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# Gene regulation in *Aspergillus oryzae* promotes hyphal growth and flavor formation in soy sauce koji

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**Running title:** *A. oryzae* genes in growth and flavor of koji

**Abstract**

*Aspergillus oryzae* 100-8 and the parental strain *A. oryzae* 3.042 are used in soy sauce fermentation in China. The growth rate of *A. oryzae* 100-8 is faster than *A. oryzae* 3.042, and the soy sauce flavors obtained with *A. oryzae* 100-8 fermentation are better than those obtained with *A. oryzae* 3.042. In this study, comparisons were made through biomass, reactive oxygen species (ROS) and gas chromatography–mass spectrometry (GC-MS) measurements, and the reasons for these differences were investigated through transcriptome and qRT-PCR analysis. The analysis indicated that several unique genes are closely associated with hyphal growth and flavor formation, as demonstrated by changes in the expression levels of these genes. These unique genes regulated hyphal growth and flavor formation in soy sauce koji fermentation.

**Keywords:** *Aspergillus oryzae*, RNA-Seq, hyphal growth, flavor, koji

## Introduction

Soy sauce is made from a mixture of soybeans and wheat using a two-step fermentation process that involves koji fermentation and brine fermentation. *Aspergillus oryzae* is always used for koji fermentation; it has earned GRAS (generally recognized as safe) status and is of significant economic importance. *A. oryzae* 100-8, a mutated strain obtained through an $N^+$ ion implantation mutagenesis method, can grow faster than the parental strain *A. oryzae* 3.042, and the faster growth rate is crucial to the development of this multicellular organism. The genomes of these two strains were sequenced and compared in our previous studies [1-3]. The growth rate of *A. oryzae* can affect the koji ripening time and koji flavors in soy sauce koji fermentation. *A. oryzae* has the ability to form sexual spores and mycelia, suggesting that it may be able to survive in the environment. Spore formation is a primitive system of cell differentiation [4], and is a trait that is typical of filamentous fungi. While the regulated mechanism for promoting hyphal growth and spore formation in *A. oryzae* is regarded as one of the unsolved mysteries of fungal biology, it is clear that it is associated with differences in the expression levels of some genes.

Various studies of the flavors in traditional soy sauce had been reported [5, 6]; however, research on the volatile flavors in soy sauce koji had not been systematically conducted. *A. oryzae* has the inherent ability to secrete degrading enzymes, such as protease, cellulase and amylase. Raw materials are decomposed to sugars and peptides, and other flavor compounds are synthesized within *A. oryzae* and then transferred to the extracellular environment during koji fermentation. Koji flavors, as soy sauce flavor precursors, play a decisive role in forming the desired flavor compounds in soy sauce.

The lack of knowledge regarding gene regulation in *A. oryzae* strains induced us

to further elucidate the differences between *A. oryzae* 100-8 and *A. oryzae* 3.042. The transcriptome sequencing approach had provided insights into the biology of several species, leading to the development of functional transcriptome analysis and to high-throughput approaches for determining phenotypes [7]. We analyzed the transcriptomes of *A. oryzae* 100-8 and *A. oryzae* 3.042 at different stages of fermentation, and demonstrated the potential of such analysis to elucidate variability in the genes associated with growth and flavor to provide further understanding of the general biology of this filamentous organism. The analysis revealed several genes that are important in mycelial growth and flavor formation.

## Materials and methods

### Strains and growth conditions

*A. oryzae* 100-8 and 3.042 were obtained from the Strain Collection Center of Tianjin University of Science and Technology (China). The mycelia of these two strains were collected after cultivation of 30 h, 36 h and 42 h in the soy sauce fermentation process, and RNA-Seq and qRT-PCR samples were prepared.

### Biomass and ROS measurements

Spores of *A. oryzae* 100-8 and 3.042 were counted using optical microscopy, and $2\times10^6$ spores were inoculated and grown in a 200-mL liquid culture of rice-juice medium [8]. The biomass of each organism (100-8 and 3.042) was measured after 30 h, 36 h and 42 h. The mycelia were air-dried overnight at 60°C.

ROS production was also estimated in this study [9]. Strains grown at 28°C for 30 h were incubated with the ROS indicator H2DCFDA (dichlorodihydrofluorescein diacetate; Invitrogen, OR, USA) (20 μM in phosphate-buffered saline). The

dichlorofluorescin (DCF) produced by the two strains was assessed using a Nikon 90i fluorescence microscope (Nikon Corp, Tokyo Japan) [10].

**Isolation of volatiles and GC-MS**

Samples of soybeans, wheat and water in the proportions 6:4:12 were inoculated separately with *A. oryzae* 100-8 and *A. oryzae* 3.042 at 30°C. The sniffing port had previously been cleared by heating the gas chromatography injection port at 250°C for 30 min until there were no miscellaneous peaks. Fermented koji samples were extracted three times via headspace solid-phase micro-extraction (HS-SPME) for 30 min (Supelco Co., Bellefonte, PA, USA) [11]. The gas chromatography–mass spectrometry (GC-MS) system (Varian, Walnut Creek, CA, USA) was equipped with a VF-5ms capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness). The injector temperature was 250°C and the transfer line and iron source temperature were set to 280°C and 220°C, respectively. The column was held isothermally at 40°C for 3 min, then raised to 150°C for 1 min, and finally raised to 250°C for 6 min. The flow rate of the carrier gas helium through the column was a constant 1 ml/min, and 1 μl of sample was injected with a split mode of 5:1 (v/v). Ions were generated by electron impact ionization (EI) at 70 eV, and were recorded over a mass range of 50-1,000 m/z. The compounds detected in the GC-MS analysis were identified by comparing the mass spectra of the unknown peaks with the MS library of the National Institute of Standards and Technology (NIST05).

**Transcriptome sequencing and analysis**

Samples of the two strains were frozen in liquid nitrogen and treated with TRIzol solution, DNaseI and Sera-Mag Oligo(dT)-coated magnetic beads (Illumina) to

extract messenger RNA according to the manufacturers' protocols. cDNA libraries were generated according to the Massively Parallel Signature sequencing protocol after reverse transcription; the cDNA was end-repaired, amplified, denatured and then sequenced with an Illumina Genome Analyzer IIx using proprietary reagents. RNA-Seq libraries were constructed using a SOLiD Total RNA-Seq Kit, and the reads were mapped to the genomes of *A. oryzae* 3.042 and *A. oryzae* 100-8. Gene expression levels were measured in terms of "fragments per kilobase of exon model per million mapped reads" (FPKM) values [12]. Genes for which the expression levels changed more than 2-fold ($p < 0.05$) were considered to show changes in transcription level.

**qRT-PCR for the gene expression test**

Genes (Ao3042_08242, Ao3042_07372, Ao3042_09608, Ao3042_09643, Ao3042_00917, Ao3042_00961, Ao3042_01056, Ao3042_11843 and Ao3042_06476) involved in hyphal growth and flavor formation in all three fermentation stages were chosen for qRT-PCR. The total RNA of the *A. oryzae* 100-8 and *A. oryzae* 3.042 strains were extracted using TRIzol (Invitrogen) and digested with RNase-free DNase-I (Fermentas). Reverse transcription of RNA was then performed following the protocol of the M-MLV Rtase cDNA Synthesis Kit (TaKaRa Biotech). 2 microgram of cDNA template, 10 μL of 2 × Mix, and 0.5 μM of forward and reverse primers (Generay Biotech) were mixed, and quantitative real-time PCR was performed with 35 cycles of amplification at 95°C for 15 s and 57°C for 30 s in an Applied Biosystems PCR machine. We used 18S rRNA as the internal control in the PCR amplification [13].

## Accession numbers

The raw RNA-Seq data had been deposited at the DNA Data Bank of Japan (DDBJ), with the accession numbers DRA000600, DRA000887 and DRA000888 for samples of *A. oryzae* 3.042 cultivated for 30 h, 36 h and 42 h, and DRA000889, DRA000890 and DRA000891 for *A. oryzae* 100-8 cultivated for 30 h, 36 h and 42 h, respectively.

## Results and discussion

### General view of the transcriptome analysis

The transcriptomes were sequenced, producing $2.8 \times 10^7$, $2.2 \times 10^7$, $1.7 \times 10^7$, $2.9 \times 10^7$, $2.3 \times 10^7$ and $3.1 \times 10^7$ reads (100 bp per read). The mapping rates of the six samples were 62.34%, 54.61%, 54.19%, 63.72%, 57.89% and 62.82%, respectively. Gene expression levels were measured in terms of FPKM. Overall, the differential transcription of genes was observed at the 30 h, 36 h and 42 h growth stages (Table S1). As shown in Figure 1, these genes were grouped into Clusters of Orthologous Groups of proteins (COGs) and were putatively involved in a wide variety of energy production, amino acid metabolism, nucleotide metabolism, carbohydrate metabolism, coenzyme metabolism and lipid metabolism processes at the 36 h growth stage. This result indicated that a fast mycelial growth rate plays a major role in regulating metabolism, and more than 200 genes were found through the comparison to be associated with hyphal growth and flavor formation ($p < 0.05$) (Table S2).

### Differences in *A. oryzae* 100-8 and *A. oryzae* 3.042 morphology

Comparison of the fermentation and morphology characteristics of these two *A. oryzae* strains had been performed previously [8]. Moreover, the appearance of these two strains was compared before the mycelia of 3.042 entered the reproductive period,

and the conidia were able to grow in either a liquid or a solid culture medium (rice juice was used as a natural medium). Furthermore, comparison of the biomass values of *A. oryzae* 100-8 and *A. oryzae* 3.042 (dry weight) following culture under identical conditions for 42 h revealed that the biomass yield of 100-8 (1.39 g/100 ml) was almost twice that of 3.042 (0.76 g/100 ml) (Figure 2). The ROS levels were shown to be lower in *A. oryzae* 100-8 than in *A. oryzae* 3.042 (Figure S1). Measurement of the ROS levels using $H_2$DCF-DA showed that *A. oryzae* 3.042 cells displayed stronger fluorescence intensity than *A. oryzae* 100-8 cells after 30 hours of growth. The genes encoding alkyl hydroperoxide reductase (AO1008_07372 and AO1008_09223), which is the primary scavenger of ROS, were highly expressed in *A. oryzae* 100-8 [14], while the genes encoding glycolate oxidase (AO1008_10905, AO1008_05009 and AO1008_11979), which modulates the production of ROS [15], were expressed at low levels (Table S2).

The expression levels of some genes associated with $Ca^{2+}$ in hyphal growth were clearly lower in *A. oryzae* 100-8 than in *A. oryzae* 3.042 (Figure 3, Table S2). It had been proposed that $Ca^{2+}$ ions regulate and coordinate the process of hyphal growth [16]. $Ca^{2+}$ ions may cross-link with the carbohydrates and macromolecules of the cell wall and make the cell wall more rigid. $H^+$ ions may promote $Ca^{2+}$ dissociation to give cell wall plasticity. $Ca^{2+}$ and $H^+$ ions thus regulate the balance between rigidity and plasticity. A relatively low concentration of cytoplasmic $Ca^{2+}$ may play a role in increasing plasticity and thus promoting hyphal growth, with the fungi responding to the balance between $Ca^{2+}$ and $H^+$ (Figure S2).

During the dynamic phase of protein secretion and hyphal growth, the energetic requirements of *A. oryzae* were increased; *A. oryzae* 100-8 required more energy to balance these processes than *A. oryzae* 3.042. The mechanism for regulating cellular

energy metabolism had been postulated on the basis of the reversible control of respiration, closely related to oxidative phosphorylation. Genes which were up-regulated in *A. oryzae* 100-8 than 3.042 were listed in Table S2. The NADH:ubiquinone oxidoreductase (complex I) (AO1008_01771, AO1008_10474, AO1008_06499, AO1008_08911, AO1008_03516) catalyzes the first step in the mitochondrial respiratory chain [17], involving the entry of electrons from NADH. Complex II participates in the electron transport chain; electrons are delivered from ubiquinol to cytochrome c by cytochrome $bc_1$ (complex III) (AO1008_08130). Cytochrome oxidase (complex IV) (AO1008_05880) generates a transmembrane proton gradient, and electrons are transferred to the active site. Complexes I, II, III and IV are the electron transfer complexes, while complex V (AO1008_05587, AO1008_01244, AO1008_02044) is an energy-conserving complex that catalyzes ATP-Pi exchange and ATP hydrolysis (Figure S3).

The comparative analysis of the transcriptomes of *A. oryzae* 3.042 and *A. oryzae* 100-8 conducted in this study suggests that some genes are involved in hyphal growth (Table S2). The Ras-like GTPase is involved in the apical polarization of the actin cytoskeleton, a determinant of growth direction [18]. RNA helicase is required for cell growth and proliferation [19]. Dual-specificity phosphatase (DSP) appears to be selective for dephosphorylating the critical phosphothreonine and phosphotyrosine residues within mitogen-activated protein kinases related to programmed cell death [20, 21]. The mitotic spindle biogenesis protein and septin proteins may be important proteins in mitosis [22], and the cAMP-dependent protein kinase in a G protein signaling pathway regulates morphological transition in *A. oryzae* [23].

The fungal cell wall is a dynamic organelle that allows for cell growth and cell division during the life cycle of *A. oryzae*. The enzyme 1,3-β-glucanosyltranferase

plays an active role in the biosynthesis of the cell wall, and cell wall glucanase is important for cell wall stability [24]. Glycosyltransferase, transglycosidase and glycosidase generate cell wall polysaccharides [25], while glycosyl-phosphatidylinositol (GPI) anchor proteins are cell wall proteins that direct glycoproteins to the secretory pathway and glycosylation sites [26]. The key enzymes for the synthesis of sterol or ergosterol as components of cell membranes are 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, SAM-dependent methyltransferase, C-4 sterol methyl oxidase and C-8,7 sterol isomerase. Fatty acid desaturase plays a key role in the maintenance of the correct structure and functioning of biological membranes [27]. Phosphatidylinositol synthase catalyzes the synthesis of the phospholipid phosphatidylinositol, which is not only a major constituent of biological membranes but also an active participant in the control of diverse cellular functions [28]. Sphingoid base 1-phosphate phosphatase is a key regulator of the metabolism of sphingolipids, which are critical structural components.

It is generally assumed that *A. oryzae* spores are formed asexually. This study found that spore formation by *A. oryzae* 100-8 was lower than *A. oryzae* 3.042 (the phenotype comparisons shown in Figure S4), indicating that this process is influenced by a mutant gene (AO1008_05602). The gene that encodes meltrin protein had been reported to play an important role in the process of fertilization in other organisms [29]. The levels of expression of other genes associated with spore formation had also been shown to be significantly decreased (Table S2). The genes encoding α-1,3-glucanase (AO1008_01791) and the early sexual development (esdC) protein (AO1008_08823) are necessary for sexual development. Furthermore, 17-β-hydroxysteroid dehydrogenase (AO1008_04266) has the ability to interconvert estrogens and androgens and also androstenedione and testosterone [30]. The *brlA* gene

(AO1008_07995) mediates the developmental switch from the apical growth pattern of vegetative cells to the budding growth pattern of conidiophores [31].

**Volatile components by GC-MS**

Volatile compounds in koji at 30 h, 36 h and 42 h were identified by GC-MS analysis. The volatile compounds were divided into eight categories according to the general flavors of soy sauce: ketones, aldehydes, alcohols, esters, furan compounds, phenols, hydrocarbons and acids. *A. oryzae* 100-8 produced larger amounts of ketones, aldehydes, alcohols, esters and furan compounds, but smaller amounts of phenols and hydrocarbons than *A. oryzae* 3.042 in the different periods (Table 1). Hydrocarbons are the precursors of flavors. The large amounts of hydrocarbons in *A. oryzae* 3.042 koji remain to be used.

Of the ketones detected, benzophenone was the most notable. It has a distinctive odor, sweet and fragrant, somewhat like a rose or a bay leaf, and was detected in the soy sauce koji fermented by both *A. oryzae* 100-8 and *A. oryzae* 3.042 during all three periods. The benzophenone content detected for *A. oryzae* 100-8 was 19.69% at 30 h, 3.83% at 36 h and 1.33% at 42 h, while that for *A. oryzae* 3.042 was 3.90% at 30 h, 2.00% at 36 h and 0.71% at 42 h. Based on its concentrations and low odor threshold values, benzophenone may partially contribute to the strongly fragrant odor of the koji of *A. oryzae* 100-8, especially at around 24 h. The threshold value was defined as the lowest concentration of a compound that can still be directly recognized by its odor [32].

The high aldehyde content contributed greatly to the overall volatile flavors of *A. oryzae* 100-8. Remarkably, the aldehydes accounted for as much as 70.18% of the total odorants produced by *A. oryzae* 100-8 at 36 h and 42.61% at 42 h, giving it an

overwhelming advantage over *A. oryzae* 3.042. Most of the aldehydes detected were aromatic, such as benzeneacetaldehyde, 2-phenyl-2-butenal, 2,4,6-trimethylbenzaldehyde, 2-phenylcrotonaldehyde, 5-methyl-2-phenyl-2-hexenal and so on. Benzeneacetaldehyde was one of the most typsical aromatic aldehydes and contributed significantly to the high aldehyde content, producing a significant sweet and fruity fragrance; it is likely to be a major contributor to the strong aromatic and sweet flavors in soy sauce koji obtained through fermentation with *A. oryzae* 100-8, especially at 36 h and 42 h.

Furan compounds, characterized by a strong scented, sweet and in some cases burnt odor, are generally recognized as important aromatic substances that contribute greatly to the flavors of soy sauce. *A. oryzae* 100-8 produced more total furan compounds in koji than did *A. oryzae* 3.042, with a content of 17.65% at 30 h, 1.64% at 36 h and 0.73% at 42 h for *A. oryzae* 100-8 and 4.49% at 30 h, 0.55% at 36 h and 0.23% at 42 h for *A. oryzae* 3.042. The furan compounds detected were primarily 2-pentylfuran, 2,3-dihydrobenzofuran and 2-*N*-octylfuran. In particular, 2-pentylfuran was found in all three periods of koji fermentation in both *A. oryzae* 100-8 and *A. oryzae* 3.042. In view of their high threshold values, the higher percentage of volatile furan compounds in *A. oryzae* 100-8 koji may contribute significantly to the overall pleasant odor of soy sauce [33].

The flavors arising from phenols, primarily guaiacol, 4-vinylguaiacol and 2,6-ditert-butyl-4-methylphenol, were lower in *A. oryzae* 100-8 koji than in *A. oryzae* 3.042 koji. Guaiacol, with its sweet “potpourri” flavor, and 4-vinylguaiacol, with its clove and smoke flavors, were regarded as two important phenols in soy sauce fermentation; they could be generated from fiber or lignin in the materials. *A. oryzae* 3.042 koji had higher levels of phenols, with 35.82% at 30 h, 64.69% at 36 h and

90.20% at 42 h, while *A. oryzae* 100-8 koji had 17.19% at 30 h, 1.25% at 36 h and 27.87% at 42 h. The distribution of phenols in *A. oryzae* 100-8 tended to generate a more harmonious and pleasant combination of odors.

Acids were seldom found in the flavors of koji fermented with *A. oryzae* 100-8 and *A. oryzae* 3.042, except that 4-methyl-2-oxovaleric acid was detected at 36 h for *A. oryzae* 100-8 and both 4-methyl-2-oxovaleric acid and 2-methylbutyric acid were found at 36 h for *A. oryzae* 3.042. The acids found in the koji were primarily produced by redox reactions of the degradation products of amino acids. Considering the overall fermentation process, the acids contained in the soy sauce were mainly produced from the action of saccharomycetes and lactobacilli in brine fermentation.

Flavor formation in soy sauce koji is mainly the result of the metabolism of proteins, sugar and lipids. *A. oryzae* 100-8 produced flavors with a more balanced structure in terms of varieties and levels than did *A. oryzae* 3.042. As shown in Table S2, *A. oryzae* 100-8 secreted significantly more acid proteases (including endopeptidases and aminopeptidases) than *A. oryzae* 3.042. Different parts of the peptides are hydrolyzed to amino acids by proteases, and the metabolism of these amino acids is the major source of the flavors. The branched-chain acids valine, leucine and isoleucine can be converted into acetoacetate and isobutanoate. The aromatic amino acids tyrosine, tryptophan and phenylalanine can be converted into phenylacetaldehyde, anthranilate and phenylacetate. Sulfuric flavors may be due to methionine and cysteine. Most of the genes involved in the metabolism of amino acids were highly expressed (Table S2).

Transcriptome analysis revealed that the genes associated with glycolysis were highly expressed in *A. oryzae* 100-8 (Table S2). Phosphofructokinase is a key regulatory enzyme in glycolysis; 3-phosphoglycerate kinase, a glycolytic enzyme,

315 catalyzes the reciprocal transformation of 1,3-bis-phosphoglycerate and
316 3-phosphoglycerate [34]. Enolase (AO1008_10057) is a ubiquitous enzyme that
317 catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis
318 [35], while pyruvate kinase (AO1008_06139) catalyzes the conversion of
319 phosphoenol-pyruvate to pyruvate and ATP in glycolysis.

320 The β-oxidation of fatty acids produces volatile flavor compounds and is of
321 particular importance for the overall flavor system [36]. Fatty acids are broken down to
322 acetyl-coenzyme A (CoA), which is used in ketone formation [37]. Some types of
323 β-oxidation enzyme, for example 3-hydroxyacyl-CoA dehydrogenase, were highly
324 expressed in *A. oryzae* 100-8. P-type ATPase is the enzyme of lipid pumps. The
325 acyl-CoA synthetase catalyzes substrates to their CoA esters, which then enter the
326 β-oxidation spiral (Table S2).

327 After the characterization of the transcriptomes, six genes were randomly
328 selected to confirm the results via qRT-PCR. The results of the qRT-PCR experiments
329 showed that changes in the levels of expression of these genes followed similar trends
330 to the transcriptome expression (Figure 4).

331

332 **Conclusion**

333 In this study, we compared the hyphal growth rates and koji flavors of *A. oryzae* 100-8
334 and *A. oryzae* 3.042. *A. oryzae* 100-8 grew faster and produced higher concentrations
335 of ketones, aldehydes, alcohols, esters and furan compounds, and lower amounts of
336 phenols, than *A. oryzae* 3.042. Genes that were found to be associated with the
337 regulation of hyphal growth and flavor formation, respectively, were identified by
338 transcriptome and qRT-PCR analysis. The levels of expression of the genes associated
339 with the formation of reactive oxygen species (ROS), intracellular $Ca^{2+}$ concentrations

and spore formation in *A. oryzae* 100-8 were lower than in *A. oryzae* 3.042. However, the genes associated with glycolysis, oxidative phosphorylation, amino acid metabolism, glycolysis and β-oxidation were twice as high in *A. oryzae* 100-8 as in *A. oryzae* 3.042. Our data clearly demonstrated that changes in gene expression levels can regulate the hyphal growth rate and the formation of flavor compounds.

These results may assist us to improve soy sauce flavors and shorten koji fermentation times in industrial production. However, the relationship between koji flavors and soy sauce flavors has not yet been fully ascertained. There are plans for further research in this area. In addition, the taste of koji should also be investigated alongside the volatile flavors to provide fuller information about the flavors; this is to be addressed shortly in another paper from our laboratory.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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425 **Table 1** Volatile compounds of soy sauce koji fermented by *A. oryzae* 100-8 and
426 3.042 from GC-MS analysis

| Flavors | *A. oryzae* 3.042 | | Area (%) | *A. oryzae* 100-8 | | Area (%) |
|---|---|---|---|---|---|---|
| | 30 h | 36 h | 42 h | 30 h | 36 h | 42 h |
| Ketone | 3.94 | 2.11 | 0.83 | 36.99 | 4.03 | 1.49 |
| Aldehyde | 13.87 | 11.74 | 0.1 | 4.16 | 70.18 | 42.61 |
| Alcohol | 3.86 | 5.58 | 3.18 | 12.11 | 20 | 10.49 |
| Ester | 0.3 | 0.2 | 0.56 | 0.19 | 1.4 | 1.65 |
| Furan compound | 4.49 | 0.55 | 0.23 | 17.65 | 1.64 | 0.73 |
| phenol | 15.82 | 44.69 | 60.2 | 17.19 | 1.25 | 27.87 |
| Hydrocarbon | 32.47 | 10.47 | 4.91 | 9.79 | 1.22 | 5.39 |
| Acid | - | 0.06 | - | - | 0.15 | - |

427

428 **Figure Captions**

429 **Figure 1.** COG functional analyses of differentially expressed genes of *A. oryzae*
430 3.042 and *A. oryzae* 100-8. A fast mycelial growth rate plays a major role in
431 regulating energy production, amino acid metabolism, nucleotide metabolism,
432 carbohydrate metabolism, coenzyme metabolism and lipid metabolism.

433

434 **Figure 2.** Comparison of the biomass yield (dry weight) of *A. oryzae* 3.042 and *A.*
435 *oryzae* 100-8 strains under identical conditions grown for 30 h, 36 h and 42 h.

436

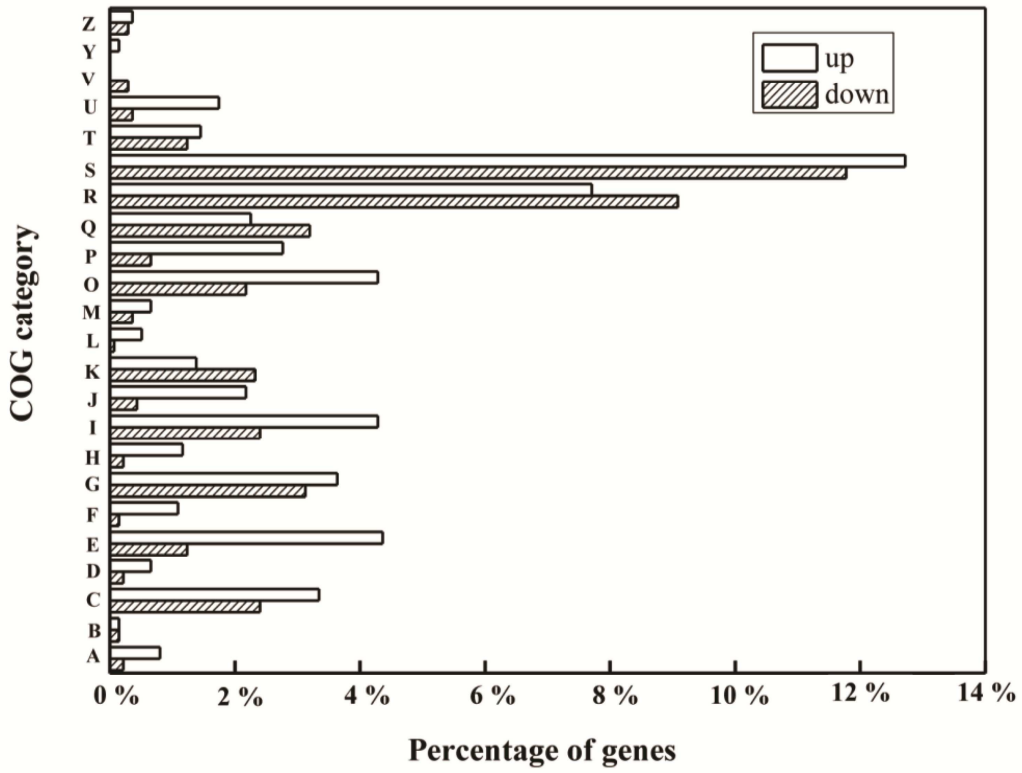
437 **Figure 3.** Comparison of the expression levels of $Ca^{2+}$ ions related genes which
438 regulate and coordinate the process of hyphal growth. a-e show the results for the
439 genes AO1008_03409, AO1008_09659, AO1008_09961, AO1008_07155 and
440 AO1008_04017, respectively.

441

442 **Figure 4.** Comparison of the expression levels of *A. oryzae* 100-8 and *A. oryzae* 3.042
443 via qRT-PCR analyses of six randomly selected genes (AO1008_10905,
444 AO1008_04017, AO1008_03499, AO1008_10057, AO1008_10013, AO1008_05605)
445 to confirm the transcriptomic results in the three fermentation stages (30 h, 36 h, 42
446 h).

447

448 **Figure 1**

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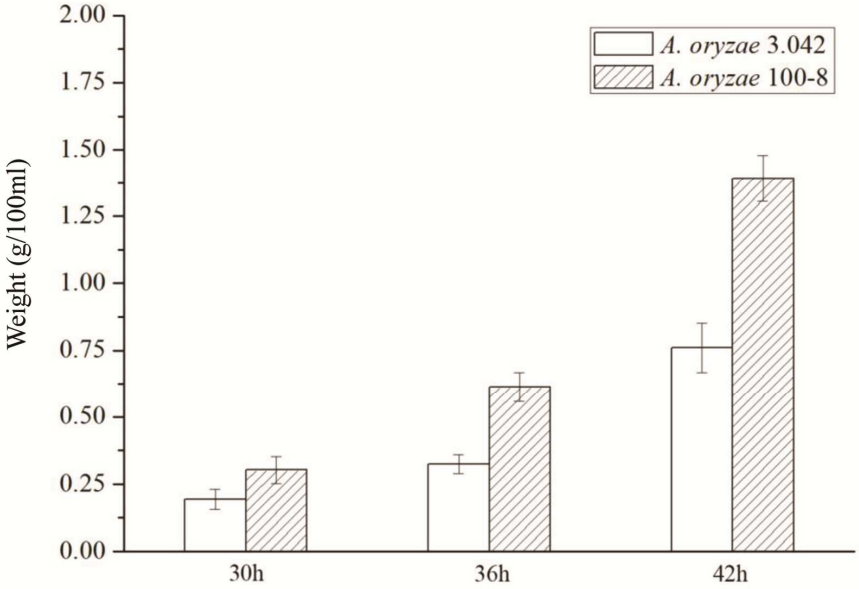
452 **Figure 2**

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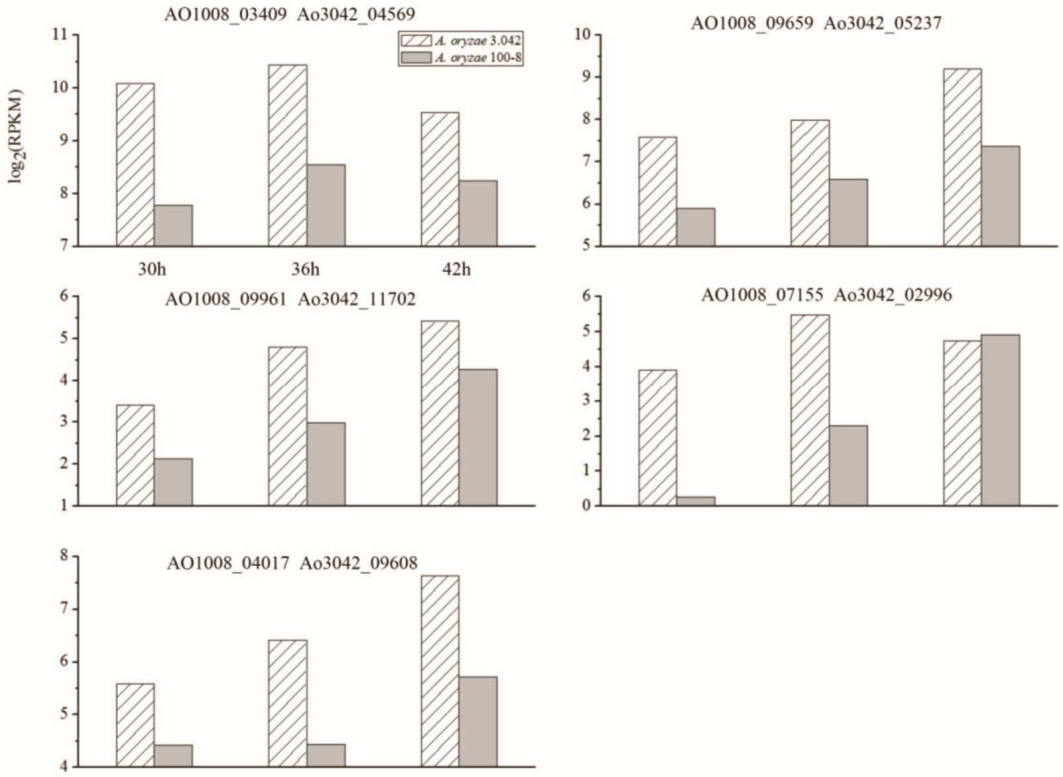
454

455 **Figure 3**

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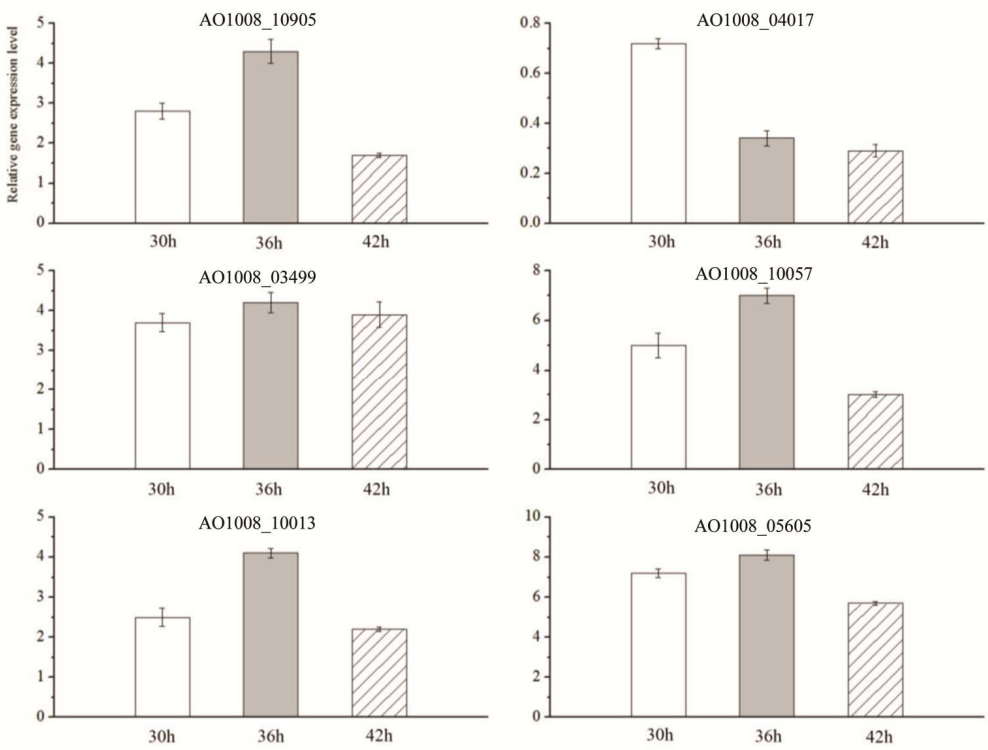
458 **Figure 4**

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