Appl Microbiol Biotechnol (2014) 98:5589–5597
DOI 10.1007/s00253-014-5763-y

GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Comparative transcriptomic analysis of the heat stress response in the filamentous fungus *Metarhizium anisopliae* using RNA-Seq

Zhang-Xun Wang · Xia-Zhi Zhou · Hui-Min Meng · Yu-Jun Liu · Quan Zhou · Bo Huang

Received: 16 January 2014 / Revised: 20 March 2014 / Accepted: 7 April 2014 / Published online: 26 April 2014

Abstract The entomopathogenic fungus *Metarhizium anisopliae* is widely used for biological control of a variety of insect pests. The effectiveness of the microbial pest control agent, however, is limited by poor thermotolerance. The molecular mechanism underlying the response to heat stress in the conidia of entomopathogenic fungi remains unclear. Here, we conducted high-throughput RNA-Seq to analyze the differential gene expression between control and heat treated conidia of *M. anisopliae* at the transcriptome level. RNA-Seq analysis generated 6,284,262 and 5,826,934 clean reads in the control and heat treated groups, respectively. A total of 2,722 up-regulated and 788 down-regulated genes, with a cutoff of twofold change, were identified by expression analysis. Among these differentially expressed genes, many were related to metabolic processes, biological regulation, cellular processes and response to stimuli. The majority of genes involved in endocytic pathways, proteosome pathways and regulation of autophagy were up-regulated, while most genes involved in the ribosome pathway were down-regulated. These results suggest that these differentially expressed genes may be involved in the heat stress response in conidia. As expected, significant changes in expression levels of genes encoding heat shock proteins and proteins involved in trehalose accumulation were observed in conditions of heat stress. These results expand our understanding of the molecular mechanisms of the heat stress response of conidia and provide a foundation for future investigations.

Keywords *Metarhizium anisopliae* · Heat stress · Transcriptome · RNA-Seq · Differential expression

Introduction

Metarhizium anisopliae is an entomopathogenic fungus widely used for biological control of a variety of insects that cause significant economic losses in agriculture (Frazzon et al. 2000; Lord 2005). Infection of insect hosts by *M. anisopliae* is initiated by conidia that penetrate the insect cuticle (St Leger and Wang 2010). Entomopathogenic fungi conidia, which are responsible for dispersal and environmental persistence, are often used as the inoculum in biological control programs (Fernandes et al. 2012; St Leger and Wang 2010). These fungal biopesticides, however, constitute only a small percentage of the total insecticide market (Kim et al. 2011), due to a short shelf-life and inconsistencies in virulence (Liao et al. 2013). The short shelf-life results from the low thermotolerance of conidia, which display a reduced percentage of conidial germination after exposure to high temperatures (Rangel et al. 2008). Fungal conidia exposed to high temperatures during storage, distribution, or in field applications, lose viability due to the effects of heat stress. Detailed knowledge of the heat stress response in entomopathogenic fungi conidia, however, is limited, hindering improvements in conidia thermotolerance by means of genetic engineering.

The description of gene pathways associated with thermotolerance and the grouping of these genes into functional

Zhang-Xun Wang and Xia-Zhi Zhou contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5763-y) contains supplementary material, which is available to authorized users.

Z.-X. Wang · X.-Z. Zhou · H.-M. Meng · Y.-J. Liu · Q. Zhou · B. Huang (✉)
Anhui Provincial Key Laboratory of Microbial Pest Control, Anhui Agricultural University, Hefei 230036, China
e-mail: bhuang@ahau.edu.cn

Z.-X. Wang
School of Plant Protection, Anhui Agricultural University, Hefei 230036, China

categories is essential for an understanding of the mechanisms of thermotolerance in fungal conidia and improving thermal resistance. The genome of *M. anisopliae*, comprised of 39 million bases and 10,582 protein coding genes, was recently sequenced (Gao et al. 2011). With the completion of genome sequencing, transcriptomic analysis of *M. anisopliae* will become an important research tool (Gao et al. 2011). The availability of a high quality genome sequence and annotations provides a reliable foundation for comparative analyses of transcriptomes. In-depth studies on thermotolerance of conidia from *M. anisopliae*, including the application of transcriptomics, will not only identify defense-related genes involved in the response to heat stress, but may also elucidate the role of metabolism in the heat stress response.

RNA-Seq has recently become a popular tool for transcriptome profiling in studies monitoring transcriptional responses in varying experimental conditions (Wang et al. 2009; Nookaew et al. 2012; Oshlack et al. 2010). With the development of high-throughput sequencing platforms, such as Illumina RNA-Seq, genome-wide expression profiles have been studied in various filamentous fungi, including *Trichoderma reesei*, *Magnaporthe oryzae*, and two *Aspergillus* species (Lin et al. 2013; Ries et al. 2013; Rokas et al. 2012; Soanes et al. 2012). Previous studies of filamentous fungi identified a number of genes involved in the response to high temperature stress (Albrecht et al. 2010; Ruoff et al. 1999). In addition, a large number of studies have shown that these genes involved in metabolic and regulatory processes are expressed in conidia of filamentous fungi (Gokce et al. 2012; Lamarre et al. 2008). To date, there have been no reports of comparative transcriptomic analysis in conidia of filamentous fungi during the heat stress response.

Recent studies on heat tolerance of entomogenous fungi, including *Metarhizium* and *Beauveria*, were limited to "key" individual genes. Overexpression of *hsp25*, a gene encoding a small heat shock protein, improved the resistance of *Metarhizium robertsii* to heat and other abiotic stresses (Liao et al. 2013). Reduced expression of the neutral trehalase gene (*ntl*), resulting in the accumulation of trehalose, increased the thermotolerance of *Metarhizium acridum* (Leng et al. 2011). In the genus *Beauveria*, a gene encoding a conidial protein (*cp15*) is associated with fungal tolerance to thermal and oxidative stresses (Ying and Feng 2011). Moreover, Δ*Bbgas1* (GPI-anchored β-1,3-glucanosyltransferase) conidial spores displayed decreased germination after 1–4 h of heat shock at high temperatures (>40 °C) as compared to wild-type (Zhang et al. 2011). Our study aimed to assess differential gene expression in the conidia of *M. anisopliae* under conditions of varying temperatures. To accomplish this, we utilized a high-throughput sequencing platform to obtain comparative transcriptomic profiling. The results provide insight into the heat stress response mechanism in conidia.

Materials and methods

M. anisopliae growth and harvest

M. anisopliae strain ARSEF 23 (ATCC No. MYA-3075) was generously provided by Dr. Chengshu Wang. Stock cultures were grown on potato dextrose agar (PDA; 20 % potato, 2 % dextrose and 2 % agar, w/v) in the dark at 28 °C for 10 days to obtain conidia. Conidia were harvested in a 0.05 % Tween 80 aqueous solution, and the conidial suspension was filtered through sterile non-woven fabrics to remove mycelia. After collection, all freshly isolated samples were immediately used for extraction of total RNA or stored in liquid nitrogen for further analysis.

Conidial germination assays

The treatment time of 120 min for conidia in the water bath was selected according to previous reports (Kim et al. 2011; Plesofsky-Vig and Brambl 1985). The effects of heat on conidial germination were monitored using a previously described protocol (Rangel et al. 2006). Briefly, 1-ml aliquots of conidial suspensions in 1.5-ml Eppendorf tubes were exposed to a water bath at 28 °C (control), 30 °C, 35 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C or 45 °C for up to 120 min. After 120 min of heat exposure, 100 μl of the conidial suspension from each tube was inoculated on PDA plates. After 24 h incubation at 28 °C, conidial germination on each plate was observed by microscopy (400× magnification), and conidia with visible germ tubes were considered to be germinated. A total of 300 conidia per plate were evaluated and the relative percentage of germination was calculated by comparing the germination of the control and heat treated conidia. Each treatment was performed in three independent experiments.

RNA extraction, library preparation and sequencing

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To maximize target coverage, equal amounts of total RNA from the three replicates of control or heat treated conidia were pooled for RNA-Seq library construction. Total RNA (5 μg) was prepared for Illumina RNA-Seq according to previously reported methods (Li et al. 2011). Briefly, poly(A) mRNA from the total RNA was purified using oligo(dT) beads. Following purification, mRNA was fragmented into small pieces. The first cDNA strand was synthesized using random hexamer primers for reverse transcription with cleaved RNA fragments serving as templates. The second strand cDNA was synthesized using RNase H and DNA polymerase I, and the sequencing library constructed following the manufacturer's instructions (Illumina, San Diego, CA,

USA). The cDNA library was sequenced using the Illumina HiSeq 2000 with a single-end (single reads of 50 bases) sequencing strategy at the Beijing Genomics Institute (BGI), Shenzhen, China. Raw data were deposited in the National Center for Biotechnology Information (NCBI) database under the SRA Study accession number SRP034836.

Mapping of RNA-Seq reads and quantitative analysis of gene expression

Clean reads were obtained by removing raw reads that contained the adaptor, unknown or low-quality sequences. Clean reads were used for mapping to the *M. anisopliae* reference genome with the SOAP (Short Oligonucleotide Alignment Program), allowing up to two base mismatches (Gao et al. 2011; Li et al. 2009). For quantification, gene expression levels were calculated using the reads per kilobase per million reads (RPKM) method (Mortazavi et al. 2008), thereby limiting the effects of different gene lengths and sequencing levels. Rigorous algorithms were applied to identify differentially expressed genes (DEGs) between control and heat treated samples at the BGI based on previously described methods (Audic and Claverie 1997). DEGs were identified using a false discovery rate (FDR) ≤0.001 and an absolute value of the $\log_2$ ratio ≥1 as the threshold.

Gene Ontology (GO) functional classification and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis of DEGs

To further characterize the biological functions and metabolic pathways of DEGs, the DEGs were subjected to a Gene Ontology (GO) functional analysis (Blast2GO, http://www.blast2go.org/) (Ye et al. 2006) and a KEGG pathway enrichment analysis (KEGG database; Kanehisa et al. 2008). The significant KEGG pathways for DEGs were selected using a p value cutoff of ≤0.05.

Validation of RNA-Seq data by quantitative real-time PCR

cDNA was synthesized from 1 μg total RNA, extracted as described above, using the Moloney murine leukemia virus reverse transcriptase (TaKaRa, Dalian, China), and subsequently used as a template for RT-PCR. All primers used for qPCR are listed in Table S1 in the Supplementary Material. The qPCR reaction was carried out using a 7500 Real-Time PCR System (Applied Biosystems) using a SYBR Green kit (SYBR Premix Ex Taq II; TaKaRa) according to the manufacturer's instructions. Amplification conditions used for qPCR were 95 °C for 5 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. All PCR reactions were run in triplicate. The threshold cycle (C_T) was determined using the default threshold settings. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative gene expression levels (Livak and Schmittgen 2001), using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (EFY96862) as an internal control for each sample similar to the procedure reported by Fang and Bidochka (2006). All data were analyzed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Data are expressed as the mean ± SE (standard error) of three independent experiments.

Results

Effects of temperature on conidia of *M. anisopliae*

The relative percentage of germination of *M. anisopliae* at different treatment temperatures was assessed to determine an optimal temperature for transcriptomic analysis of the heat stress response (Table 1). Results showed that the viability of conidia was greater than 90 % at temperatures ≤38 °C suggesting heat stress at these temperatures was non-lethal. Viability declined dramatically when the temperature was increased beyond 38 °C (Table 1). Furthermore, it has been reported that the upper thermal limit for *M. anisopliae* conidial germination is approximately 37 °C (Walstad et al. 1970). Therefore, a 38 °C treatment temperature was selected for transcriptomic analysis in our study.

Overview of RNA-Seq data

RNA-Seq was performed to compare gene expression profiles of control and heat treated conidia from *M. anisopliae*. Raw data have been deposited in the NCBI database under the SRA Study accession number SRP034836. After removal of

Table 1 Percent germination of *M. anisopliae* conidia following temperature treatment

Treatment temperature[a]	Percent germination (%)[b]
28 °C→28 °C (control)	100
28 °C→30 °C	93.0±0.44
28 °C→35 °C	92.2±2.05
28 °C→37 °C	90.8±0.67
28 °C→38 °C	90.3±0.52
28 °C→39 °C	69.5±1.36
28 °C→40 °C	70.0±2.41
28 °C→41 °C	48.5±0.67
28 °C→45 °C	19.6±0.51

[a] Treatment at each temperature was undertaken for 120 min in a water bath

[b] Data are expressed as the mean ± SE (standard error) of three independent experiments

adaptor sequences, unknown, and low-quality sequences, a total of 6,284,262 and 5,826,934 clean reads were obtained from the RNA of control and heat treated samples, respectively, and the percentage of clean reads in raw tags of the two libraries was 98.67 % and 98.46 %, respectively (Fig. S1). Of the total reads, over 90 % of the reads from both samples were uniquely mapped to the reference genome (Table 2). Sequences with at least one read matched to approximately 86.0 % of the 10,582 coding genes in the *M. anisopliae* genome (Table 2 and Table S2), suggesting that the sequencing was sufficient.

Global analysis of DEGs between control and heat treated samples

In the present study, we used FDR ≤0.001 and an absolute value of the $\log_2$ ratio ≥1 as the threshold to determine the significance of gene expression differences. Based on this criterion, we determined a total of 3,510 genes were differentially expressed between control and heat treated samples. Of these, a total of 2,722 genes were significantly up-regulated under conditions of heat stress while 788 genes were significantly down-regulated (Table S3 in the Supplementary Material). The global gene expression profiles of control and heat treated groups are shown in Fig. 1. Furthermore, GO functional classification for the 3,510 DEGs was performed to reveal the potential functions of the DEGs during heat stress. The results showed that the 3,510 DEGs could be categorized into 37 functional groups, belonging to three main GO domains: biological processes (19); cellular components (8); and molecular functions (10; Fig. 2 and Table S4). Metabolic process, cell, and catalytic activity were the most common annotation terms in each of the three GO term categories, respectively. Among these groups, we found a high percentage of genes in the categories of metabolic processes, cellular processes, biological regulation, response to stimulus, catalytic activity, binding, cell and cell part. The results of the GO functional annotation indicate that multiple biological processes are involved in the response to heat stress in *M. anisopliae* conidia.

Table 2 Summary of RNA-Seq data and mapping

Sample	Control	Heat treated
Total clean reads (%)	6,284,262 (100.00 %)	5,826,934 (100.00 %)
Total mapped reads (% mapped)	5,781,356 (92.00 %)	5,303,037 (91.01 %)
Total unmapped reads (%)	502,906 (8.00 %)	523,897 (8.99 %)
Unique match (% uniquely mapped reads)	5,772,373 (91.85 %)	5,288,982 (90.77 %)
Detected genes (% 10,582 coding genes)	9,095 (85.95 %)	9,081 (85.82 %)

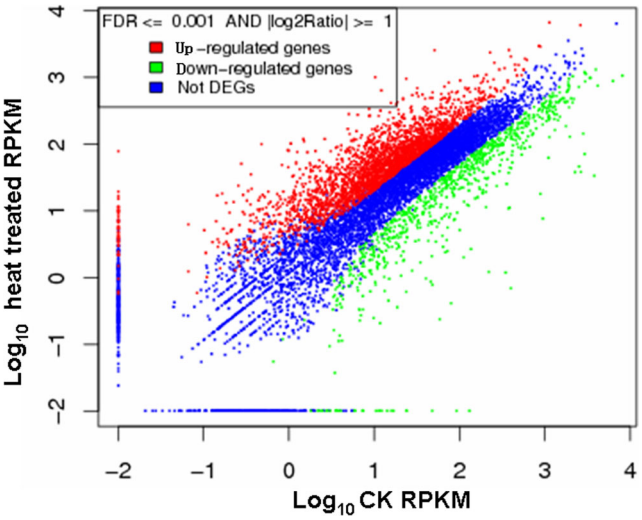

Fig. 1 Comparison of gene expression levels in control and heat treated groups. The expression level of each gene was normalized as reads per kilobase per million reads (*RPKM*). The up-regulated and down-regulated genes are shown in *red* and *green*, respectively. Genes that were not differentially expressed between control and heat treated groups are shown in *blue*. The *x*-axis represents the $\log_{10}$ RPKM of control (CK) samples. The *y*-axis represents the $\log_{10}$ RPKM of heat treated samples

KEGG pathway enrichment analysis of DEGs

To further assess the functions of DEGs in the response to heat stress, the KEGG database was used to analyze pathways. The results of KEGG pathway enrichment analysis are displayed in Table S5 in the Supplementary Material. A total of 14 different metabolic pathways were identified with at least 13 related DEGs. The ribosome pathway was the most significantly enriched pathway. There were 81 DEGs involved in the ribosome pathway. Of these, 72 genes were significantly down-regulated, including genes encoding 40S ribosomal proteins and 60S ribosomal proteins. The endocytosis pathway was also highly represented. In total, 44 DEGs were involved in the endocytosis pathway (Fig. 3 and Table S5), of which 36 genes were up-regulated, including *clathrin* (EFZ00769), *stam* (EFZ02693, EFZ02528), *tsg101* (EFZ03268), *vps22* (EFZ02916), *vps25* (EFZ02631), *vps36* (EFY96269, EFY98186), *chmp3* (EFZ01379), *chmp5* (EFY99429), *chmp6* (EFZ01425), *vps4* (EFY98000, EFY99553, EFZ00023), *vta1* (EFY95550), *arf6* (EFZ01558), *pip5k* (EFY99982) and *pld* (EFZ04055). The third group of metabolic pathways involved in the response to heat stress was the proteasome pathway (Fig. S2 and Table S5), in which all 28 genes were up-regulated.

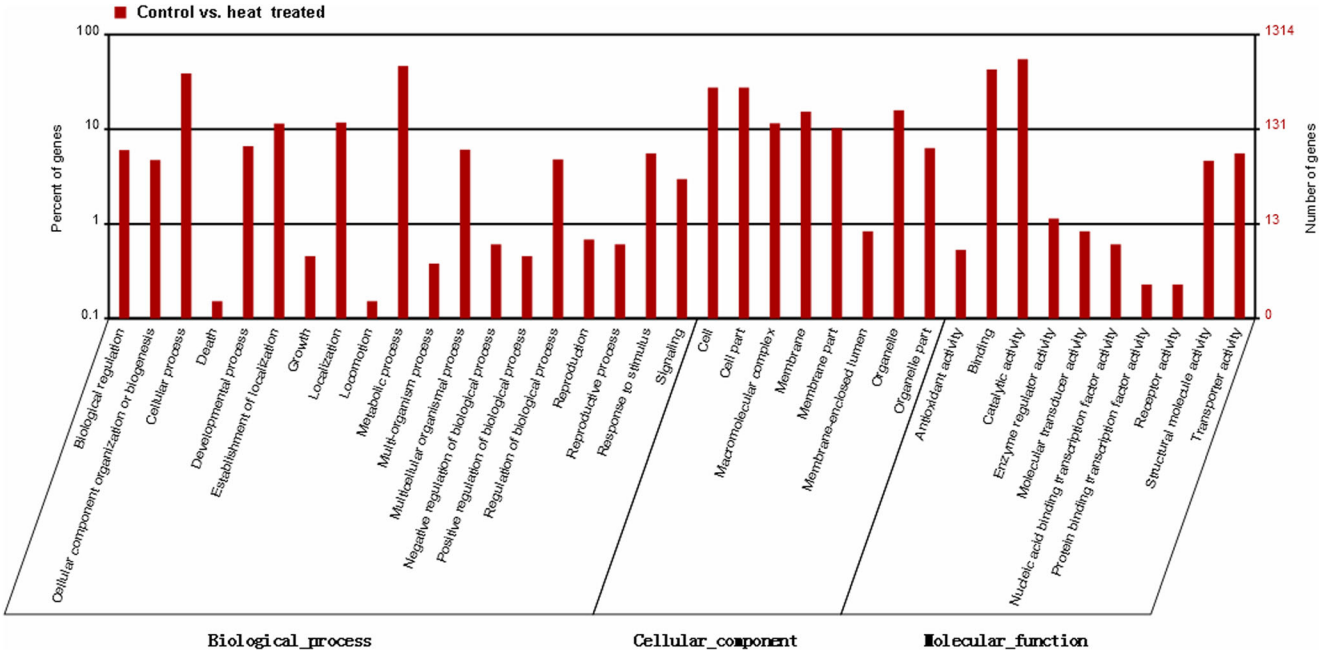

Fig. 2 GO functional classification of DEGs. The DEGs were placed into the three main GO categories: biological process, cellular component and molecular function

Other representative pathways regulated by heat stress include those involved with the phagosome, regulation of autophagy, glycerophospholipid metabolism, inositol phosphate metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, ether lipid metabolism and propanoate metabolism (Table S5). Moreover, one pathway involved in signal transduction, the phosphatidylinositol signaling system, was enriched in heat induced genes.

Expression levels of heat shock protein genes and trehalose accumulation-related genes were significantly altered in heat treated conidia

Previous studies of the heat shock response have found that heat shock proteins function as molecular chaperones to aid in the folding and unfolding of other proteins, and that heat shock proteins are also involved in degradation or reactivation of damaged proteins (Parsell and Lindquist 1993), and acquisition of thermotolerance in filamentous fungi (Montero-Barrientos et al. 2007; Liao et al. 2013). As expected, 11 genes encoding heat shock proteins showed differential expression under conditions of heat stress in our transcriptomic analysis. Most DEGs (10/11) associated with heat shock proteins were significantly up-regulated in heat treated conidia (Table S3), although an orthologous gene of *hsp70* (EFZ03582) was down-regulated. The DEGs encoding heat shock proteins included *hsp101* (EFZ00938), *hsp70* (EFZ03582) and *hsp78* (EFZ00288), which play a leading role in protein folding, unfolding and thermotolerance. These findings are in agreement with recent studies that report high expression of genes encoding heat shock proteins in *Metarhizium* conidia (Barros et al. 2010; Su et al. 2013).

In our study, up-regulation of several orthologous genes of trehalose-6-phosphate synthases was observed, although expression of the gene encoding neutral trehalase was not significantly changed under conditions of heat stress based on our criteria for identification of DEGs (Table S3). These significant expression changes in trehalose-related genes ultimately result in the accumulation of trehalose, which improves the thermotolerance of conidia. The results are in agreement with the current hypothesis that the role of trehalose is primarily as a protectant against stress in filamentous fungi (Al-Bader et al. 2010; Doehlemann et al. 2006).

Validation of the gene expression profiles by qRT-PCR

To verify the quantitative results of the RNA-Seq experiments, a subset of eight genes was selected for analysis by qRT-PCR. The genes were selected based on their expression levels according to the RNA-Seq data and also on their important regulation patterns by heat stress. Among them, two genes were significantly down-regulated (*rpl31* EFZ03449, *rps3* EFZ01054), four genes were significantly up-regulated (*gebgalp* EFY98323, *pxdp* EFY98533, *hsp101* EFZ00938 and *tps* EFY96650), and two genes (*tublin* EFZ00524, *mdmp34* EFY98744) were unchanged by heat stress. The results shown in Fig. 4 confirm that the expression profiles of

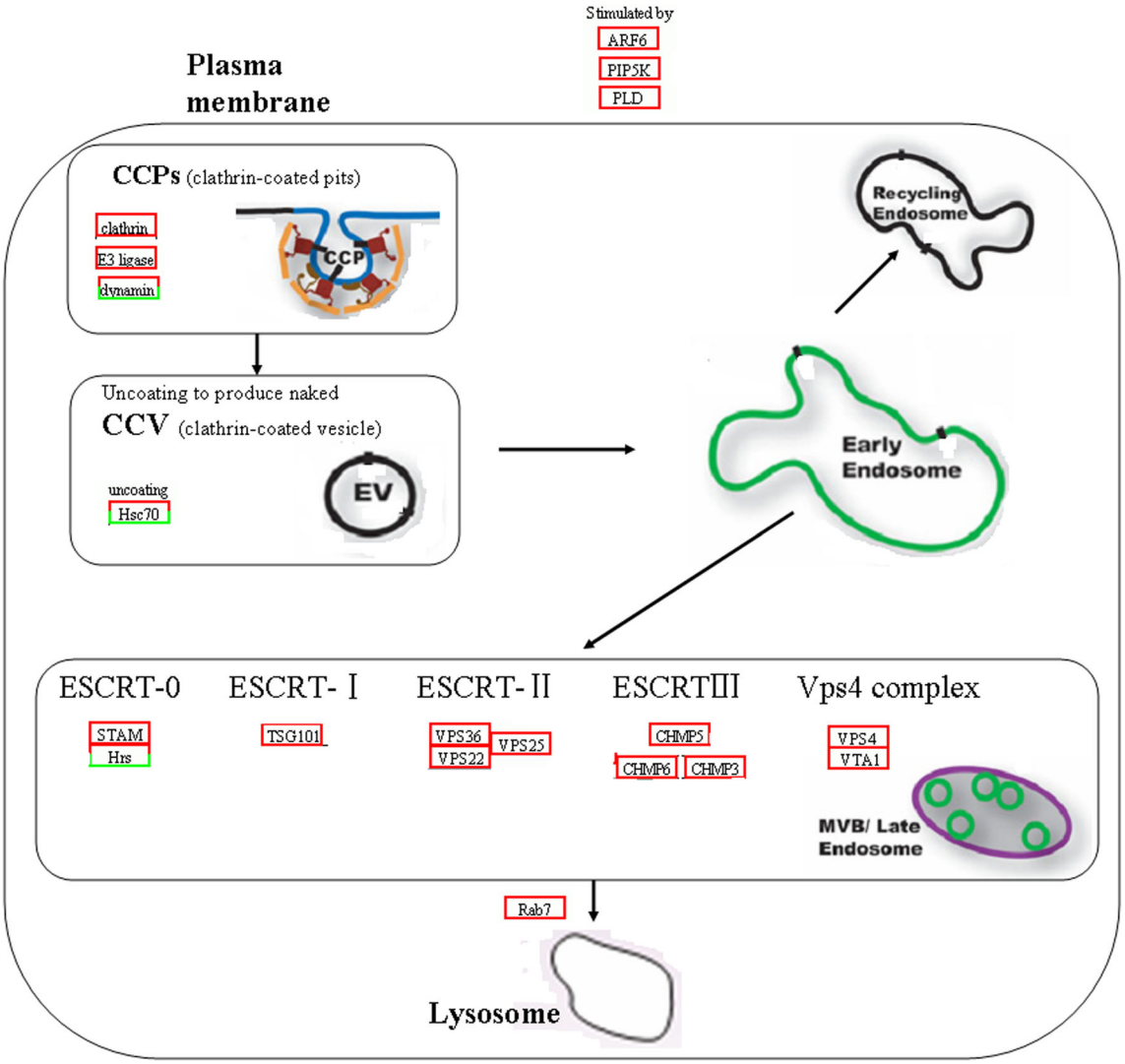

Fig. 3 Simplified pathways in clathrin-dependent endocytosis generated by KEGG enrichment analysis of DEGs. Up-regulated and down-regulated genes are shown in *red* and *green boxes*, respectively. Genes shown in *boxes lined with both red and green* are annotated by different transcripts, which may be either up-regulated or down-regulated

genes from control and heat treated samples were similar as determined by RNA-Seq transcriptomic analysis or by qRT-PCR, supporting the validity of our transcriptomics results.

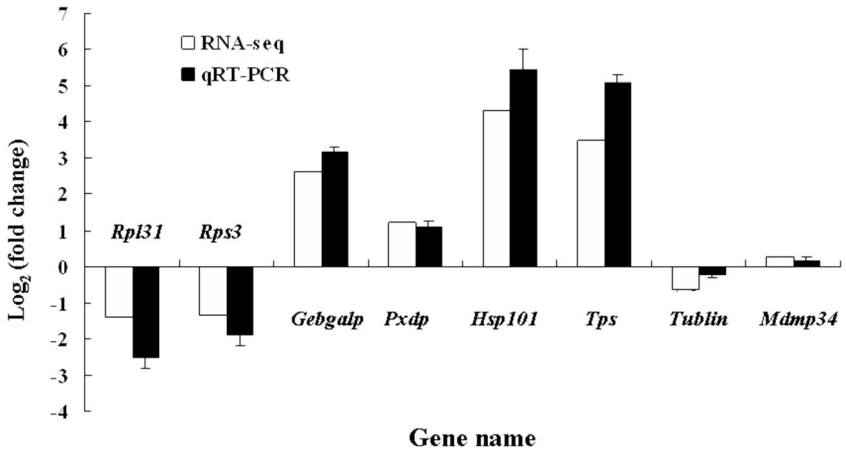

Fig. 4 qRT-PCR validation of DEGs identified in RNA-Seq analysis. The qRT-PCR data represents the mean ± SE (standard error) of three biological replicates. All primers and gene abbreviations are listed in Table S1

Discussion

Previous studies have reported that heat stress commonly represses general protein synthesis, in part, by down-regulating genes encoding ribosomal proteins (Liang et al. 2013; Cherkasov et al. 2013). In the present study, our pathway enrichment analysis for DEGs showed that the ribosome pathway was the most significantly enriched pathway. As expected, most DEGs (72/84) associated with the ribosome pathway were significantly down-regulated in heat treated conidia, possibly suggesting an overall slowdown of protein biosynthesis and decreased metabolism in heat treated conidia. These findings suggest that the modification of primary metabolism, including decreased protein biosynthesis, may be a strategy for heat stress management in *M. anisopliae* conidia.

Endocytosis is another highly represented pathway, in which 81.8 % (36/44) of associated genes were significantly up-regulated by heat stress. Endocytosis is a process by which cells absorb molecules (such as proteins) by engulfing them (Marsh and McMahon 1999). Endocytic pathways can be subdivided into two major categories: clathrin-dependent and clathrin-independent endocytosis (Miaczynska and Stenmark 2008). Clathrin-mediated endocytosis, which was the first described endocytic pathway, is well characterized and has been identified as evolutionarily conserved from humans to fungi (McMahon and Boucrot 2011). As expected, we found that *clathrin*, a key gene in clathrin-dependent endocytosis (McMahon and Boucrot 2011), was significantly up-regulated by heat stress. The principal components of the endocytic pathway are early endosomes, late endosomes and lysosomes (Miaczynska and Stenmark 2008). Late endosomes are also known as multivesicular bodies (MVBs). Biogenesis of MVBs is a process in which ubiquitin tagged proteins enter organelles called endosomes via the formation of vesicles. This process is essential for cells to destroy misfolded and damaged proteins (Miaczynska and Stenmark 2008). The endosomal sorting complex required for transport (ESCRT) plays a vital role in the MVB biogenesis (Miaczynska and Stenmark 2008). In our study, a series of genes involved in the ESCRT pathway, including ESCRT-0 (*stam*), ESCRT-I (*tsg101*), ESCRT-II (*vps22*, *vps25* and *vps36*), ESCRT-III (*chmp3*, *chmp5* and *chmp6*), and vps4 complex (*vps4* and *vta1*), were found to be up-regulated by heat stress. Additionally, *rab7* (EFZ04415), a late endosome marker (Miaczynska and Stenmark 2008), was also found to be up-regulated by heat stress. These results suggest that the endocytic processes involved in destroying misfolded and damaged proteins are significantly activated.

Autophagy is a highly conserved process that maintains intracellular homeostasis by degrading damaged proteins or organelles through the actions of lysosomes or vacuoles in all eukaryotes (Voigt and Pöggeler 2013). In the present study, certain genes involved in the pathway associated with regulation of autophagy, including a series of critical autophagy-related genes, were strongly up-regulated by heat stress (Table S5). Our results suggest a role for autophagy in the heat stress response, which is consistent with autophagy playing an important role in the mammalian stress response, where it can serve as an alternative degradation pathway in the ubiquitin–proteasome system (Ryter and Choi 2013). While no previous studies have examined the role of autophagy in the heat stress response in filamentous fungi (Pollack et al. 2009), a recent report showed a link between autophagy and fungal survival, development and virulence (Duan et al. 2013; Nitsche et al. 2013).

The third group of metabolic pathways involved in the response to heat stress is the proteasome pathway, of which all 28 DEGs involved in the pathway were significantly up-regulated. The primary function of the proteasome is to degrade proteins (Adams 2003). For a protein to be recognized and ultimately degraded by the proteasome, a small ubiquitin molecule must first be attached to the protein substrate (Shang and Taylor 2011). The selective degradation of various forms of damaged proteins by the ubiquitin–proteasome pathway is an important protein quality control mechanism (Shang and Taylor 2011). The proteasome, specifically the 26S proteasome, is a multiprotein complex composed of a 20S catalytic core and a 19S regulatory particle (Shang and Taylor 2011). Among the DEGs in the proteasome pathway, our results show that the majority of heat induced genes encoding components of the 20S catalytic core and 19S regulatory particle were up-regulated (Fig. S2 and Table S5). For example, 12 of the 14 different genes that encode components of the 20S core particle (Adams 2003), including all α1–7 and β subunits with the exception of β1 and β4, were found to be up-regulated. In the 19S regulatory particle, 13 of the 19 different genes were up-regulated, including four genes encoding distinct AAA-family ATPases (*Rpt1* EFY97316 and EFZ00381, *Rpt2* EFZ01529 and EFY99802, *Rpt4* EFZ00101 and *Rpt5* EFZ03187) and nine genes encoding Rpn proteins (*Rpn2* EFY97637, *Rpn3* EFZ03471, *Rpn5* EFZ04494, *Rpn6* EFY96798, *Rpn7* EFZ02660, *Rpn9* EFY99530, *Rpn10* EFZ00164, *Rpn13* EFZ00282 and *Rpn15* EFY95850). In addition to the up-regulation of several genes encoding the ubiquitin-conjugating enzyme, ubiquitin–protein ligase, and ubiquitin-activating enzyme (Table S3), the ubiquitin–proteasome pathway is significantly induced by heat stress, suggesting the degradation and modification of misfolded or unfolded proteins occurs in conditions of heat stress.

As expected, multiple genes encoding heat shock proteins and those involved in trehalose accumulation were significantly up-regulated, which could provide cellular protection through protein chaperones and trehalose (Al-Bader et al. 2010; Parsell and Lindquist 1993). Heat stress, however, can cause irreversible misfolding or damaged proteins that

molecular chaperones cannot remedy (Parsell and Lindquist 1993). Such unwanted proteins need to be removed through proteolysis (Kultz 2005). The majority of protein degradation is achieved via two pathways: the lysosomal pathway and the ubiquitin–proteasome pathway (Shang and Taylor 2011). Recent studies indicate that the ubiquitin–proteasome pathway and lysosomal degradation pathway work closely in a coordinated manner (Korolchuk et al. 2009). The majority of membrane-bound or organelle-associated proteins are degraded via the lysosomal pathway, either through endocytosis or autophagy mechanisms, whereas the ubiquitin–proteasome pathway is the primary cytosolic proteolytic machinery for the selective degradation of various forms of damaged proteins (Shang and Taylor 2011). In the present study, the ribosome, endocytosis and proteasome pathways were the first, second and third most active metabolic pathways involved in the response to heat stress, respectively. We speculate that there may be two strategies utilized by *M. anisopliae* conidia for heat stress tolerance: the first strategy is to down-regulate genes encoding the ribosomal protein to reduce general protein biosynthesis, and the second strategy is to up-regulate specific stress-responsive genes.

In conclusion, our study provides a comprehensive investigation of the gene expression profiles in heat treated *M. anisopliae* conidia compared with a control. Transcriptomic analysis has identified many heat regulated genes that are key components of crucial biological processes and pathways such as metabolic processes, the ribosome, proteasome, and endocytosis pathways, and autophagy. The DEGs and pathways identified in this study could facilitate further investigations into the detailed molecular mechanisms and provide a foundation for future studies of the response to heat stress in *M. anisopliae* conidia. Furthermore, manipulation of these genes may be a new tool for strain improvement of thermotolerance in *M. anisopliae*.

Acknowledgments This work was supported by the Special Fund for Forestry Scientific Research in the Public Interest (Grant No. 201204506), the National Natural Science Foundation of China (Grant Nos. 31201568 and 31272096) and the Key Project for Natural Science Research of Anhui Provincial Higher School (Grant No. KJ2014A012).

References

Adams J (2003) The proteasome: structure, function, and role in the cell. Cancer Treat Rev 29:3–9

Al-Bader N, Vanier G, Liu H, Gravelat FN, Urb M, Hoareau CMQ, Campoli P, Chabot J, Filler SG, Sheppard DC (2010) Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. Infect Immun 78:3007–3018

Albrecht D, Guthke R, Brakhage AA, Kniemeyer O (2010) Integrative analysis of the heat shock response in *Aspergillus fumigatus*. BMC Genomics 11:32

Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7:986–995

Barros BHR, Silva SH, Marques EDR, Rosa JC, Yatsuda AP, Roberts DW, Braga GUL (2010) A proteomic approach to identifying proteins differentially expressed in conidia and mycelium of the entomopathogenic fungus *Metarhizium acridum*. Fungal Biol 114: 572–579

Cherkasov V, Hofmann S, Druffel-Augustin S, Mogk A, Tyedmers J, Stoecklin G, Bukau B (2013) Coordination of translational control and protein homeostasis during severe heat stress. Curr Biol 23: 2452–2462

Doehlemann G, Berndt P, Hahn M (2006) Trehalose metabolism is important for heat stress tolerance and spore germination of *Botrytis cinerea*. Microbiol SGM 152:2625–2634

Duan Z, Chen Y, Huang W, Shang Y, Chen P, Wang C (2013) Linkage of autophagy to fungal development, lipid storage and virulence in *Metarhizium robertsii*. Autophagy 9:538–549

Fang W, Bidochka MJ (2006) Expression of genes involved in germination, conidiogenesis and pathogenesis in *Metarhizium anisopliae* using quantitative real-time RT-PCR. Mycol Res 110:1165–1171

Fernandes EKK, Bittencourt VREP, Roberts DW (2012) Perspectives on the potential of entomopathogenic fungi in biological control of ticks. Exp Parasitol 130:300–305

Frazzon APG, Vaz ID, Masuda A, Schrank A, Vainstein MH (2000) *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet Parasitol 94:117–125

Gao QA, Jin K, Ying SH, Zhang YJ, Xiao GH, Shang YF, Duan ZB, Hu XA, Xie XQ, Zhou G, Peng GX, Luo ZB, Huang W, Wang B, Fang WG, Wang SB, Zhong Y, Ma LJ, St Leger RJ, Zhao GP, Pei Y, Feng MG, Xia YX, Wang CS (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7: e1001264

Gokce E, Franck WL, Oh Y, Dean RA, Muddiman DC (2012) In-depth analysis of the *Magnaporthe oryzae* conidial proteome. J Proteome Res 11:5827–5835

Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y (2008) KEGG for linking genomes to life and the environment. Nucleic Acids Res 36:D480–D484

Kim JS, Kassa A, Skinner M, Hata T, Parker BL (2011) Production of thermotolerant entomopathogenic fungal conidia on millet grain. J Ind Microbiol Biotechnol 38:697–704

Korolchuk VI, Menzies FM, Rubinsztein DC (2009) A novel link between autophagy and the ubiquitin–proteasome system. Autophagy 5:862–863

Kultz D (2005) Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol 67:225–257

Lamarre C, Sokol S, Debeaupuis JP, Henry C, Lacroix C, Glaser P, Coppée JY, Francois JM, Latgé JP (2008) Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. BMC Genomics 9:417

Leng Y, Peng G, Cao Y, Xia Y (2011) Genetically altering the expression of neutral trehalase gene affects conidiospore thermotolerance of the entomopathogenic fungus *Metarhizium acridum*. BMC Microbiol 11:32

Li R, Yu C, Li Y, Lam TW, Yiu SM, Kristiansen K, Wang J (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25:1966–1967

Li Z, Zhang Z, Yan P, Huang S, Fei Z, Lin K (2011) RNA-Seq improves annotation of protein-coding genes in the cucumber genome. BMC Genomics 12:540

Liang WD, Bi YT, Wang HY, Dong S, Li KS, Li JS (2013) Gene expression profiling of *Clostridium botulinum* under heat shock stress. Biomed Res Int:760904

Liao X, Lu HL, Fang W, St Leger RJ (2013) Overexpression of a *Metarhizium robertsii* HSP25 gene increases thermotolerance and survival in soil. Appl Microbiol Biotechnol 98:777–783

Lin JQ, Zhao XX, Zhi QQ, Zhao M, He ZM (2013) Transcriptomic profiling of *Aspergillus flavus* in response to 5-azacytidine. Fungal Genet Biol 56:78–86

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25:402–408

Lord JC (2005) From Metchnikoff to Monsanto and beyond: the path of microbial control. J Invertebr Pathol 89:19–29

Marsh M, McMahon HT (1999) The structural era of endocytosis. Science 285:215–220

McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12:517–533

Miaczynska M, Stenmark H (2008) Mechanisms and functions of endocytosis. J Cell Biol 180:7–11

Montero-Barrientos M, Cardoza RE, Gutierrez S, Monte E, Hermosa R (2007) The heterologous overexpression of *hsp23*, a small heat-shock protein gene from *Trichoderma virens*, confers thermotolerance to *T. harzianum*. Curr Genet 52:45–53

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5:621–628

Nitsche BM, Burggraaf-van Welzen AM, Lamers G, Meyer V, Ram AFJ (2013) Autophagy promotes survival in aging submerged cultures of the filamentous fungus *Aspergillus niger*. Appl Microbiol Biotechnol 97:8205–8218

Nookaew I, Papini M, Pornputtapong N, Scalcinati G, Fagerberg L, Uhlen M, Nielsen J (2012) A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: a case study in *Saccharomyces cerevisiae*. Nucleic Acids Res 40:10084–10097

Oshlack A, Robinson MD, Young MD (2010) From RNA-Seq reads to differential expression results. Genome Biol 11:220

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496

Plesofsky-Vig N, Brambl R (1985) Heat shock response of *Neurospora crassa*: protein synthesis and induced thermotolerance. J Bacteriol 162:1083–1091

Pollack JK, Harris SD, Marten MR (2009) Autophagy in filamentous fungi. Fungal Genet Biol 46:1–8

Rangel DEN, Butler MJ, Torabinejad J, Anderson AJ, Braga GUL, Day AW, Roberts DW (2006) Mutants and isolates of *Metarhizium anisopliae* are diverse in their relationships between conidial pigmentation and stress tolerance. J Invertebr Pathol 93:170–182

Rangel DEN, Alston DG, Roberts DW (2008) Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. Mycol Res 112:1355–1361

Ries L, Pullan ST, Delmas S, Malla S, Blythe MJ, Archer DB (2013) Genome-wide transcriptional response of *Trichoderma reesei* to lignocellulose using RNA sequencing and comparison with *Aspergillus niger*. BMC Genomics 14:541

Rokas A, Gibbons JG, Zhou X, Beauvais A, Latgé JP (2012) The diverse applications of RNA-Seq for functional genomic studies in *Aspergillus fumigatus*. Ann N Y Acad Sci 1273:25–34

Ruoff P, Vinsjevik M, Mohsenzadeh S, Rensing L (1999) The Goodwin model: simulating the effect of cycloheximide and heat shock on the sporulation rhythm of *Neurospora crassa*. J Theor Biol 196: 483–494

Ryter SW, Choi AM (2013) Autophagy: an integral component of the mammalian stress response. J Biochem Pharmacol Res 1:176–188

Shang F, Taylor A (2011) Ubiquitin–proteasome pathway and cellular responses to oxidative stress. Free Radic Biol Med 51:5–16

Soanes DM, Chakrabarti A, Paszkiewicz KH, Dawe AL, Talbot NJ (2012) Genome-wide transcriptional profiling of appressorium development by the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog 8:e1003604

St Leger RJ, Wang C (2010) Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests. Appl Microbiol Biotechnol 85:901–907

Su Y, Guo Q, Tu J, Li X, Meng L, Cao L, Dong D, Qiu J, Guan X (2013) Proteins differentially expressed in conidia and mycelia of the entomopathogenic fungus *Metarhizium anisopliae* sensu stricto. Can J Microbiol 59:443–448

Voigt O, Pöggeler S (2013) Self-eating to grow and kill: autophagy in filamentous ascomycetes. Appl Microbiol Biotechnol 97: 9277–9290

Walstad JD, Anderson RF, Stambaugh WJ (1970) Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarhizium anisopliae*). J Invertebr Pathol 16: 221–226

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63

Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, Wang J, Li S, Li R, Bolund L, Wang J (2006) WEGO: a web tool for plotting GO annotations. Nucleic Acids Res 34:W293–W297

Ying SH, Feng MG (2011) A conidial protein (CP15) of *Beauveria bassiana* contributes to the conidial tolerance of the entomopathogenic fungus to thermal and oxidative stresses. Appl Microbiol Biotechnol 90:1711–1720

Zhang S, Xia Y, Keyhani NO (2011) Contribution of the *gas1* gene of the entomopathogenic fungus *Beauveria bassiana*, encoding a putative glycosylphosphatidylinositol-anchored β-1,3-glucanosyltransferase, to conidial thermotolerance and virulence. Appl Environ Microbiol 77:2676–2684