

Transcriptome Analysis of Two Strains of *Aspergillus oryzae* with Different Responses to Light

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Abstract

The production process of such traditional Japanese fermented foods as miso, shoyu and sake using *Aspergillus oryzae* has been highly optimized over a long history. However, new technology is needed to achieve further improvements in productivity or production efficiency. Light affects the gene expression and metabolic pathways of fungi. Thus, the development of technology that takes advantage of the light response of *A. oryzae* is important. In a previous study, we found that *A. oryzae* strains RIB40 and RIB1187 showed opposing manners of growth and conidiation in response to light. To elucidate the effect of light on genome-wide gene expression for both strains, we conducted transcriptome analysis by RNA sequencing. We also identified 453 differentially expressed genes (DEGs) between both strains by bioinformatic analysis. Of these 453 DEGs, we identified a total of 67 light-responsive DEGs between RIB40 and RIB1187. We also analyzed the expression patterns of fermentation-related digestive enzyme genes.

Discipline: Biotechnology

Additional key words: RNA sequence, gene expression, koji fermentation

Introduction

Aspergillus oryzae produces and secretes large amounts of many kinds of digestive enzymes during the production of such traditional Japanese fermented foods as miso, shoyu and sake. Grain fermented with *A. oryzae* is called *koji*. The balance of these enzymes in *koji* has a critical effect on the final product, and the balance of glycolytic and proteolytic enzyme activity during *koji* fermentation by *A. oryzae* can be adjusted to a considerable extent by temperature control. The production process of traditional Japanese fermented foods has been highly optimized over a long history of revision by predecessors, and thus achieving further improvements in productivity by extension of the prior art of temperature control is difficult. Additional artificially controllable parameters are also necessary.

Many studies have reported that filamentous fungi are responsive to light. The saprotrophic fungi *A. nidulans* is thought to live in soil in nature and use light as a signal of the soil surface environment (Rodriguez-Romero et al. 2010). When *A. nidulans* hyphae grow upward and reach the surface of the soil, fungi are exposed to UV radiation, desiccation or significant temperature changes by

sunlight. Light affects the gene expression and metabolic pathways of fungi (Tisch and Schmoll 2010). For example, *A. nidulans* produces asexual spores (conidia) under conditions of light, whereas it produces sexual spores (ascospores) under dark conditions (Rodriguez-Romero et al. 2010; Ruger-Herreros et al. 2011). There have been many studies on the molecular mechanisms of *A. nidulans* light response. Velvet proteins (fungus-specific regulators) make complexes and shuttle from the cytosol to nuclei according to the light conditions (Bayram et al. 2008; Bayram et al. 2010). White collar complex (WCC), the blue-light receptor, and phytochrome, the red-light receptor, can physically interact, and blue and red light induce conidiation in a comparable extent of white light by an additive effect (Purschwitz et al. 2009; Purschwitz et al. 2006). *A. nidulans* is a model organism of the genus *Aspergillus*, and most species in this genus such as *A. fumigates* (Kevin, K. et al. 2016), *A. flavus* (Calvo et al. 1999), and *A. parasiticus* (Calvo et al. 1999) conserve similar mechanisms of light response, and produce more conidia under light conditions than under dark conditions. In a study of *A. oryzae*, Hatakeyama et al. (2007) reported that *A. oryzae* RIB40 (a genome-sequenced strain of *A. oryzae*) conserved light signaling molecules and can

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respond to light, but in a manner opposite to that of other *Aspergillus* species (as *A. oryzae* produces more conidia under dark conditions than under light conditions). Previously, we showed that the light response of *A. oryzae* is strain-dependent, and that there are three types of light response among *A. oryzae* strains (Pushpa et al 2018): i) the same manner as other *Aspergillus* species; ii) the opposite manner to other *Aspergillus* species; and iii) no significant response. RIB40 belongs to type ii). Kumagai (1978) reported that the manner of light response of plant pathogens could be classifiable into three categories. Some plant pathogenic fungi show different light responses among strains of the same species (Kihara et al. 1997). By comparative genomics with other *Aspergillus* species, the ancestor of *A. oryzae* was assumed to be a plant pathogen (Kobayashi et al. 2007). Our previous results showed that *A. oryzae* strain RIB1187, isolated from soy sauce koji, makes more conidia under light conditions, that is, RIB1187 belongs to type i) (Pushpa et al 2018). The reason why RIB1187 and RIB40 behave in opposite manners in response to light remains unclear. Variations of the gene expression pattern induced by light stimuli are presumed to lead to the opposite behavior of the two strains. Transcriptome studies enable an investigation of genome-wide gene expression. RNA sequencing (RNA-seq) was recently used for transcriptome analysis of *A. oryzae* (He et al. 2018, Zhong et al. 2018). Here, we conducted transcriptome analysis of RIB1187 and RIB40 to elucidate the effect of light illumination on the genome-wide gene expression pattern of both strains of *A. oryzae*, and elucidate such metabolic changes as the enzyme production caused by light.

Materials and methods

1. Strains and media

A. oryzae maintained at the National Food Research Institute, Japan (NFRI 1599 = RIB40) and obtained from the National Research Institute of Brewing (RIB1187) were used in this study. Spore suspensions were prepared using sterile water solution containing 0.002% (v/v) Tween 80 and 0.5% (w/v) NaCl. Plate (2% agar) and liquid cultures were conducted on Czapek Dox (0.6% NaNO₃, 0.1% KH₂PO₄, 0.05% KCl, 2 mM MgSO₄, 1% glucose, and a 0.1% trace element solution consisting of 0.1% FeSO₄·7H₂O, 0.88% ZnSO₄·7H₂O, 0.04% CuSO₄·5H₂O, 0.01% Na₂B₄O₇·10H₂O, and 0.005% (NH₄)₆Mo₇O₂₄·4H₂O).

2. Light exposure conditions

White fluorescent light (Panasonic, FHF24SEW) was used for light illumination experiments. The photon

flux density at the surface of the culture was approx. 25 μmol m⁻² s⁻¹.

3. Total RNA preparation

The RIB40 and RIB1187 strains were incubated with liquid surface culture on CD liquid medium at 30°C for 34 h in the dark. Total RNA was isolated by ISOGEN (Nippon Gene, Tokyo, Japan) from 34-h mycelia exposed to white light (25 μmol m⁻² s⁻¹) for 10 min. or kept in the dark as per the manufacturer's instructions.

4. RNA sequencing

Library preparation, illumina sequencing (75-bp paired-end reads), and bioinformatic analysis were conducted by Bioengineering Lab. Co., Ltd. (Atsugi, Japan). Sequencing was performed by an Illumina NextSeq 500 with 2 × 76 bp. And sequencing reads were quality filtered by Sickle (ver. 1.33). Reads were mapped on the genome of *A. oryzae* (https://www.ncbi.nlm.nih.gov/assembly/GCF_000184455.2) by HISAT 2 (ver. 2.1.0). Reads mapped on exons were counted by featureCounts (ver. 1.5.3), and reads were normalized using reads per kilobase of exon per million mapped reads (RPKM) by iDEGES, with differentially expressed genes (DEGs) being identified by DESeq. The false discovery rate (FDR) was < 0.05.

The Venn diagram was drawn by Venny 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

Results and discussion

In a previous study, we showed that *A. oryzae* strain RIB1187 produces more conidia under light conditions than under dark conditions, whereas *A. oryzae* strain RIB40 produces more conidia under dark conditions than under light conditions. To elucidate the effect of light illumination on the genome-wide gene expression pattern of the two strains (RIB40 and RIB1187), RNA sequencing was performed on cultures of each strain kept in the dark for 34 h or illuminated by white light for 10 min. following 34 h of culture in the dark. More than nine million high-quality reads per sample were generated and aligned on the whole reference genomic sequence. More than 97% high-quality reads were mapped on the genome of *A. oryzae*.

Clustering analysis of the total gene expression of each sample was conducted (Fig. 1). The cluster dendrogram showed that the difference between both strains was greater than the difference between culture conditions. From the dendrogram, it was expected that more differentially expressed genes (DEGs) would be detected by comparing strains under the same culture

condition than by comparing different culture conditions of the same strain. DEGs between both strains were identified by statistical analysis. Under the dark culture condition, 237 genes were identified as DEGs between RIB40 and RIB1187, and under the light-stimulated culture condition, 216 genes were identified as DEGs. Under the dark culture condition, 106 genes were expressed at higher levels in RIB1187 than in RIB40, while under the light-stimulated culture condition, 100 genes were expressed at higher levels in RIB1187 than in RIB40. However, the genes expressed at higher levels in RIB1187 than in RIB40 under both dark and light-

stimulated culture conditions shared 90 common genes, and the genes expressed at higher levels in RIB40 than in RIB1187 under both dark and light-stimulated conditions shared 103 common genes. This showed that the 90 and 103 common DEGs mentioned above may result from variation of the strains, and thus may have less relevance to the light response. Omitting these genes, 67 genes were identified as light-responsive DEGs between RIB40 and RIB1187 (Fig. 2). These results were concordant with the results of clustering analysis (i.e., a greater difference between both strains than between culture conditions).

To estimate the differences in biological function of each strain under dark and light-stimulated culture conditions, the 67 light-responsive DEGs were analyzed by gene ontology (GO) enrichment analysis. GO terms were classified into three categories: biological process (Fig. 3), cellular component (Fig. 4), and molecular function (Fig. 5). Biological process (GO:0008150), cellular component organization (GO:0016043), and molecular function (GO:0003674) were prominent among all conditions and showed a similar pattern. The oxidation–reduction process (GO:0055114) was most enriched in DEGs that were expressed at higher levels in RIB40 than in RIB1187 under the dark culture condition. Conversely, the oxidation–reduction process was enriched to a similar extent in DEGs that were expressed more in RIB1187 than in RIB40 under both dark and light-stimulated culture conditions. Regulation of transcription, DNA-templated (GO:0006355), sterigmatocystin biosynthetic process (GO:0045461), DNA binding (GO:0003677), electron carrier activity (GO:0009055), heme binding (GO:0020037), iron ion binding (GO:0005506), oxidoreductase activity acting on

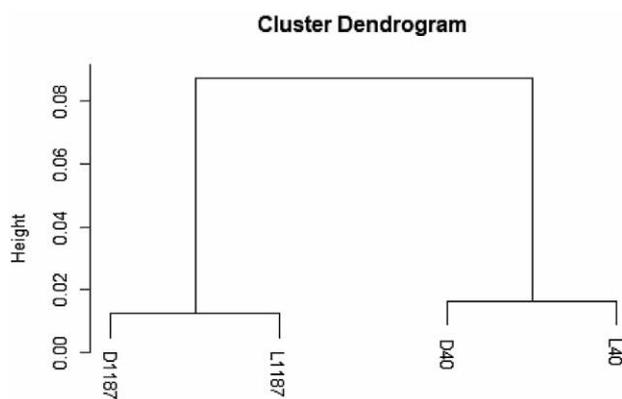


Fig. 1. Dendrogram of clustering analysis of whole gene expression pattern of four samples

1187D: gene expression pattern of strain RIB1187 under dark condition; 1187L: gene expression pattern of strain RIB1187 under light-stimulated condition; 40D: gene expression pattern of strain RIB40 under dark condition; 40L: gene expression pattern of strain RIB40 under light-stimulated condition.

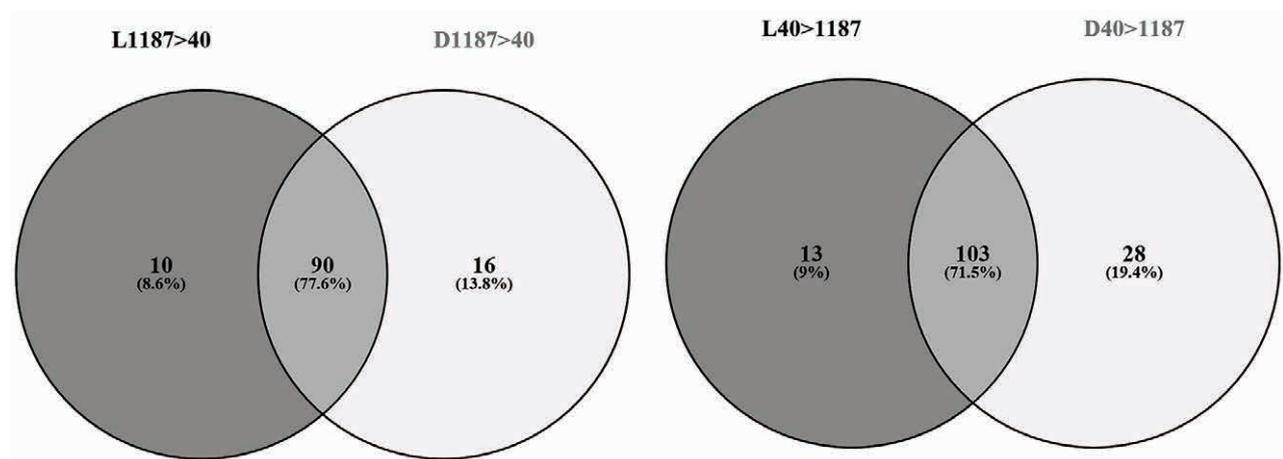


Fig. 2. Venn diagrams of DEGs

L1187 > 40: DEGs expressed at higher levels in RIB1187 than in RIB40 under light-stimulated condition; D1187 > 40: DEGs expressed at higher levels in RIB1187 than in RIB40 under dark condition; L40 > 1187: DEGs expressed at higher levels in RIB40 than in RIB1187 under light-stimulated condition; D40 > 1187: DEGs expressed at higher levels in RIB40 than in RIB1187 under dark condition. L1187 > 40 shared 90 DEGs with D1187 > 40. L40 > 1187 shared 103 DEGs with D40 > 1187.

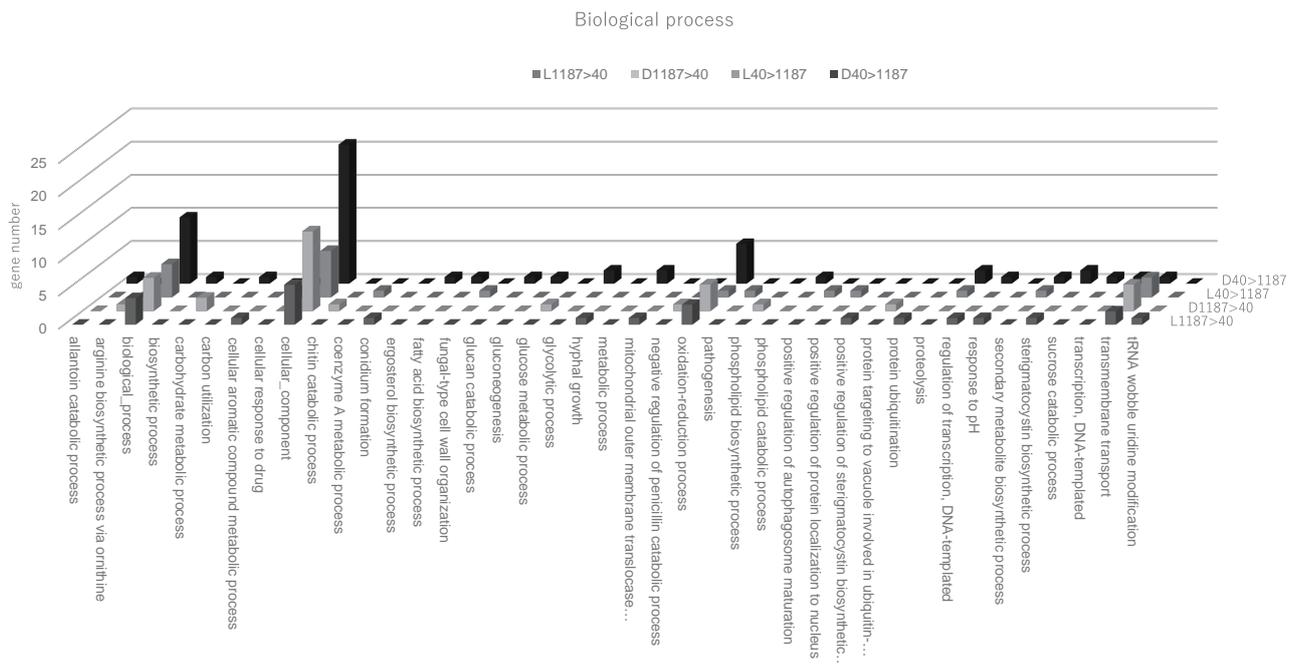


Fig. 3. GO (biological process) enrichment analysis of light-responsive DEGs between RIB40 and RIB1187
The frequency of each GO term for individual DEGs is shown.

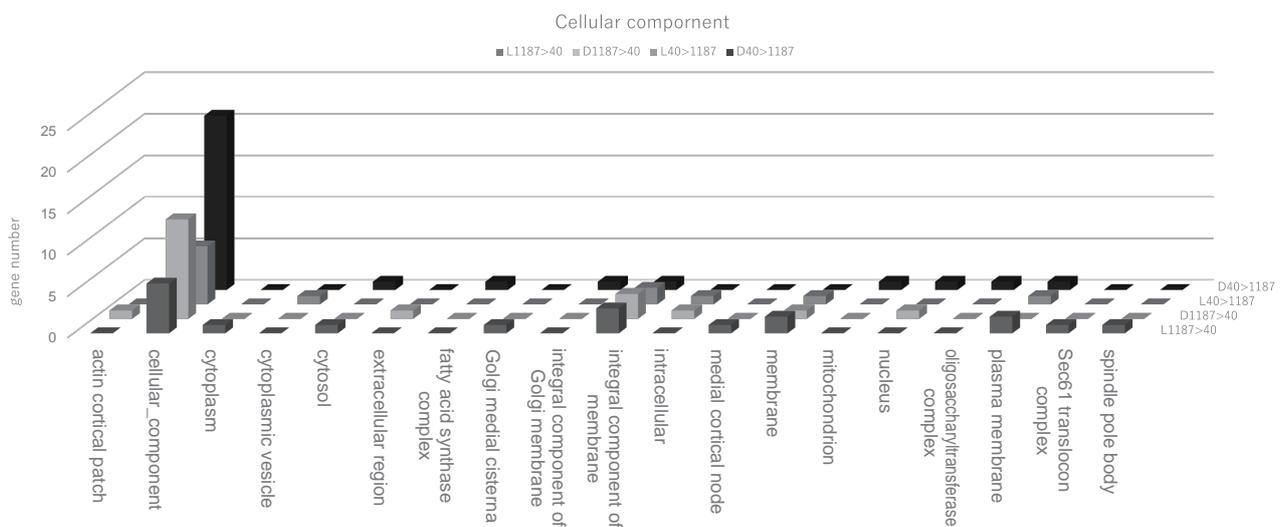


Fig. 4. GO (cellular component) enrichment analysis of light-responsive DEGs between RIB40 and RIB1187
The frequency of each GO term for individual DEGs is shown.

paired donors with incorporation or reduction of molecular oxygen (GO:0016708), and zinc ion binding (GO:0008270) were enriched in both DEGs expressed at higher levels in RIB1187 than in RIB40 under the light-stimulated culture condition, and in RIB40 than in RIB1187 under the dark culture condition. As RIB1187 produces conidia under the light-stimulated condition and RIB40 produces conidia under the dark condition, these processes are likely to have some relation to

conidiation.

To study the effect of light on the fermentation process, the expression of genes coding for fermentation-related digestive enzymes was analyzed. The descriptions of mapped and normalized data were used to determine 214 genes encoding these enzymes. There were 23 amylolytic genes, 95 other glycolytic genes excluding amylolytic genes, 68 proteolytic and glutaminase genes, and 28 other genes including lipases and phosphatases.

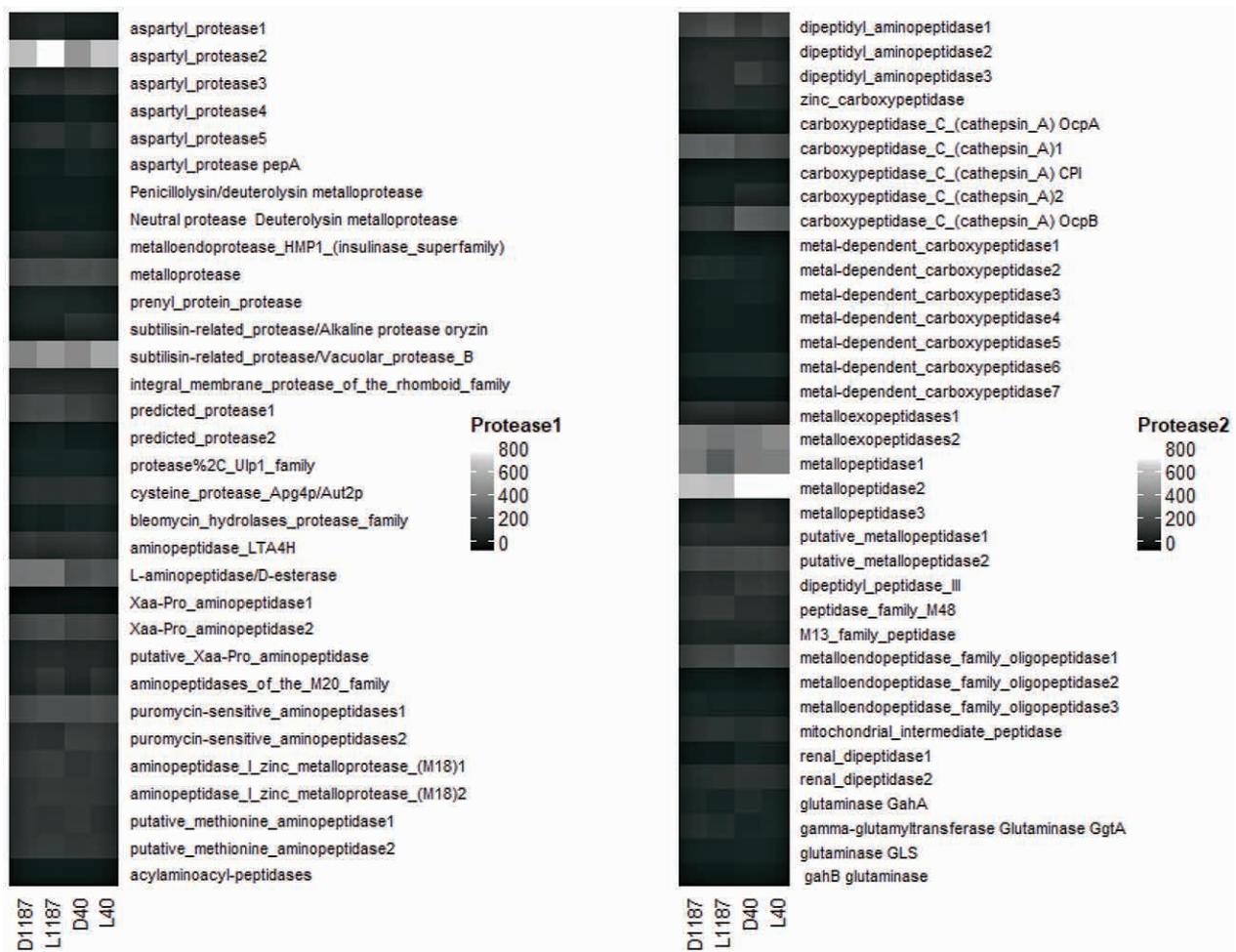


Fig. 7. Heatmap of the expression of proteolytic genes and glutaminase genes

highly expressed genes in RIB1187 (aspartyl protease 2 and subtilisin-related protease) were upregulated under the light-stimulated condition in both strains. The RPKM of aspartyl protease 2 in RIB1187 was 585 under the dark condition and 718 under the light-stimulated condition. The RPKM of aspartyl protease 2 in RIB40 was 458 under the dark condition and 602 under the light-stimulated condition. The metallopeptidases including metallopeptidase 2—the second most highly expressed gene in RIB1187—showed a tendency of higher expression under the dark condition in RIB1187. For example, the RPKM of metallopeptidase 1 in RIB1187 was 337 under the dark condition and 260 under the light-stimulated condition. It seems that most carboxypeptidases were constantly expressed, regardless of dark or light-stimulated condition. Figure 6 B shows the expression of the glycolytic genes, excluding the amylolytic genes. The expression patterns of genes in this category were randomly distributed, making it difficult to find any regularity. Figure 8 shows the expression of phosphatases, lipases, and other enzymes. Eleven of the 28 genes were

upregulated under the light-stimulated condition in both strains, while 13 of the 28 genes were downregulated under the light-stimulated condition in both strains. Figure 9 shows the expression of the top 50 high expressed genes (excluding amylase) in RIB1187 under the light-stimulated condition. Seven of the 50 genes including various kinds of enzymes were upregulated in RIB1187 and downregulated in RIB40 under the light-stimulated condition, whereas 14 of the 50 genes including nine peptidases and two proteases were downregulated in RIB1187 and upregulated in RIB40 under the light-stimulated condition. However, not all peptidases and proteases showed a similar tendency of expression. The expression analysis showed that considerable numbers of genes encoding fermentation-related enzymes were upregulated or downregulated by light.

In conclusion, we have shown the possibility of controlling the enzyme activity of *A. oryzae* during the production of fermented foods by changing the light conditions, because light can control the gene expression of enzymes of *A. oryzae*. Industrial cellulase producer

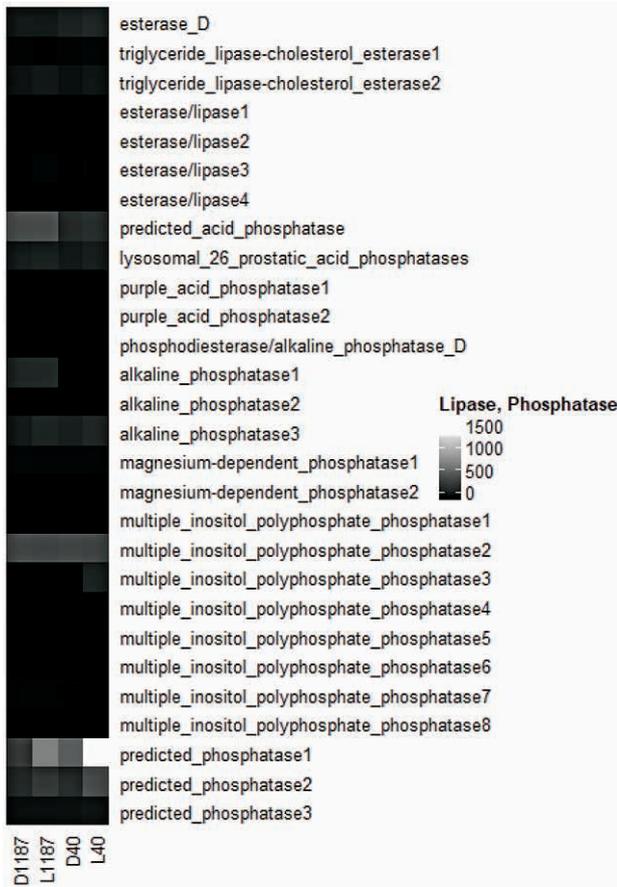


Fig. 8. Heatmap of the expression of phosphatases, lipases, and other enzyme genes

Trichoderma reesei expresses a greater amount of cellulase under dark conditions than under light, and adenylate cyclase and protein kinase A are involved in light-modulated cellulase gene expression (Schuster et al. 2012). Cellulase is also regulated by light in *Neurospora crassa* via the blue-light receptor WCC (Schmoll et al. 2012). Taking these studies into account, our results contribute not only to the production of traditional Japanese fermented food, but also to the enzyme industry. We used RNA from liquid surface cultures to ensure that we obtained the first RNA-seq data under dark and light conditions; however, traditional Japanese fermented foods are made by solid state fermentation (SSF) on grain. In future studies, we plan to obtain transcriptome data from SSF. In this study, we showed the RNA sequence data without replication. Overall gene expression tendency did not conflict with other gene expression data of *A. oryzae* (i.e., extremely high-level expression of amylases). We used the DESeq method of the R/Bioconductor package for statistical analysis of our RNA sequence data. DESeq allows an analysis of experiments with no biological replicates under one or both of the conditions (Anders and Huber, 2010).

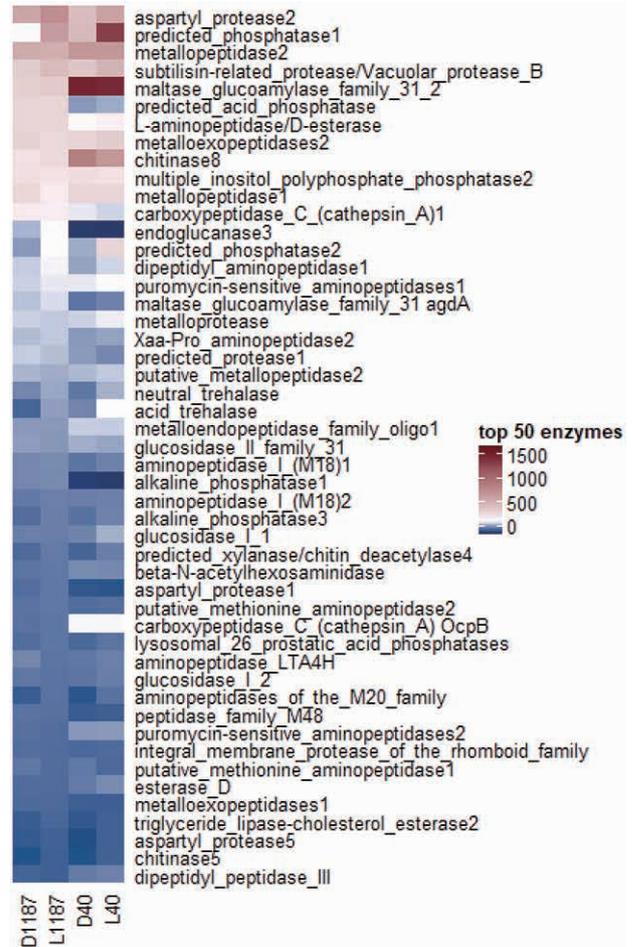


Fig. 9. Heatmap of the expression of top 50 high expressed genes (excluding amylase) in RIB1187 under light-stimulated condition

Although more biological replications may improve the accuracy of statistical analysis, our data is still useful for the first screening of light-regulated genes of *A. oryzae*. We have obtained a great deal of data on the effect of light on all 12,000 genes of *A. oryzae*, which will give us insights into the molecular basis of the variation of light response among the strains of *A. oryzae*.

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