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Aspergillus fumigatus transcriptome response to a higher temperature during the earliest steps of germination monitored using a new customized expression microarray

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***Aspergillus fumigatus* transcriptome response to a higher temperature during the earliest steps of germination monitored using a new customized expression microarray**

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Abstract

Aspergillus fumigatus is considered to be the most prevalent airborne pathogenic fungus and can cause invasive diseases in immunocompromised patients. It is known that its virulence is multifactorial although the mechanisms of pathogenicity remain unclear. With the aim of improving our understanding of these mechanisms, we have designed a new expression microarray covering the entire genome of *A. fumigatus*. In this first study, we have analyzed the transcriptomes of this fungus at the first steps of germination after being grown at 24 and 37°C. The microarray data revealed that 1,249 genes were differentially expressed with growth at these two temperatures. According to our results, *A. fumigatus* modifies significantly the expression of genes related to metabolism to adapt to new conditions. The high percentages of genes that encode hypothetical or unclassified proteins differentially expressed imply that many as yet unknown genes are involved in the establishment of *A. fumigatus* infection. Furthermore, among the genes implicated in virulence up-regulated at 37°C on the microarray, we found those that encoded proteins mainly related to allergens (Asp F1, Asp F2 and MnSOD), gliotoxin biosynthesis (GliP and GliZ), nitrogen (NiiA and NiaD) or iron (HapX, SreA, SidD and SidC) metabolism. However, the gene expression in iron and nitrogen metabolisms might be influenced not only by the heat shock but also by the availability of nutrients in the medium, as shown with the addition of fresh medium.

Introduction

Aspergillus fumigatus is a saprophytic mold and is considered to be the most prevalent airborne pathogenic fungus. This is particularly due to the fact that it spreads widely and easily disperses thanks to its conidia, which are extremely well-suited for air dispersal. Their small size (2-3 μm), hydrophobicity, thermotolerance and melanin content allow them to withstand a wide range of temperatures and to remain airborne for long periods (O’Gorman, 2011). Furthermore, their small size allows them to overcome the defense mechanisms of the nasal cavity and other structures of the upper respiratory tract to reach the lung alveoli. On average, it is estimated that a person may inhale up to 200 conidia per day (Latgé, 1999).

Despite this high exposure of humans to conidia, they are not responsible for illnesses in immunocompetent hosts, as conidia levels are controlled by immune mechanisms. However, in immunocompromised individuals conidia can overcome immune defense mechanisms resulting in their establishment in the lung (Latgé, 1999). Once there, the conidia germinate, and subsequently, start to colonize or even invade the surrounding tissue (Dagenais & Keller, 2009) causing invasive aspergillosis (IA) with mortality rates of 70-90%. These high mortality rates are mainly due to the weakened immune system of the patients, the virulence of the fungus and late diagnosis (Del Palacio *et al.*, 2003).

A crucial event in the infectious process of this pathogen is conidial germination, given that at this stage conidia have to adapt to the host environment and to overcome the immune system as well as to germinate. It is known that the first step in conidial germination consists of breaking the spore dormancy followed by isotropic swelling, establishment of cell polarity, formation of a germ tube and maintenance of polar growth (Barhoom & Sharon, 2004; Harris & Momany, 2004). However, despite the knowledge about the different stages of germination, little is known about the molecular aspects of the processes taking place during this period.

To date, several studies have demonstrated that the pathogenesis of this fungal species is multifactorial due to a combination of its biological characteristics and the immune status of patients (Beauvais & Latgé, 2001; Tekaiia & Latgé, 2005; Osherov, 2007; Abad *et al.* 2010). Furthermore, it is generally assumed that some physiological characteristics of *A. fumigatus* allow it to be an efficient opportunist pathogen, with thermotolerance playing an important role (Rementeria *et al.*, 2005). *A. fumigatus* has developed mechanisms that allow it to grow at 37°C and to tolerate temperatures of over 60°C, which might contribute to the virulence of this pathogen.

The availability of the whole genome sequence of *A. fumigatus* since 2005 has enabled the design of microarrays, and these have been used to analyze the transcriptome of the fungus under different growth conditions. With the aim of improving our understanding of the adaptation of *A. fumigatus*

to host environments and its mechanisms of pathogenicity, we have designed and validated a new specific expression microarray (AWAFUGE, Agilent Whole *A. fumigatus* Genome Expression 44K v.1), which could provide useful information about the transcriptome of this pathogen. As germination is a crucial event in the development of the infection, in this study, we focused on the effect of host temperature during the first steps of germination. Specifically, after being cultured at 24 or 37°C, the transcriptomes of the conidia were compared to identify functional and gene expression changes potentially linked to virulence in order to shed light on the mechanisms of adaptation and virulence of this fungus during this early stage of infection. We also studied whether the expression changes detected were only due to the temperature of incubation, analyzing the expression of selected genes after the addition of fresh medium when the isotropic swelling had taken place.

Materials and Methods

Aspergillus fumigatus strain, media and growth conditions

The *A. fumigatus* strain Af-293 was used in this study and grown on potato dextrose agar (Cultimed, Castellar de Vallés, Spain) at 28°C for 7 days. Conidia were harvested and washed twice with saline-Tween solution (0.9% NaCl, 0.02% Tween 20). The concentration of conidia was determined using an hemocytometer and was adjusted to 10^7 conidia/ml. Further, the viability was evaluated in terms of the percentage of conidia that were able to grow on Sabouraud glucose agar (Cultimed) considering colony forming units per ml (CFU/ml) after culture at 37°C for 24 hours, assessed by recounting the conidia with the hemocytometer.

The germination rate was determined on the basis of three independent assays. *A. fumigatus* conidia at a concentration of 10^7 conidia/ml were grown in 50 ml of RPMI 1640 medium (Sigma Aldrich, Madrid, Spain) and samples were incubated at 24 or 37°C, both with shaking at 120 rpm in a continuous incubation. In addition, to study the effect of nutrient consumption of the medium, cultures incubated for 16 h at 24°C were centrifuged for 5 minutes at 2,500 rpm and then, fresh medium, preheated at 24 or 37°C, was added and incubated at 24°C and 37°C, respectively. Once inoculated, aliquots of 500 µl of each sample were fixed with 50 µl of fixing solution (10% formaldehyde/10% SDS) and stained with calcofluor white (Becton Dickinson and Company, Madrid, Spain) at different times. Cultures with 15-30% and 40-60% germination rates were centrifuged for 5 minutes at 2,500 rpm and washed twice with saline-Tween solution. The final pellet of these samples was weighed, placed in 10 µl/mg of RNeasy Lysis Buffer (Qiagen, Valencia, CA, USA) and stored at -20°C until RNA extraction. Cultures from the continuous incubation with 15-30% germination rates were used in the transcriptomic studies. To assess whether the medium retained the same characteristics despite the different lengths of culture used for the two temperatures, the pH of the medium was measured at starting and harvesting time.

RNA isolation

Three biological replicates of germinated conidia from each condition were used for RNA extraction. Samples of 100 mg (wet weight) were ground three times using an agate mortar in the presence of liquid nitrogen. Afterwards, total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen). Samples were treated with DNase I amplification grade (Sigma Aldrich) and RNA concentration and quality were measured with a NanoPhotometer (Implen, Schatzbogen, Germany). Then, RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) by the Advanced Research Facilities Service (SGIker) at the University of the Basque Country (UPV/EHU). The 2100 Bioanalyzer generated an RNA integrity number (RIN)

that indicated the suitability of RNA samples for gene expression analysis. Finally, extracted RNA was stored at -80°C until use.

Expression microarray design

The Agilent Whole *A. fumigatus* Genome Expression 44K (AWAFUGE) microarray v.1 was designed using the eArray system (Custom Microarray Design, Agilent Technologies: <http://earray.chem.agilent.com/erray/>). The AWAFUGE microarray (v. 1) includes 28,890 probes to monitor the expression of the 9,630 *A. fumigatus* genes (three 60-bp probes per gene). In addition, 62 quality control genes were incorporated, also with 3 targets for each gene and each repeated 5 times on the microarray, to correct the variations caused by biological and technical factors. Of these control genes, 52 were chosen from the 9,630 genes of *A. fumigatus*, 5 from *Mus musculus* and the other 5 from *Homo sapiens* (Table S1). Lastly, the eArray system automatically included positive and negative controls, selected by Agilent for use in commercial microarrays, resulting in a final microarray of 45,220 probes. The format adopted was four microarrays per glass slide (4x44K).

Transcriptome profiling studies

The transcriptional profiling of this study was carried out by Tecnia Research & Innovation (Biscay, Spain). RNA samples obtained from the continuous cultures with 15-30% germination rates were used in the transcriptomic studies. The profiling was based on three independent RNA samples obtained from each of the conditions, 24 and 37°C, and on two technical replicates. Total RNA (1 µg) from each sample was amplified and labeled with Cy3-fluorescent dye using an Amino Allyl MessageAmpTM II aRNA Amplification Kit (Ambion, Austin, TX, USA) and hybridized to individual arrays (one sample per array) in accordance with the manufacturer's recommendations (Agilent Technologies). After washing, microarray slides were scanned using a Gene Pix 4100A scanner (Axon Instruments, Union City, CA, USA) and image analysis was performed using associated software, GenePiX Pro 6.0 (Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription quantitative PCR (RT-qPCR)

In order to validate the results obtained using the microarray, three independent RNA samples from growth in each of the two conditions, different from those used in microarray analysis, were subjected to RT-qPCR by the SGIker at the UPV/EHU. Twenty of the most up-regulated genes as well as 8 genes with significant differences that have been related to virulence were selected for the validation. In addition to these, two other genes, actin 1 (*act1*) and the glyceraldehyde 3-phosphate dehydrogenase (*gpdA*), were included as housekeeping genes. Specific primer pairs were designed using Primer 3 (version 0.4.0: <http://bioinfo.ut.ee/primer3-0.4.0/>) software to amplify each gene (Table 1).

The cDNA was generated from 1 µg of total RNA using an AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) in a total volume of 20 µl. Afterwards, 10 µl of each sample were set up using Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies) in which 300 nM of ROX, 10 ng of cDNA and 500 nM of primers were added per reaction.

RT-qPCR experiments were performed in 96-well plates in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR amplification conditions were 3 minutes at 95°C followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The specificity of the primer pairs was verified by melting curve analysis following the last amplification cycle. No-template controls and minus-reverse transcriptase controls (samples in which no *reverse-transcriptase* was added) were also included.

This analysis was also carried out using BioMark HD Nanofluidic qPCR System technology (Fluidigm, San Francisco, CA, USA) combined with GE 48.48 Dynamic Arrays IFC (Fluidigm) in samples in which fresh medium was added. Briefly, the cDNA was preamplified using the QIAGEN Multiplex PCR Kit (Qiagen) and 50 nM of primers. The PCR amplification conditions were 15 minutes at 95°C followed by 14 cycles of 95°C for 15 sec and 60°C for 4 minutes, and then treated with Exonuclease I (New England Biolabs, Hertfordshire, UK). Gene expression analysis of the preamplified amplicons was performed with 48.48 Dynamic Array IFC using the Master Mix SsoFastTM Evagreen[®] Supermix with Low Rox (Bio-Rad, Madrid, Spain) in which 2.7 µl of preamplified cDNA and 500 nM of primers were added. The PCR amplification conditions consisted on 40 minutes at 70°C, 30 sec at 60°C, and 1 minute at 95°C followed by 35 cycles of 5 sec at 96°C and 20 sec at 60°C. After the final amplification cycle, a melting curve analysis was carried out. No-template controls and minus-reverse transcriptase controls were also included in these assays.

Data analysis

Gene expression data obtained after microarray hybridizations were analyzed using the GeneSpring GX software (version 12; Agilent Technologies) and the Babelomics server (<http://www.babelomics.org>) (Medina *et al.*, 2010). The normalization procedure was carried out using the GeneSpring GX software and specific recommendations for one-color hybridizations. Basically, after subtracting the background intensity and log₂ data transformation, a global normalization (75th percentile shift normalization) was performed followed by a baseline transformation to the median of all samples. Each gene was considered to be positively expressed if in the three independent samples all the specific probes designed for it were hybridized. Afterwards, levels of expression were compared using the Student's t-test, to identify genes differentially expressed in *A. fumigatus* conidia cultured in the two conditions studied, namely 24 and 37°C. Multiple testing with Benjamini-Hochberg correction was applied in order to control for

the occurrence of false positives. A gene was considered to be differentially expressed if $p < 0.05$. Results were expressed as $\log_2$ fold-change, representing the $\log_2$ at 24°C minus the $\log_2$ at 37°C. Differentially expressed genes were classified into functional groups using FungiFun (Priebe *et al.*, 2011; Priebe *et al.*, 2014). The enrichment p-value of each category was performed establishing FunCat method, as the classification ontology, and Fisher's test, and Benjamini-Hochberg adjustment method as the parameters to carry out the procedure. A category was considered significant if $p < 0.05$.

The analysis of RT-qPCR results was carried out using the SDS 2.4 software (Applied Biosystems) or Analysis Software v. 3.1.3 (Fluidigm). In both cases, the data were normalized with results for the *act1* and the *gpdA* housekeeping genes using GenEx (version 5.4; MultiD, Gothenburg, Sweden). Then, relative quantification of gene expression was performed by the $\Delta\Delta C_t$ method, and the significance of differences was assessed using the Student's t-test ($p < 0.05$). Finally, fold changes were standardized by $\log_2$ transformation, and compared to microarray data using Pearson's correlation. The $\log_2$ fold change thresholds were set over 1 and under -1, meaning an increase of the expression in more than twice at 24°C and an increase in more than twice at 37°C, respectively.

Microarray accession number

The AWAFFUGE microarray (v.1) design was submitted to the ArrayExpress database at the European Bioinformatics Institute (EBI, <http://www.ebi.ac.uk/miamexpress/>) under accession number A-MEXP-2352. The *A. fumigatus* gene expression data obtained following the hybridization with the microarray have also been deposited in the ArrayExpress under accession number E-MTAB-1910.

Results

Viability and germination of conidia

The viability of *A. fumigatus* Af-293 conidia was $75.82 \pm 14.50\%$. To study the early expression of this fungus, a germination rate of 15-30% was selected given the size of the germinated conidia (Fig. 1a). This was faster at 37 than at 24°C. Specifically, in cultures at 37°C, cells reached these germination rates after 6.5 hours of incubation, whereas at 24°C these rates were achieved after 18 hours of incubation (Fig. 1b). Measurements showed no differences in pH between starting (pH=7.2) and harvesting times in either of the cultures (pH=7.1±0.11). Hence, for the microarray analysis and its validation by RT-qPCR, germinated cells were obtained after different lengths of incubation at 37 and 24°C (namely, 6.5 and 18 h respectively). In addition, to study the effect of the nutrient source depletion, this expression analysis was also performed following the addition of fresh medium. In this case, cultures after 16 hours of incubation at 24°C, which showed swelling and 1-4% germination rates, were used. After the addition of fresh medium, samples with 15-30% and 40-60% germination rates were harvested, which were reached after 1.5 and 3 hours of incubation at 37°C and 3 and 6 hours at 24°C (Fig 1). The total RNA from each condition was obtained, transcribed into cDNA and used in hybridization or RT-qPCR studies, as indicated in each case.

Gene expression in response to temperature

Three samples from the growth of *A. fumigatus* at each temperature were hybridized with the microarray. The data obtained in these transcriptomic studies were deposited in the ArrayExpress through the MIAMEExpress tool (accession number E-MTAB-1910). Following hybridizations, normalized data were graphically represented (Fig. 2a) showing similar expression at the two temperatures. On the other hand, the statistical comparisons showed that 1,249 genes were differentially expressed (Fig. 2b). Negative or positive log₂ fold changes meant up-regulation at 37 or 24°C respectively. Overall, 591 genes were up-regulated at 37°C and 658 at 24°C (Table S2). Among them 385 were unclassified proteins and 326 hypothetical proteins. According to the functional classification and the enrichment p-values of each functional group, 8 categories were significantly enriched at 37°C, being C-compound and carbohydrate metabolism, secondary metabolism, lipid, fatty acid and isoprenoid metabolism, non-vesicular cellular import, NAD/NADP binding, non-ribosomal peptide synthesis, fatty acid metabolism and degradation of leucine. At 24°C, 8 categories appeared to be enriched as well, although in this case the functional groups were rRNA processing, ribosome biogenesis, rRNA synthesis, RNA binding, rRNA modification, tRNA processing, tRNA modification and RNA degradation (Table 2).

We also analyzed the location of the significantly expressed genes along *A. fumigatus* chromosomes, but no specific distribution was observed. The ratio of up-regulated genes found at 24°C versus those found at 37°C was around 1 in each chromosome (data not shown).

In addition, several genes involved in metabolic pathways related to virulence appeared to be up-regulated at 37°C. These genes are involved with gliotoxin biosynthesis, nitrogen and iron metabolism and encoding allergens. Specifically, the up-regulated genes related to gliotoxin biosynthesis included those that encode GliP, GliJ, GliZ and a GliP-like proteins, while in relation to iron metabolism, there was up-regulation of the gene which encodes bZIP transcription factor HapX and genes related to siderophores biosynthesis and transporters (SidD, SidC, MirB and MirC). With regard to nitrogen metabolism, up-regulation was found in three genes which encode the nitrite reductase NiiA and two nitrate reductases, one of these being the nitrate reductase NiaD and the other one AFUA_5G10420. Finally, nine genes that encode allergens were up-regulated at 37°C. Among them, those that code for the Mn superoxide dismutase (MnSOD), the major allergen Asp F2 and the major allergen and cytotoxin Asp F1 were those which showed the highest up-regulation. In contrast, only two genes related to iron metabolism, those which code for the siderophore transcription factor SreA, and the homoaconitase LysF, and a cell wall glucanase/allergen F16-like protein, showed up-regulation at 24°C (Table 3).

Reverse transcription quantitative PCR (RT-qPCR) validation of microarray data

The 20 genes that showed the greatest differential expression in the microarrays, 10 of them up-regulated at 37°C and the other 10 at 24°C, as well as 8 genes related to virulence, whose expressions are shown in Table S2, were selected for RT-qPCR analysis to validate the results obtained with the microarray experiments. The specific primer pairs designed for each selected gene are listed in Table 1. In addition, *act1* and *gpdA* genes were used as housekeeping genes. RT-qPCR results were normalized.

Genes showing a negative $\log_2$ fold change was interpreted as meaning that they were up-regulated at 37°C, while a positive $\log_2$ fold change was taken to indicate an up-regulation at 24°C. RT-qPCR results were compared and correlated with the data obtained from the same genes on the microarray (Fig 3a). The Pearson's correlation coefficient between microarray and RT-qPCR data was high, with a value of 0.91 (Fig. 3b).

The expression of these genes was analyzed after the addition of fresh medium and compared with the results obtained in the validation of microarray data to determine whether the temperature was the only factor affecting. This analysis showed that 14 genes agreed with microarray, being the Pearson's correlation between these results 0.86. Only 4 genes appeared to have different expression pattern related to microarray analysis, and they were those that encode the NACHT

domain protein, the ATP/GTP-binding protein, the nitrite reductase NiiA and the nonribosomal peptide synthase SidD, and 10 genes had fold changes below the thresholds previously established (Table S3). In samples with 40-60% germination rates after the addition of fresh medium, 17 genes agreed with the expression found on the microarray. Nevertheless, 5 genes showed different expression, which encode for the Zn^{2+} dependent hydrolase, the ATP/GTP-binding protein, the FAD binding monooxygenase, the glycosyltransferase and the Mn superoxide dismutase MnSOD (Table S3).

Focusing on the genes related to virulence, 3 out of 9 genes agreed with microarray data in samples with 15-30% germination rates. Among the genes involved with gliotoxin biosynthesis only *gliZ* appeared to be up-regulated at 37°C in all cases. In iron metabolism, there was up-regulation of the nonribosomal peptide synthase SidD at 24°C in 15-30% germination rate samples, whereas in nitrogen metabolism, up-regulation at 37°C was found only in 40-60% germination samples. Finally, among the allergens analyzed, only AspF2 and MnSOD were up-regulated at 37°C in 15-30% germination rate samples (Fig. 4).

299 Discussion

300 The infection of *A. fumigatus* starts with conidia inhalation, which in immunocompromised
301 individuals, results in conidia establishment in the lung, germination and invasion of the
302 surrounding lung tissue. Taking the host environment into account, conidial germination and
303 growth requires the activation of nutrient sensing, nutrient uptake and acquisition, and biosynthetic
304 pathways, knowledge of which would be essential for understanding the pathogenesis of *A.*
305 *fumigatus* (Dagenais & Keller, 2009).

306 Due to the multifactorial virulence of *A. fumigatus*, apart from direct site mutagenesis, the best
307 ways to identify genes related to virulence appear to be transcriptomic and proteomic analysis. In
308 line with this, various different transcriptomic experiments, such as exposure to antifungal drugs
309 (Ferreira *et al.*, 2006), high temperatures (Nierman *et al.*, 2005), germination and hyphae
310 development (Sugui *et al.*, 2008) and even the infection of human cells (Oosthuizen *et al.*, 2011;
311 Morton *et al.*, 2011) have been carried out in an attempt to shed light on the pathogenesis of *A.*
312 *fumigatus*. However, the mechanisms of pathogenicity of *A. fumigatus* remain unclear. To improve
313 our understanding of the mechanisms that enable this fungus to cause infection, we designed an *A.*
314 *fumigatus* microarray using the information from all the mRNA sequences of available from the
315 NCBI database and the eArray system developed by Agilent Technology. The strain selected for
316 the design of the microarray as well as for this study was *A. fumigatus* Af-293 due to the fact that
317 this strain was isolated from a lung biopsy from a neutropenic patient with IA (Anderson &
318 Denning, 2001), it was the first strain of *A. fumigatus* whose genome was sequenced (Nierman *et*
319 *al.*, 2005) and because it has been used in both *in vitro* and *in vivo* experiments (Power *et al.*, 2006,
320 Gravelat *et al.*, 2008, Lamarre *et al.*, 2008, McDonagh *et al.*, 2008, Fraczek *et al.*, 2010, Morton *et*
321 *al.*, 2011, Farnell *et al.*, 2012).

322 Although aspergillosis can be attributed to a combination of the immune status of the patient and
323 the biological characteristics of the fungus, it is believed that some physiological characteristics of
324 *A. fumigatus* allow it to be an efficient opportunistic pathogen (Abad *et al.*, 2010). Among these
325 characteristics, its ability to survive high temperatures is one of the most cited, since it tolerates
326 temperatures higher than 50°C, and its optimal temperature is around 37°C (Krijgheld *et al.*,
327 2013). Hence, it is plausible that the mechanisms of thermotolerance involve the expression of
328 stress resistance genes that, at the same time, confer unique virulence properties to the fungus
329 (Bhabhra & Askew, 2005). Consistent with this, Araujo & Rodrigues (2004) demonstrated that
330 temperature plays a crucial role in the correlation between the germination rate and pathogenicity,
331 showing how germination is a crucial step in the development of aspergillosis, and concluding that
332 *A. fumigatus* is the most able to adapt to extreme changes in environmental conditions. To date,
333 several studies have analyzed the transcriptomic changes at different temperatures (Nierman *et al.*,

2005; Low *et al.*, 2011; Krishnan *et al.*, 2014), without considering the different growth rate reached in each temperature. Taking this into consideration, we decided to investigate the adaptive changes of *A. fumigatus* to host temperature, using the AWAFUGE microarray (v.1) to study the transcriptome at 24°C, representing the environmental temperature, and at 37°C, reflecting the host temperature. We focused on the first steps of the germination to identify those genes differentially expressed at both temperatures when 15-30% of conidia had germinated at both temperatures, owing to all germinated conidia were of a similar size, (Fig. 1a), and because using samples with the same germination rate might minimize the expression changes that are due to the growth rate. Specifically, cultures were incubated at 37°C for 6.5 hours and at 24°C for 18 hours (Fig. 1b). It should be underlined that no changes were detected in pH, indicating that this factor had no effect on gene expression changes.

Transcriptome profiling results showed that 1,249 genes were differently expressed (Fig. 2b and Table S2). To validate these results, RT-qPCR assays were carried out with the genes found to be most differentially up-regulated and those related to virulence which showed significant differences (Table 1). The RT-qPCR results confirmed the differential expression of the 28 selected genes with a high significant Pearson's correlation value of 0.91 despite using different samples in microarray and RT-qPCR assays (Fig. 3). Overall, these results underline the effectiveness and the reliability of the AWAFUGE microarray (v.1), making it suitable for use in further studies analyzing the virulence mechanisms or the response of *A. fumigatus* to environmental changes or during murine or human cell infections.

The 9,630 genes, the whole genome of *A. fumigatus*, have been classified into 18 functional groups according to the Functional Catalogue categorization. Building on this, we explored the significant functional categories of the subset of genes found to be differentially expressed. As shown in Table 2, the predominant functional groups at 37°C were those mainly involved in metabolism. In a previous study, comparing the transcriptome of *A. fumigatus* at 25 and 37°C, up-regulated genes involved in metabolism also appeared at 37°C (Krishnan *et al.*, 2014), which was explained based on the faster growth of the fungus at 37°C. However, in our study, both cultures at 24 and 37°C had the same germination rate, so the results showed that the metabolic adjustments not only depend on the faster growth of this fungus but also on the temperature in which the germination takes place. Moreover, the significant groups at 24°C, related to rRNA processing and synthesis, tRNA processing and modification, and RNA degradation, showed that RNA pathways are down-regulated at 37°C. On the other hand, it should be pointed out the high percentage of differentially expressed genes coded for hypothetical (26.10%) or unclassified proteins (30.82%). It is true that these genes mean a limitation of the conclusions of our study, although at the same time, they stress the importance that these unknown genes might have during the germination of this fungus and point to avenues for future research.

With the addition of fresh medium, we could conclude that the temperature was the main factor that determined the expression changes of the 50% of the genes (14/28 genes) analyzed in samples with 15-30% germination rates, with a high Pearson's correlation (0.86). Focusing on the 20 most expressed genes detected by microarray data, those genes that showed different expression patterns in this analysis encoded unclassified proteins, and with no statistical differences encoded proteins mainly related to secondary metabolism and C-compound and carbohydrate metabolism, (Table S4). As secondary metabolism is affected by several nutritional conditions, such as amino acid, nitrogen and iron sources (Bayram & Braus, 2012), it is understandable that the addition of fresh medium could incorporate the consumed nutrients, decreasing the need to stimulate different metabolisms. These results highlighted the importance that the isotropic swelling and the formation of a germ tube had since the beginning of the germination, altering the medium conditions even when the majority of the conidia had not germinated yet.

Several studies have been carried out during the last decade studying the germination of *Aspergillus* species (Araujo & Rodrigues, 2004; Lamarre *et al.*, 2008; Leeuwen *et al.*, 2013). However, the comparison of findings between studies is very difficult owing to the use of different fungal species or time intervals. On the other hand, in previous studies, some metabolic pathways have been found to be related to virulence namely those for gliotoxin (Scharf *et al.*, 2012; Bok *et al.*, 2006), iron metabolism (Schrettl *et al.*, 2007; Schrettl *et al.*, 2010; Haas, 2012) and allergens (Chaudhary & Marr, 2011; Low *et al.*, 2011). Analyzing these findings together with our results, we found significant differences in expression for some genes involved in these pathways, and the results were validated by RT-qPCR assays (Fig. 3a).

Our results showed that some genes involved in gliotoxin biosynthesis were up-regulated at 37°C (Table 3), such as *gliZ*, a transcription factor which regulates gliotoxin biosynthesis, and *gliP*, a non-ribosomal peptide synthetase which catalyses the first step of gliotoxin biosynthesis. Furthermore, the analysis following the addition of fresh medium showed a clear up-regulation at 37°C of the gene that encodes GliZ, demonstrating that the expression of this gene is directly influenced by the heat shock. However, the expression pattern of the gene that encodes GliP indicated that the heat shock did not directly stimulate the up-regulation of this gene (Fig. 4ab). According to the literature, *gliZ* is a positive regulator of *gliP* and its over-regulation enhances the production of gliotoxin (Bok, *et al.*, 2006). Nevertheless, Dhingra *et al.* (2012) showed different expression patterns among *gliZ* and *gliP* when *veA*, a major fungal regulatory gene, was deleted, which was explained based on a possible influence of *veA* on *gliP* by unknown mechanisms. Therefore, the expression of *gliP* could be controlled by other regulators, apart from *gliZ*, which might have been affected by the addition of fresh medium.

Genes involved in iron metabolism were also found to be differentially expressed in our study. In order to maintain iron homeostasis, two transcriptional factors are necessary, *sreA* and *hapX*. The first one, *sreA*, represses the expression of high-affinity iron uptake when iron concentrations are high enough (Schrettl *et al.*, 2008). The second one, *hapX*, mediates adaptation to iron starvation conditions, in which the iron uptake through siderophores is activated (Schrettl *et al.*, 2010) and, recently, other authors have demonstrated a dual role in high and low iron conditions (Gsaller *et al.*, 2014). Our microarray results showed that *sreA* was up-regulated at 24°C, whereas *hapX*, SidD and SidC siderophores as well as MirB and MirC siderophores transporters were up-regulated at 37°C. Interestingly, after the addition of fresh medium, *sreA* expression levels did not up-regulate, but *sidD* showed an up-regulation at 24°C, unlike microarray data (Fig. 4c). Therefore, the iron uptake through siderophores appears not to be affected by the heat shock, but rather by a possible decrease of iron availability in the culture medium during the isotropic swelling and germ tube formation at 37°C.

Another metabolic pathway whose genes were found to be differentially expressed in our microarray results and that has been related to virulence was nitrogen metabolism. *Aspergillus* can use a wide range of nitrogen sources and contribute significantly to global nitrogen recycling via nitrate assimilation (Dagenais & Keller, 2009). According to them, three genes involved in nitrogen metabolism were up-regulated at 37°C. Two of them encode nitrate reductases (NiaD and AFUA_5G10420), and the other, the nitrite reductase NiiA, which was the most up-regulated gene (Table 3). With the addition of fresh medium, the results showed that in 15-30% germination rate samples only the gene that encode the nitrite reductase NiiA appeared to be up-regulated at 24°C, whereas in 40-60% germination samples the genes that code for the nitrite reductase NiiA and the nitrate reductase NiaD were up-regulated at 37°C (Fig. 4ef). These expression changes detected in our study could mean that the uptake of nitrogen is up-regulated in line with the growth of *A. fumigatus* at 37°C and a possible consumption of nitrogen sources as in the iron case.

Finally, *A. fumigatus* may be responsible for various allergic diseases such as extrinsic allergic alveolitis, asthma, allergic sinusitis, and allergic bronchopulmonary aspergillosis (ABPA) (Park & Mehrad, 2009). More than 20 allergenic molecules are known to be produced by this fungus, and some of them, in addition to their allergenic activities, are also related to virulence (Abad *et al.*, 2010). Our results reveal that nine genes that encode allergens were highly up-regulated when *A. fumigatus* germinated at 37°C, the most up-regulated allergens being the MnSOD (Asp F6), the major allergen Asp F2 and the major allergen and cytotoxin Asp F1. Only one gene that codes for a cell wall glucanase/allergen F16-like appears to be up-regulated at 24°C (Table 3). According to the addition of fresh medium results, the expression of allergens seems to take place at the beginning of the germination. In 15-30% germination samples, Asp F2 and MnSOD were also up-regulated at 37°C, being only MnSOD up-regulated at 24°C in 40-60% germination rates samples

(Fig. 4ghi). Furthermore, the up-regulation in 15-30% germination rates not only highlighted the importance of these allergen production during the first steps of germination but the effect of a higher temperature. However, AspF1 showed no statistical differences in 15-30% germination rates samples, pointing out the effect of the medium in the production of this allergen, which might be stimulated by a depletion of nutritional sources.

This is the first study that compares the transcriptomes of germinated conidia at 24 and 37°C taking into account the germination rate. According to our results, *A. fumigatus* modifies the expression of genes mainly related to metabolism and down-regulates RNA pathways in order to adapt to the new conditions at 37°C. Moreover, the high percentages of differentially expressed genes that encode hypothetical or unclassified proteins imply that many of these unknown genes must be essential during the germination of this fungus whose study might improve the understanding of the infection caused by the fungus. Among the genes related to virulence up-regulated at 37°C, we found genes coding for allergenic proteins and several genes involved in gliotoxin biosynthesis, nitrogen and iron metabolism. Furthermore, the addition of fresh medium has demonstrated the importance of the isotropic swelling, which can alter the growth medium even before reaching the 15-30% germination rate. Gene expression in iron and nitrogen metabolism appears to be influenced by the availability of these nutrients in the medium, whereas in allergen production, the temperature, the germination stage and the medium conditions seem to be involved. Therefore, the expression changes detected in our study, might demonstrate not only the rapid heat shock adaptation of *A. fumigatus* but also its efficient ability to uptake the nutrients of the medium during the earliest steps of germination, which might be another aspect that allows it to germinate easily in the host environment.

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586

587 **Table 1.** Genes selected and primers used in the reverse transcription quantitative PCR.

Target ^a	Primer names	Sequence (5′→3′) ^b	Tm ^c	%GC ^d	Amplicon (pb) ^e
Most Up-regulated at 37°C					
L-amino acid oxidase Laoa	F-LaoA	TTCAAGACAGTTGCCAGTGC	60.03	50.00	148
	R-LaoA	ATTACAGCCAATCGAGGTAG	60.10	50.00	
Extracellular proline-rich protein	F-Extracel	TCCATCCACAGTCACGGTAA	59.96	50.00	120
	R-Extracel	CCAGTGCCAGTACCAGGATT	59.99	55.00	
NACHT domain protein	F-NACHT	ATAGGCGACGTGGTTGTAGG	60.02	55.00	127
	R-NACHT	CAC TTCTGCCTGCGTTCATA	60.01	50.00	
Dimethylallyl tryptophan synthase Sird-like	F-SirD	ATTTGTTTCCCCATCATCCA	59.99	40.00	121
	R-SirD	GTGCTGTACACCCGGAGATT	60.00	55.00	
CobW domain protein	F-CobW	GTCATTGAGAGCACGGGAAT	60.08	50.00	108
	R-CobW	CTCATCCACCAGCATCTCCT	60.22	55.00	
Nitrite reductase NiiA	F-NiiA	TCCTCGCTACAGGCTCAGAT	60.12	55.00	113
	R-NiiA	AGCGAACTCGATGAGACGTT	60.02	50.00	
Thioredoxin	F-Thioered	TTGATGTCGATGAGCAGGAG	59.94	50.00	103
	R-Thioered	CCCACAGCTTCACCAATCTT	60.11	50.00	
Ribonuclease H1	F-H1	CTTAAAGAACGGCCAAGACG	59.88	50.00	129
	R-H1	GCTCGTAACTCGGCTACCTG	60.04	60.00	
RTA1 domain protein	F-RTA1	CAACCTGCGTCTTTGCTACA	60.05	50.00	111
	R-RTA1	ACACGTAACCGCAATCATCA	60.00	45.00	
C4-dicarboxylate transporter/malic acid transport protein, putative	F-C4	GGACTGGCTCTTCTGATTGC	59.96	55.00	134
	R-C4	TGCAAAACGAATCTCAGCAC	60.00	45.00	
Most Up-regulated at 24°C					
Catalase	F-Catal	CCATCCAGATGTGTGGTGAG	59.95	55.00	150
	R-Catal	CGAATGGATCAAATCCCAAC	60.13	45.00	
Pyruvate dehydrogenase E1 component alpha subunit	F-E1	AATCATGAGGTGGCAGAAGC	60.23	50.00	117
	R-E1	CACGGTACGTCTCGGTACT	60.05	60.00	
NAD dependent epimerase/dehydratase family protein	F-NAD	CGGTTGCAAAGCCAACTACT	60.30	50.00	143
	R-NAD	TCTGGAAGGACTCCGTATCG	60.21	55.00	
FAD binding monooxygenase	F-FADmon	ACGGAACAGCATTTCATCCTC	60.08	50.00	123
	R-FADmon	CTGCAGCGTCTTTGACTGAG	59.92	55.00	
ATP/GTP-binding protein	F-ATP/GTP	GTGGACTACCCCAAGGAACA	59.82	55.00	128
	R-ATP/GTP	CGGAATGCGAAAGAGAAGTC	59.96	50.00	
Nmra-like family protein	F-NmrA	TTTGCTATGCTGTGCTCTGG	60.16	50.00	131
	R-NmrA	CCACCTCTCGAAATCCTTCA	60.19	50.00	
Glycosyltransferase	F-Glyco	CACTGCAACCGTCAAAGCTA	60.05	50.00	124
	R-Glyco	GTGTGCTGGCCTGATATCCT	59.96	55.00	
Zn ²⁺ dependent hydrolase	F-Zn2	TTTGGAGACCGATTCCTTTG	60.04	45.00	140
	R-Zn2	TGCCCAAGTTGAGAATAGCA	59.42	45.00	
Replication factor C subunit	F-FactorC	GTGCATGACGTCCAGATACG	60.14	55.00	139
	R-FactorC	TTTGTGCAGTGTGGTCCATT	60.01	45.00	
Conidiation-specific protein Con-10	F-Con10	ATCTCTCTGGCGGACCACTA	59.83	55.00	145
	R-Con10	AACACCCCTGGCTCTACCTC	60.51	60.00	
Related to virulence					
C6 finger domain protein GliZ	F-GliZ	GGGCATGTCTTTGAACCCTA	59.93	50.00	137
	R-GliZ	ACCAGCGTACTCCGAAACTG	60.31	55.00	
Nonribosomal peptide synthase GliP	F-GliP	TCGTGACCTTGCTCATTCTG	59.98	50.00	115
	R-GliP	GTCAATGTCGCCAAGAGAT	60.08	50.00	
Nonribosomal peptide synthase SidD	F-SidD	ACCAGGATGCTACGAACACC	60.00	55.00	134
	R-SidD	TCCACCAAGAGCGAAGAAGT	59.99	50.00	
Siderophore transcription factor SreA	F-SreA	CCTGCCTACAACAACCGAGT	60.17	55.00	126
	R-SreA	ATTCTTTCCCGCATCGTTTT	60.80	40.00	
Nitrate reductase NiaD	F-NiaD	GACGCCCTGCTAGTGAGAAG	60.16	60.00	102
	R-NiaD	CACTCGGAACCAAGGATTGT	59.97	50.00	

Major allergen and cytotoxin AspF1	F-AspF1	CCACAGCCGTGTCTGTTCTA	59.90	55.00	109
	R-AspF1	CGCTTGTCTTCCCATTGTT	60.11	45.00	
Major allergen Asp F2	F-AspF2	TCTGTGATCGCAGCTACACC	60.02	55.00	133
	R-AspF2	ACAGCAGGCACATGGTACAG	59.78	55.00	
Mn superoxide dismutase MnSOD	F-MnSOD	CCTACGTCAATGGCCTGAAT	59.96	50.00	138
	R-MnSOD	TCCAGAAGAGGGAATGGTTG	60.04	50.00	
Housekeeping genes					
Actin 1 Act1	F-Act1	TCATCATGCGCGACAGCTTA	63.95	50.00	179
	R-Act1	CGTGCTCGATGGGGTATCTG	63.86	60.00	
Glyceraldehyde 3-phosphate dehydrogenase GpdA	F-GdpA	CTCCCTCCAACAAGGACTGG	61.99	60.00	115
	R-GpdA	AGCTTGCCGTTGAGAGAAGG	61.98	55.00	

^aGenes chosen from the list of genes of the microarray.

^bSequence of each primer.

^cTm: Melting temperature of each primer.

^d%GC: Percentage of guanine and cytosine content of each primer.

^eSize of cDNA amplification product.

594 **Table 2.** Enriched FunCat categories at 24 and 37°C.

FunCat description ^a	p-value ^b	Genes/Category ^c
37°C		
C-compound and carbohydrate metabolism	2.45E-09	97 / 858
secondary metabolism	6.72E-03	77 / 870
lipid, fatty acid and isoprenoid metabolism	6.72E-03	56 / 577
non-vesicular cellular import	1.96E-02	24 / 190
NAD/NADP binding	1.99E-02	24 / 193
non-ribosomal peptide synthesis	2.06E-02	8 / 32
fatty acid metabolism	2.43E-02	19 / 142
degradation of leucine	3.99E-02	6 / 21
24°C		
rRNA processing	1.91E-47	85 / 188
ribosome biogenesis	4.61E-22	58 / 182
rRNA synthesis	4.62E-12	27 / 70
RNA binding	3.36E-11	59 / 308
rRNA modification	4.18E-05	9 / 17
tRNA processing	4.66E-02	12 / 60
tRNA modification	4.66E-02	10 / 45
RNA degradation	4.66E-02	13 / 70

^aFunctional classification according to the Munich Information Center for Protein Sequence Functional Catalogue (FunCat; <http://pedant.helmholtz-muenchen.de>)

^bEnrichment p-value of each FunCat description obtained after Fisher's test and the Benjamini-Hochberg correction.

^cNumber of up-regulated genes found in each functional group relative to the total number of each category.

Table 3. Genes related to virulence differentially expressed on the microarray.

Gene product description ^a	Locus ID ^b	24°C ^c	37°C ^c	Fold change ^d
Glutotoxin biosynthesis				
Nonribosomal peptide synthase GliP	AFUA_6G09660	-3.376	1.689	-5.065
Membrane dipeptidase GliJ	AFUA_6G09650	-4.574	-0.184	-4.390
C6 finger domain protein GliZ	AFUA_6G09630	-1.638	1.484	-3.122
Nonribosomal peptide synthase GliP-like	AFUA_3G12920	-1.174	0.189	-1.362
Iron metabolism				
MFS siderochrome iron transporter MirB	AFUA_3G03640	-2.496	2.199	-4.695
bZIP transcription factor HapX	AFUA_5G03920	-1.100	0.855	-1.955
Nonribosomal peptide synthase SidD	AFUA_3G03420	-1.094	0.690	-1.784
Nonribosomal siderophore peptide synthase SidC	AFUA_1G17200	-0.647	0.667	-1.314
Siderochrome-iron transporter MirC	AFUA_2G05730	-0.353	0.699	-1.052
Mitochondrial ornithine carrier protein AmcA/Ort1, putative	AFUA_8G02760	-0.529	0.483	-1.013
Siderophore transcription factor SreA	AFUA_5G11260	0.585	-0.632	1.218
Homoaconitase LysF	AFUA_5G08890	0.950	-1.130	2.080
Nitrogen metabolism				
Nitrite reductase NiiA	AFUA_1G12840	-4.979	2.684	-7.663
Nitrate reductase NiaD	AFUA_1G12830	-1.331	1.685	-3.017
Nitrate reductase	AFUA_5G10420	-0.273	0.941	-1.213
Allergens of <i>A. fumigatus</i>				
Mn superoxide dismutase MnSOD	AFUA_1G14550	-2.165	1.599	-3.765
Major allergen Asp F2	AFUA_4G09580	-2.865	0.166	-3.032
Major allergen and cytotoxin AspF1	AFUA_5G02330	-1.874	0.942	-2.816
Aspartic endopeptidase Pep1/aspergillopepsin F	AFUA_5G13300	-1.142	0.742	-1.884
Allergenic cerato-platanin Asp F13	AFUA_2G12630	-0.993	0.836	-1.829
Cell wall galactomannoprotein Mp2/allergen F17-like	AFUA_2G05150	-0.769	0.798	-1.567
Cell wall serine-threonine-rich galactomannoprotein Mp1	AFUA_4G03240	-0.658	0.814	-1.473
Elastinolytic metalloproteinase Mep	AFUA_8G07080	-0.722	0.447	-1.169
Extracellular cellulase CelA/allergen Asp F7-like, putative	AFUA_5G08030	-0.314	0.715	-1.029
Cell wall glucanase/allergen F16-like	AFUA_6G03230	0.561	-0.655	1.216

Note: Negative fold changes mean an up-regulation at 37°C and positive fold changes an up-regulation at 24°C.

^aGenes product description of the genes found on the microarray.

^bGenBank accession number.

^cNormalized expression data in log₂ in each condition. The normalization was carried out using GeneSpring GX software. After subtracting the background intensity and log₂ data transformation, a global normalization was performed followed by a baseline transformation to the median of all samples.

^dFold change in log₂ obtained for each gene. This value represents the log₂ at 24°C minus the log₂ at 37°C.

Legends

Fig. 1. Germination of *A. fumigatus*. (a) Calcofluor white staining of *A. fumigatus* Af-293. Each picture showed the percentage of germination and the hours of incubation. (b) Curves of germination rates of *A. fumigatus* Af-293. Each point represents the mean value $\pm$ standard deviation of three replicates of each time. Black and white arrows indicate the time points in which samples were obtained. Continuous incubation at 24°C (I and black triangles) and at 37°C (II and black circles). Incubation at 24°C (III and white triangles) and at 37°C (IV and white circles) following the addition of fresh medium after 16 hours of incubation at 24°C.

Fig. 2. Gene expression analysis. (a) Box plot representing the distribution of gene expression. Each sample corresponds to the mean expression of three independent samples obtained from the growth at 24 and 37°C, respectively. The Y-axis values represent the gene expression ($\log_2$). (b) Volcano plot showing differentially (in black) and non-differentially (in gray) expressed genes. The X-axis values represent the fold change ($\log_2$) of microarray data and the Y-axis values the statistical significance ($-\log_{10}$ p-value). Spots with negative fold change values mean an up-regulation at 37°C and with positive values an up-regulation at 24°C.

Fig. 3. Comparison of the results obtained by microarray and RT-qPCR. (a) Comparative levels of gene expression between the microarray and RT-qPCR. Negative fold changes means an up-regulation at 37°C and positive fold changes an up-regulation at 24°C. (b) Correlation analysis between microarray and RT-qPCR data. The X-axis values represent the fold change ($\log_2$) of microarray data and the Y-axis values the fold change ($\log_2$) of RT-qPCR results. Each point corresponds to the mean value from three independent samples.

Fig. 4. Gene expression levels of genes related to virulence. *gliZ* (a), *gliP* (b), *sreA* (c), *sidD* (d), *niaD* (e), *niiA* (f), Asp F1 (g), Asp F2 (h), and MnSOD (i). Negative $\log_2$ fold changes means an up-regulation at 37°C and positive fold changes an up-regulation at 24°C and the asterisk shows data above or below of the thresholds established (over 1 and under -1). Samples with continuous incubation, and with 15-30% and 40-60% germination rates after the addition of fresh medium, are shown as black-white stripe, white bars and black bars respectively.

Figure 1
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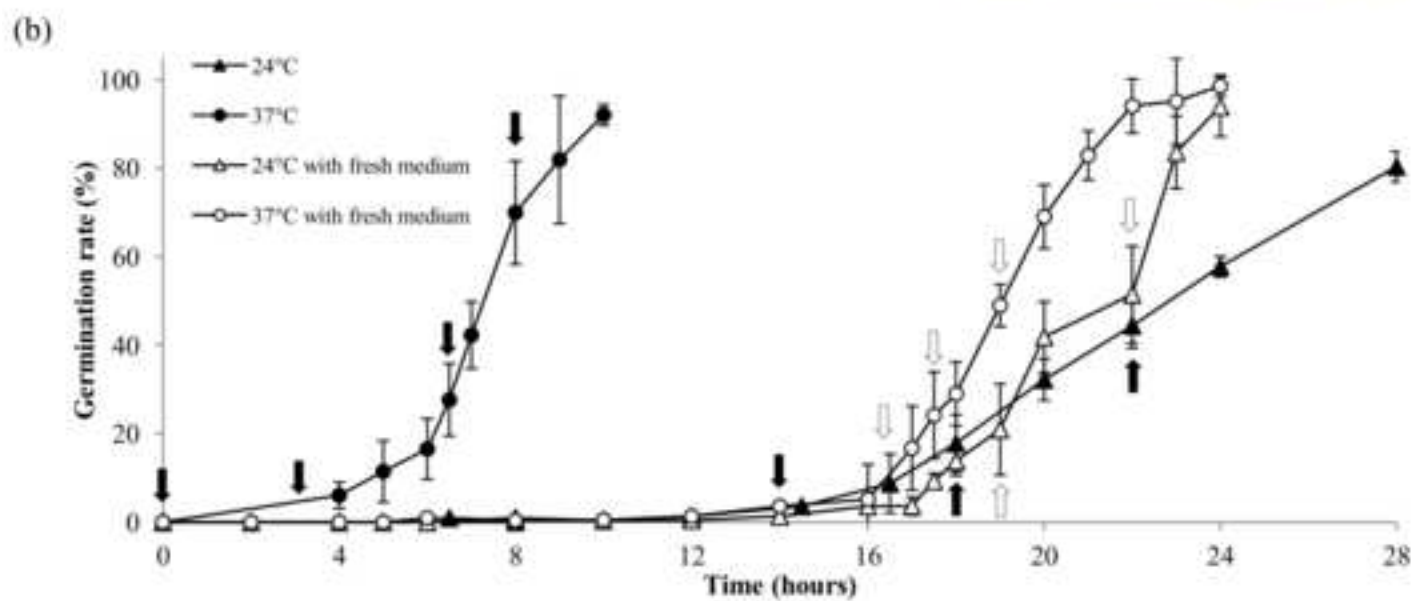
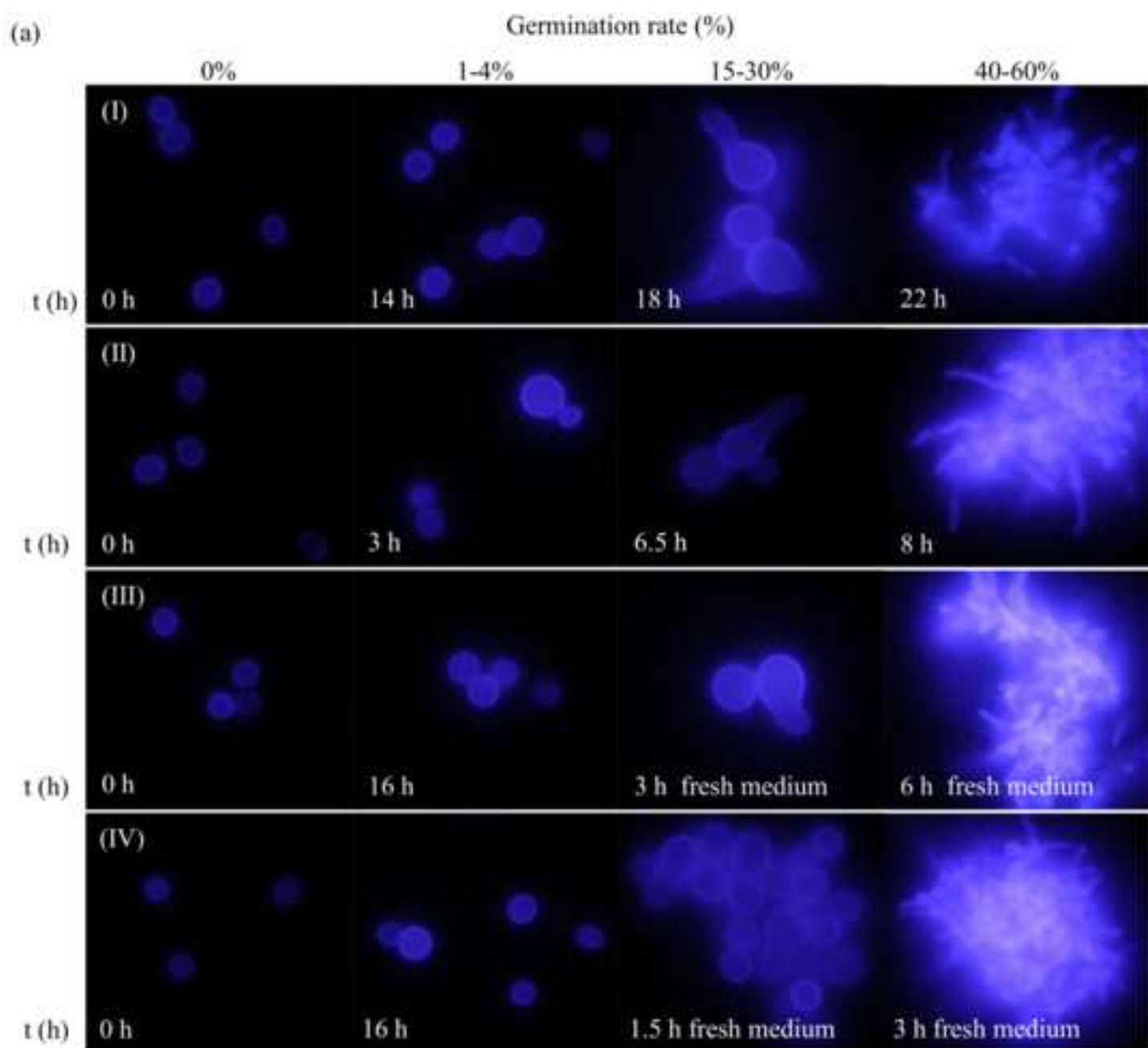


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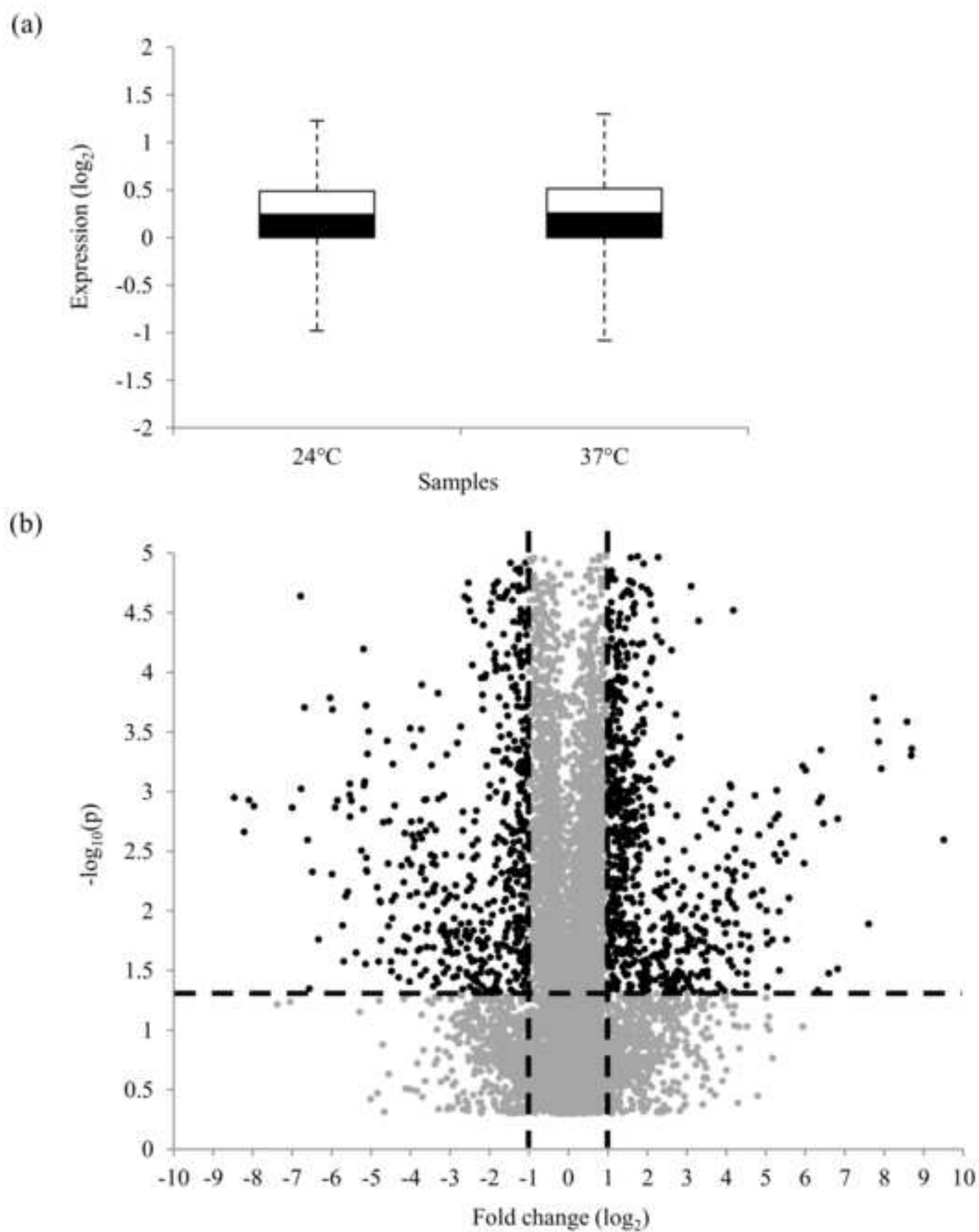


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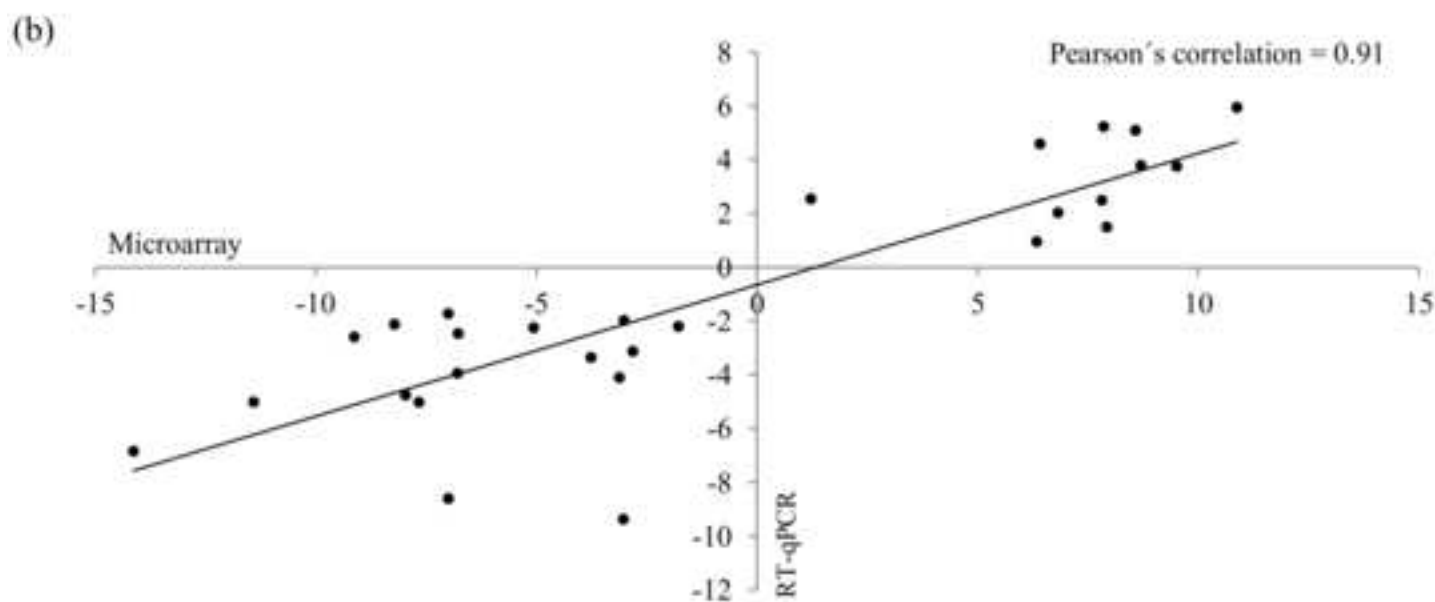
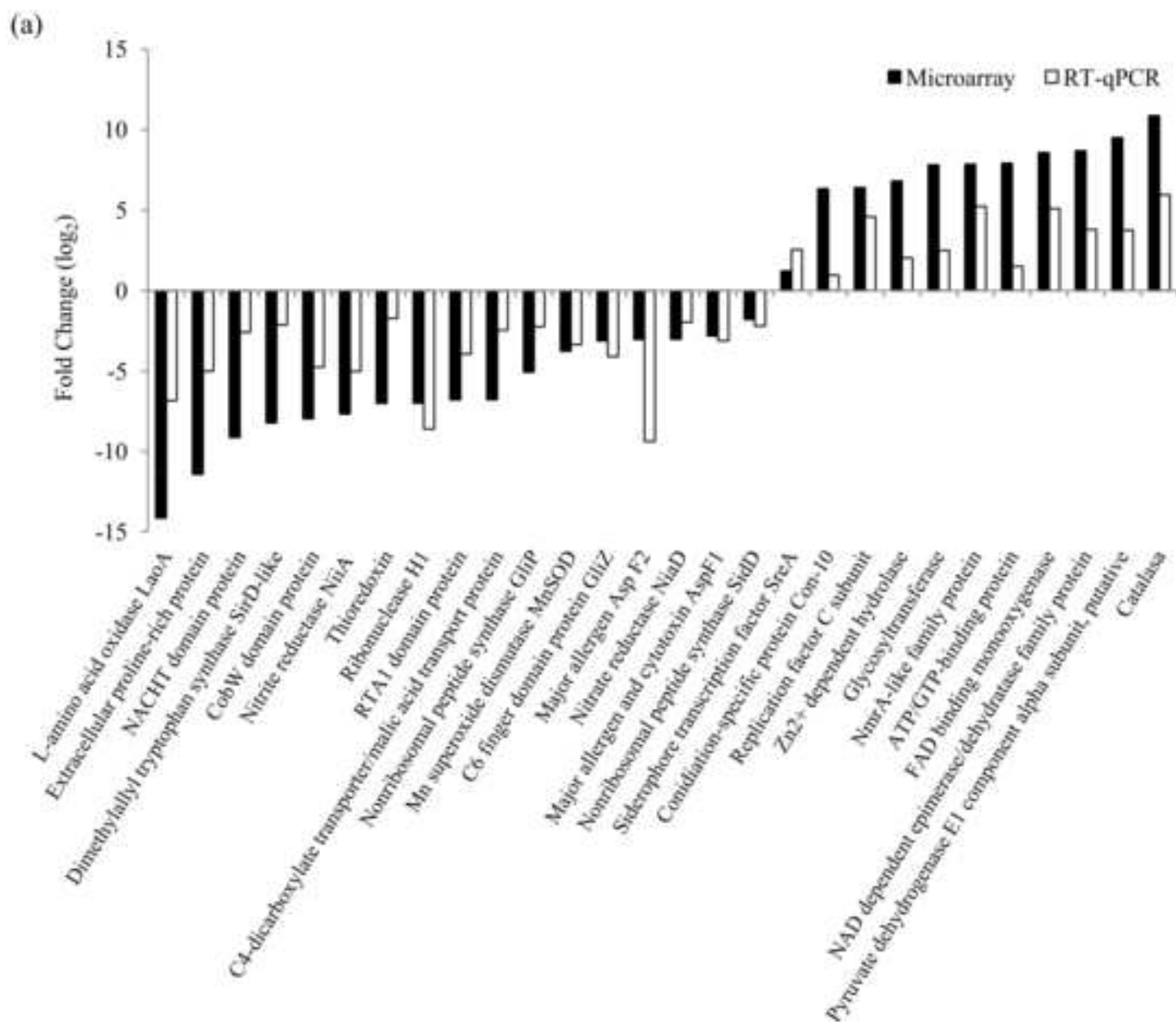
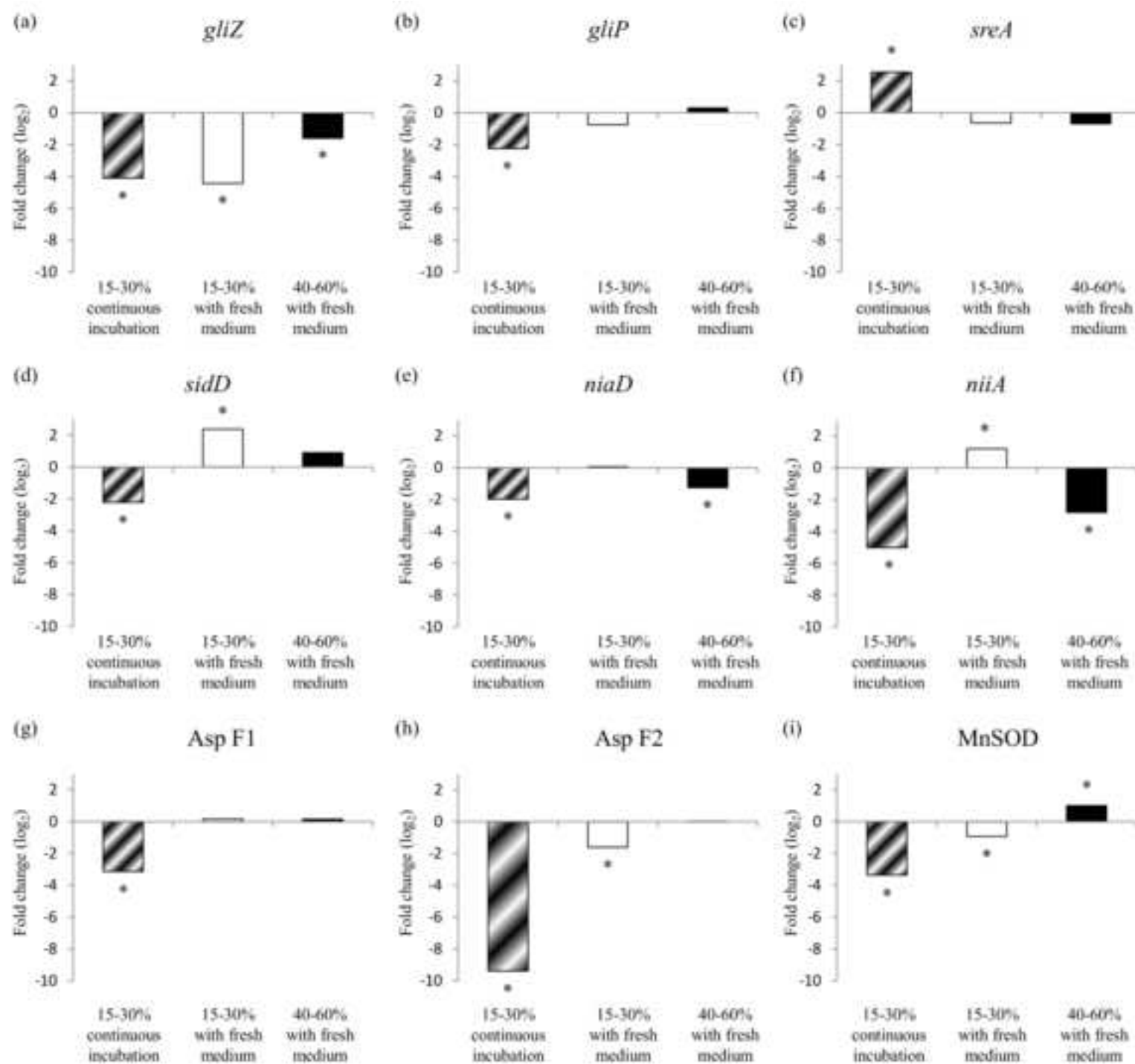


Figure 4
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Supplementary tables

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