_001727223.1	Hypothetical protein with sequence-specific DNA binding RNA polymerase II transcription factor activity	– 2.38	– 3.97
XP_003190202.1	Hypothetical protein AOR_1_1158134	– 2.55	– 2.77
XP_001727223.1	Hypothetical protein AOR_1_284194	– 2.87	– 3.93
XP_001825558.1	Hypothetical protein AOR_1_66064, located in extracellular region	3.84	2.56

tebuconazole and terbinafine treatment groups, we also identified DEGs for tebuconazole vs terbinafine treatment involved in ergosterol biosynthesis. Six ergosterol biosynthesis genes were identified including *ERG4/ERG24*, *AoERG24*, *AoERG25*, *AoERG6*, *AoERG3A*, and *AoERG3B*. It is interesting that *AoERG3A* and *AoERG3B* showed different expression pattern in response to tebuconazole and terbinafine treatment (Table 6).

Discussion

Ergosterol as well as intermediates of ergosterol pathway, such as lanosterol and zymosterol, is of great economic value. For example, lanosterol and zymosterol can be used as emulsifiers for cosmetics and precursors for the production of cholesterol

lowering substances [23]. Therefore, it is of great value to study ergosterol biosynthesis. However, there has only been limited research on ergosterol biosynthesis in *A. oryzae*. In this study, we used bioinformatics and transcriptome analysis to identify genes involved in ergosterol biosynthesis and those regulated after treatment with ergosterol biosynthesis inhibitors.

Ergosterol biosynthesis genes in *A. oryzae* 3.042

The ergosterol biosynthesis pathway was found to be conserved in *A. oryzae*; however, multiple paralogues exist for almost all the biosynthesis genes: a situation that is not observed in *S. cerevisiae*. For example, *AoERG10* has six paralogues, and *AoHMG* and *AoERG20* have five paralogues each. Moreover, the amino acid sequences are not well conserved. Treatment

with inhibitors showed that *A. oryzae* 3.042 was also sensitive to terbinafine (target site is ERG1) and tebuconazole (target site is ERG11), which further indicates that the functions of AoERG1 and AoERG11 were conserved. Therefore, our results indicate that the ergosterol biosynthesis pathway in *A. oryzae* is conserved, but the biosynthesis enzymes and regulation mechanisms may be more complicated.

Effects of inhibitor treatment on gene transcription in *A. oryzae* 3.042

We identified 138 and 104 DEGs for non-treated vs tebuconazole and non-treated vs terbinafine, respectively. Compared with other transcriptome analyses, the number of DEGs appears to be smaller. This may be because RNA-seq was performed only 8 h after treatment with the inhibitors. However, the advantage of this is that these DEGs are more likely to be the direct target genes regulated by these inhibitors. It is interesting that among the DEGs, about half of these belong to transport- and metabolism-related genes, and the most common are those involved in amino acid and carbohydrate transport. This may be because ergosterol is an important component of the cell membrane and most transporters are located in the cell membrane. Therefore, these genes are those that respond quickly and directly to the inhibitors. As ergosterol and lipid metabolism are closely related, we also analyzed the effects of inhibitor treatment on genes involved in lipid biosynthesis and metabolism and identified 12 and 11 DEGs that were involved in lipid biosynthesis and metabolism for non-treated vs tebuconazole and non-treated vs terbinafine, respectively, including ergosterol biosynthesis genes.

Comparison of gene expression profiles following tebuconazole or terbinafine treatments

In this study, the gene expression profiles after tebuconazole and terbinafine treatment were investigated. The target site and the mechanism of action of two inhibitors are different. When the ERG11 was inhibited by tebuconazole, ergosterol biosynthesis was blocked. However, when the ERG1 was inhibited by terbinafine, it not only caused a decrease in ergosterol production in *A. oryzae* but also caused squalene accumulation in the plasma membrane, leading to increased fragility of the plasma membrane and resulting in the destruction of the structure and function of cell membrane [15]. The results of DEGs for non-treated vs tebuconazole and non-treated vs terbinafine further proved the different mechanisms. For example, among the DEGs for non-treated vs tebuconazole and non-treated vs terbinafine, there were only 17 genes that were regulated by both tebuconazole and terbinafine treatment. These 17 DEGs (16 downregulated and one upregulated genes) are likely to be the target genes directly regulated by ergosterol. The most frequently downregulated genes are classified as cell membrane and cell wall-related genes (Table 7). The possible

reason is that blocking ergosterol biosynthesis changes the composition of the cell membrane, resulting in feedback regulation of cell membrane-related gene expression. It has been reported that ergosterol can act as a signaling molecule to activate the expression of defense-related genes in tobacco plants [24]. Thus, the downregulation of cell wall-related genes may be attributed to the use of ergosterol as a signaling molecule that mediates communication between the cell membrane and cell wall, and these cell wall-related genes are the target genes directly regulated by ergosterol. We also analyzed the DEGs between terbinafine and tebuconazole and found 256 DEGs, which was far more than that for non-treated vs tebuconazole and non-treated vs terbinafine, which further proves the different expression profiles of two treatments. On the one hand, these DEGs may be regulated by squalene accumulation caused by terbinafine treatment. However, on the other hand, the intermediates between EGR1 and ERG11, such as lanosterol, also play important roles in regulating *A. oryzae* gene expression, and these DEGs may be regulated by the intermediates.

In summary, the present study identified several genes involved in ergosterol biosynthesis and the response of *A. oryzae* to ergosterol biosynthesis inhibitors. These results provide new information on *A. oryzae* ergosterol biosynthesis and regulation, which may lay the foundation for the genetic modification of the ergosterol biosynthesis pathway, as ergosterol is not only important for the fungal growth, reproduction, and stress tolerance, but also of great economic value.

Acknowledgments This study was financially supported by the National Natural Science Foundation of China (NSFC) (Grant nos. 31700068 and 31460447), International S&T Cooperation Project of Jiangxi Provincial (Grant no. 20142BDH80003), General Science and Technology Project of Nanchang City (Grant no. 3000035402), “555 Talent Project” of Jiangxi Province, Natural Science Foundation of Jiangxi Province (Grant nos. 20181BAB214001 and 20171BAB214004), and the Open Foundation of Hubei Key Laboratory of Edible Wild Plants Conservation and Utilization (Grant no. EWPL201705).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Wollam J, Antebi A (2011) Sterol regulation of metabolism, homeostasis and development. *Annu Rev Biochem* 80:885–916
2. Prasad R, Shah AH, Antifungals RMK (2016) Mechanism of action and drug resistance. *Adv Exp Med Biol* 892:327–349
3. Beni A, Soki E, Lajtha K, Fekete I (2014) An optimized HPLC method for soil fungal biomass determination and its application to a detritus manipulation study. *J Microbiol Methods* 103(4):124–130

4. Kodedova M, Sychrova H (2015) Changes in the sterol composition of the plasma membrane affect membrane potential, salt tolerance and the activity of multidrug resistance pumps in *Saccharomyces cerevisiae*. *PLoS One* 10(9):e0139306
5. Görög S (2011) Advances in the analysis of steroid hormone drugs in pharmaceuticals and environmental samples (2004–2010). *J Pharm Biomed Anal* 55(4):728–743
6. Whiteman MK, Jeng G, Samarina A, Akatova N, Martirosyan M, Kissin DM, Curtis KM, Marchbanks PA, Hillis SD, Mandel MG, Jamieson DJ (Jan 2016) Associations of hormonal contraceptive use with measures of HIV disease progression and antiretroviral therapy effectiveness. *Contraception* 93(1):17–24
7. Hu Z, He B, Ma L, Sun Y, Niu Y, Zeng B (2017) Recent advances in ergosterol biosynthesis and regulation mechanisms in *Saccharomyces cerevisiae*
8. Hayakawa H, Sobue F, Motoyama K, Yoshimura T, Hemmi H (2017) Identification of enzymes involved in the mevalonate pathway of *Flavobacterium johnsoniae*. *Biochem Biophys Res Commun* 487(3):702–708
9. Klug L, Daum G (2014) Yeast lipid metabolism at a glance. *FEMS Yeast Res* 14(3):369–388
10. Lee SH, Raboune S, Walker JM, Bradshaw HB (2010) Distribution of endogenous farnesyl pyrophosphate and four species of lysophosphatidic acid in rodent brain. *Int J Mol Sci* 11(10):3965–3976
11. Ahmad A, Khan A, Manzoor N, Khan LA (2010) Evolution of ergosterol biosynthesis inhibitors as fungicidal against *Candida*. *Microb Pathog* 48(1):35–41
12. Müller C, Staudacher V, Krauss J, Giera M, Bracher F (2013) A convenient cellular assay for the identification of the molecular target of ergosterol biosynthesis inhibitors and quantification of their effects on total ergosterol biosynthesis. *Steroids* 78(5):483–493
13. Kristan K, Rizner TL (Mar 2012) Steroid-transforming enzymes in fungi. *J Steroid Biochem Mol Biol* 129(1–2):79–91
14. Mercer EI (1993) Inhibitors of sterol biosynthesis and their applications. *Prog Lipid Res* 32(4):357–416
15. Ryder NS (2010) Terbinafine: mode of action and properties of the squalene epoxidase inhibition. *Br J Dermatol* 126(s39):2–7
16. Goldstein AS, Frye LL (1996) Synthesis and bioevaluation of delta 7-5-desaturase inhibitors, an enzyme late in the biosynthesis of the fungal sterol ergosterol. *J Med Chem* 39(26):5092–5099
17. Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto KI, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama JI, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005) Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* 438(7071):1157–1161
18. Zhao G, Yao Y, Chen W, Comparison CX (2013) Analysis of the genomes of two *Aspergillus oryzae* strains. *J Agric Food Chem* 61(32):7805–7809
19. Bhattacharya S, Esquivel BD, White TC (2018) Overexpression or deletion of ergosterol biosynthesis genes alters doubling time, response to stress agents and drug susceptibility in *Saccharomyces cerevisiae*. *mBio* 9(4):e01291–18
20. Long M, Li ZQ, Lei B et al (2016) Identification and comparative study of chemosensory genes related to host selection by legs transcriptome analysis in the tea geometrid *Ectropis obliqua*. *PLoS One* 11(3):e0149591
21. Audic S, Claverie JM (1997) The significance of digital gene expression profiles. *Genome Res* 7(10):986–995
22. Blanc G, Gallot-Lavallée L, Maumus F (2015) Provirophages in the *Bigeloviella* genome bear testimony to past encounters with giant viruses. *Proc Natl Acad Sci U S A* 112(38):5318–5326
23. Wriessnegger T, Pichler H (2013) Yeast metabolic engineering – targeting sterol metabolism and terpenoid formation. *Prog Lipid Res* 52(3):277–293
24. Mikeš V, Lochman J, Kašparovský T (2006) Ergosterol is a signal molecule that leads to the expression of defence-related genes in tobacco