

***Aspergillus Oryzae* Strain with Improved Conidiation after Light Stimulation**

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Abstract

The influence of light on the development of asexual spores is an example to elucidate the effect of environmental factors on fungi. Well-studied fungus *Aspergillus nidulans* produces more conidia under the light condition than under the dark condition, whereas RIB 40, a standard strain of *A. oryzae*, produces more conidia under the dark condition. In this study, we found that an *A. oryzae* strain (RIB 1187) responded positively to incubation under light and resulted in better conidia count as compared to that in the dark. We studied the gene expression of RIB 1187 and found that four homologous genes of *A. nidulans* (up-regulated genes by light) were also up-regulated in RIB 1187 under the light condition.

Discipline: Biotechnology

Additional key words: gene expression, light response, asexual development, filamentous fungi

Introduction

Light is one of the most crucial environmental signals for regulating the developmental and physiological processes in most organisms. Diverse filamentous fungi live in soil under dark and stable conditions. But upon reaching the surface of the soil, fungi are exposed to UV radiation, desiccation or significant temperature changes. Therefore, most fungi use light as a signal of the surface environment in adjusting to a new habitat (Rodriguez-Romero et al. 2010). In adapting to the environment, fungi drastically change both gene expression and metabolic pathways (Rodriguez-Romero et al. 2010, Tisch and Schmoll 2010). Fungi produce asexual structures toward the open air to facilitate spore dispersal in response to light (Rodriguez-Romero et al. 2010, Ruger-Herreros et al. 2011). Conidium (a fungal asexual spore) is important structure because it enables fungi to expand their habitat and survive through unfavorable times for fungi. In recent years, considerable progress has been made in elucidating the photoresponses and mechanisms responsible for induction of the asexual development in *Aspergillus nidulans* and *Neurospora crassa*

(Bayram et al. 2008, Bayram et al. 2010, Corrochano 2007, Purschwitz et al. 2009, Purschwitz et al. 2006). In the *A. nidulans* study, microarray analysis elucidated 425 up-regulated genes and 108 down-regulated genes by illumination, and most of the genes among the top 15 up-regulated genes were related to conidia or conidiation (Ruger-Herreros et al. 2011). However, not all fungi behave according to the standard manner of the “soil/surface transition model” as shown in prominent works with *N. crassa* and *A. nidulans* (Rodriguez-Romero et al. 2010). Kumagai (1978) classified the mode of light response in conidiophore induction and conidial development of several fungi including plant pathogens into three categories. Hatakeyama et al. (2007) investigated *A. oryzae* RIB 40 (a genome sequenced strain of *A. oryzae*), and found that it conserved light signaling molecules and can respond to light, but in a manner opposite to that of *A. nidulans* (as *A. oryzae* produces more conidia under the dark condition than under the light condition). In this paper, we screened *A. oryzae* strain RIB 1187 that showed a different light response to RIB 40. We also studied the expression of orthologous genes of up- and down-regulated genes by light in *A. nidulans*.

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Materials and methods

1. Strains and media

Various fungal spp. of *A. oryzae* maintained at the National Food Research Institute, Japan (NFRI 1137 and NFRI 1599 = RIB 40) and obtained from the National Research Institute of Brewing (RIB 163, 306, 430, 915, 1108, 1172, 1178, 1187) 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. Culture conditions

White fluorescent light (Panasonic, FHF24SEW) was used for light illumination experiments. The photon flux density at the surface of the culture was approximately 25 μmol m⁻² s⁻¹. Linear growth of the fungal mycelium was measured daily from the area of inoculation along four diameters, with the mean of these values being recorded.

3. Conidia counting

Mycelia disks (5 mm in diameter) from the agar plates were taken and homogenized with 1 mL of water with 0.002% (v/v) Tween 80 and 0.5% (w/v) NaCl. Conidia were counted using a hemocytometer under a light microscope. Spore data were statistically compared by analyzing variance using the student's t test for comparison. Statistically, significant mean values, indicated in the figures, are significant at $P < 0.05$.

4. Biomass measurement

The fungi were grown on a Czapek Dox (CD) liquid medium surface, the mycelial mat was filtered using Whatman filter paper No. 1 (GE Healthcare Japan, Tokyo), and then the biomass was determined after being heated at 105°C to a constant weight.

5. RNA preparation and quantitative reverse transcription polymerase chain reaction

The strain RIB 1187 was incubated with liquid surface culture on CD liquid medium at 30°C for 34 h. 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. Reverse transcription and real-time PCR were performed with a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green® (Invitrogen, Carlsbad, CA, USA). Relative quantification was done using the 2^{-ΔΔCt}

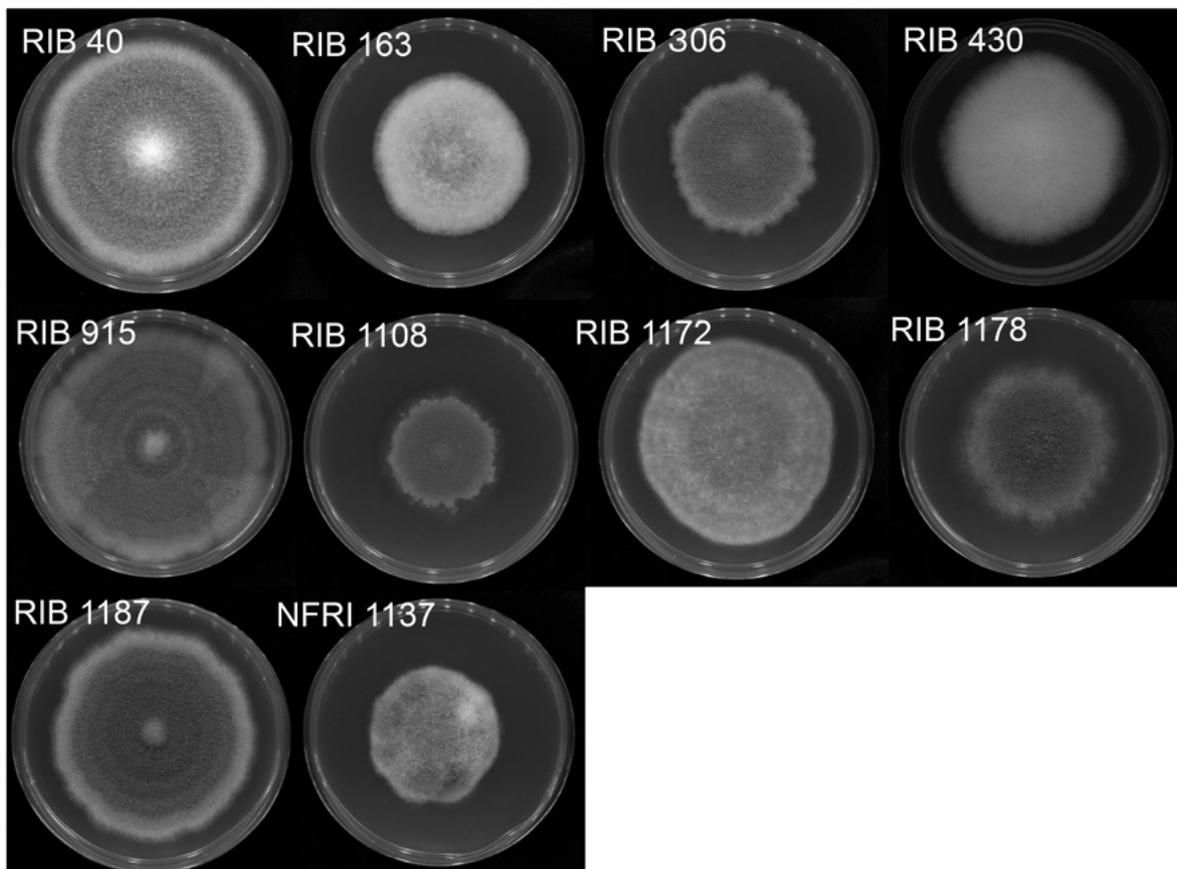
technique (ABI User Bulletin 2). Histone H1 was used as an endogenous standard. Each sample was analyzed three times. Table 1 lists the primers used in this experiment.

Results and discussion

Fungi respond to light diversely in terms of growth and metabolic pathways. In this study, we carried out 12 h / 12 h of white light and dark incubation of the growing cultures of ten *A. oryzae* strains (RIB 40, RIB 163, RIB 306, RIB 430, RIB 915, RIB 1108, RIB 1172, RIB 1178, RIB 1187 and NFRI 1137) (Fig. 1). These strains were chosen at random according to "clade" as defined by Iwashita et al. (2011). Iwashita et al. (2011) performed array comparative genomic hybridization (CGH) analysis of 55 strains of *A. oryzae* and phylogenetic analysis. According to their classification, RIB 40 and RIB 1172 belong to the "RIB 40 clade." RIB 306 belongs to the "So3 clade," RIB 915 belongs to the "So2 subclade," and RIB 1187 belongs to the "So1 subclade," which are used in soy sauce fermentation. RIB 430 belongs to the "New Sake clade," which is used in a new type of dry rice wine fermentation. RIB 163, RIB 1108 and RIB 1178 belong to a clade without any specification. NFRI 1137 was not studied by Iwashita et al. and is used in rice wine brewing. Four strains of *A. oryzae* (RIB 430, RIB 1108, RIB 1172 and RIB 1178) showed a uniform appearance or subtle changes in hyphal growth and indicated that they do not regulate conidiation according to the light condition. Conversely, the other six strains (RIB 40, RIB 163, RIB 306, RIB 915, RIB 1187 and NFRI 1137) caused the subsequent formation of a dense and weakly colored circular/ring of conidia-bearing mycelium on agar plate culture, and we selected these strains as candidates that can regulate conidiation according to the light condition. The conidia count reveals that RIB 1187 incubated in light had 65.56×10^6 per dish and that dark incubation had no conidium. The variation in conidia count between light and dark conditions was statistically significant (Fig. 2). As previously reported by Hatakeyama et al. (2007), RIB 40 produced more conidia under the dark condition (Fig. 2). The other four strains did not show any significant difference between both light and dark conditions. RIB 1187 showed the opposite light response in conidiation compared to RIB 40 (Fig. 3), thus demonstrating better growth when incubated in light than in the dark. Both point inoculation and the spread culture photo in Fig. 3 show that RIB 40 produced more conidia in the dark than in light, whereas, RIB 1187 produced more conidia in light than in the dark. Particularly regarding the spread culture of RIB 1187, we hardly observed conidia with the exception of trace amounts of conidia on the periphery of a solid medium. This observation was in agreement with the data on conidia counting shown in Fig. 2. As mentioned above,

Table 1. Primers used in qRT-PCR

Designation	Sequence in 5 → 3 order
AoconF F	GCA GAA CAA GGA TCC GAT GAG
AoconF R	CGT CTT GCT TCG TCC GAT ACA
AO090120000385F	CTGGACTTAACAAGGGTTCTTTGAC
AO090120000385R	AGA CCA CCT GCG GAC CTC TT
AO090012000521F	CAG CGA GAA CAC CGA CAA GA
AO090012000521R	CTA GCG ACT GGT GGC TGA TCA
AO090038000462F	CGG TTA AGG TGA GGG ATG TGT T
AO090038000462R	CGA GAA TAT CCT CCG CAG AAT C
AO090023001009F	TTG CTC TGG GCA CGT TTT TC
AO090023001009R	GCG CAC GAT GGT AAG TGT CA
AolaeA F	ACC GGG ATA TGG GCA ATT G
AolaeA R	GGG CTG TAT GGG AGC AAG GT
AoveA F	CAT TGC GGA GCA AGG TTG T
AoveA R	TAG TCA TCG GTG CGC TTC TCT
Histone F	GAC AAC ATC CAG GGT ATC ACT AAG C
Histone R	CGG GTC TCC TCG TAG ATC ATG GCA G

**Fig. 1. Effect of light on 10 strains of *A. oryzae***

Ten strains of *A. oryzae* were point-inoculated and cultured under 12 h light/12 h dark conditions. Top row: RIB 40, RIB 163, RIB 306, RIB 430; middle row: RIB 915, RIB 1108, RIB 1172, RIB 1178; bottom row: RIB 1187 and NFRI 1137.

RIB 1187 showed a positive light response, RIB 40 showed a negative light response, and the other eight strains showed no light response relative to the regulation of conidiation. These results showed that the effect of light illumination on conidiation is different among the *A. oryzae* strains, and that there is no apparent relation with phylogenetic “clade” in terms of light response. RIB 1187 was thus selected for further analysis.

In order to evaluate the effect of light on the growth of RIB 1187, we measured the colony diameter and biomass. The growth diameter under the light condition was 51 ± 0.2 mm as compared to 48 ± 0.1 mm under the

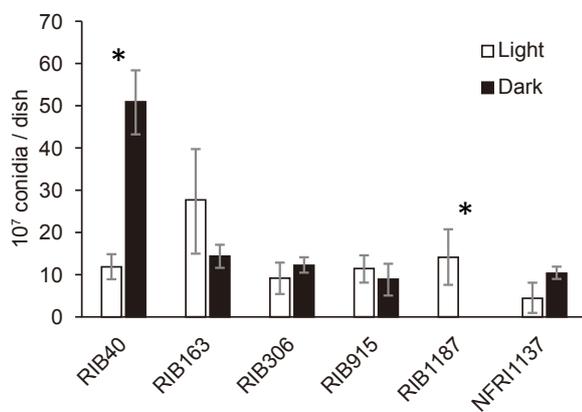


Fig. 2. Conidia counting under light and dark conditions
 Conidia were counted as described in the text. The graph shows the average of three independent experiments. Error bars represent standard deviation. White bars: conidia counting in light; Solid bars: conidia counting in the dark. The difference between the two culture conditions was evaluated in terms of statistical significance by the t-test. * denotes $0.05 > P$ value.

dark condition. The RIB 1187 biomass grown on minimal media weighed 43.2 ± 0.4 mg when grown under the light condition, and weighed 40 ± 0.1 mg when grown under the dark condition, thereby showing 7.4% ($P < 0.05$) more growth under the light condition. Light has various effects on not only asexual development but also on the growth and metabolism of fungi (Tisch and Schmoll 2010, Atoui et al. 2010). Our results also showed that light could affect not only asexual development but also the growth rate of RIB 1187.

To investigate gene regulation by light, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of total RNA from RIB 1187 was performed. We analyzed the homologues of nine up-regulated genes, a neutral gene (*laeA*), and a down-regulated gene (*veA*) as reported in *A. nidulans* (Ruger-Herrerros et al. 2011). In the *A. nidulans* study, the most up-regulated gene (*ccgB*) and second most up-regulated gene (*ccgA*) (Ruger-Herrerros et al. 2011), which are homologues of the *N. crassa ccg-1* gene, a clock-controlled and glucose-repressed gene of unknown function (Arpaia et al. 1995), were not found in the *A. oryzae* genome (Machida et al. 2008). We conducted RT-PCR on nine of the 11 genes described above, excluding *ccgA* and *ccgB*. However, homologous genes of the 9th up-regulated gene (*cetJ*) (Osheroov et al. 2002) and 13th up-regulated gene (*conJ*) (Ruger-Herrerros et al. 2011, Suzuki et al. 2013) were found in the *A. oryzae* genome, *A. oryzae* homologues of *cetJ* and *conJ* were scarcely expressed under both light and dark conditions, and were undetectable in RT-PCR (data not shown). The *A. oryzae* homologue of the 89th up-regulated gene (*conF*) (Suzuki et al. 2013) showed expression two times higher (at a relative expression ratio of 1: 0.54) in light than under the dark condition (Fig. 4).

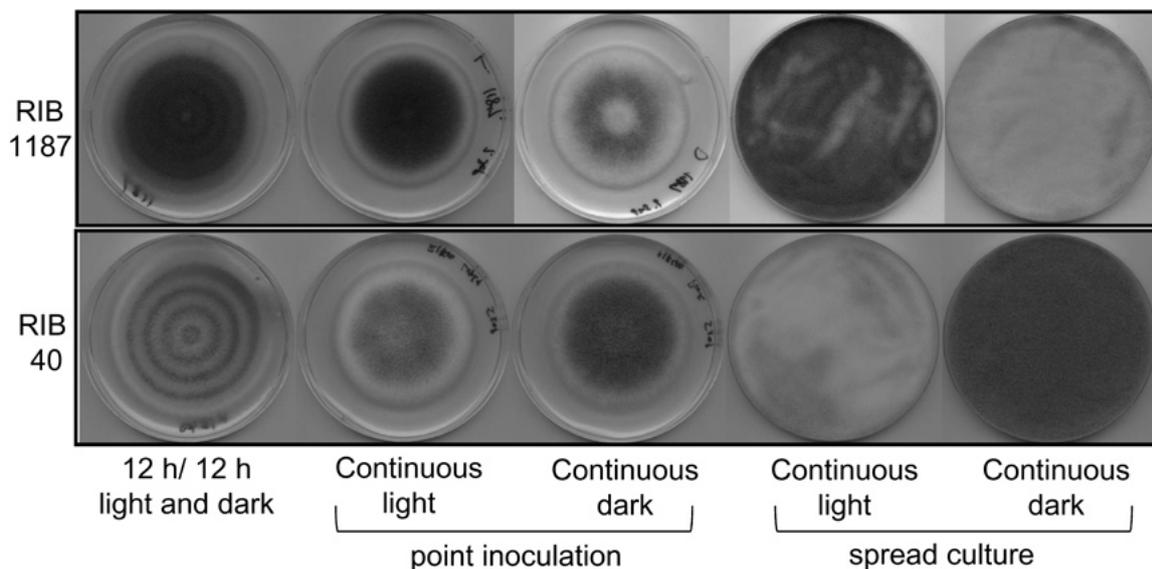


Fig. 3. Light effect on conidiation of strains RIB 1187 and RIB 40

cetJ, *conF* and *conJ* encode a conidia-specific small protein of unknown function. AO090120000385 encoding solid-state culture expressed protein (Aos23), which is the *A. oryzae* homologue of the 3rd up-regulated gene (AN0045), AO090012000521 (unknown function), which is the *A. oryzae* homologue of the 4th up-regulated gene (AN0693), and AO090038000462 (unknown function), which is the *A. oryzae* homologue of the 5th up-regulated gene (AN8339), showed moderate up-regulation under the light condition than under the dark condition (Fig. 4). The ratios of relative gene expression were 1: 0.70, 1: 0.67 and 1: 0.55, respectively. AO090023001009 (unknown function), the *A. oryzae* homologue of the 6th up-regulated gene (AN4299), showed no significant difference (at a relative expression ratio of 1: 0.95) between under both light and dark conditions. The *A. oryzae* homologue of *laeA* (a neutral gene in *A. nidulans*)

and the *A. oryzae* homologue of the most down-regulated gene (*veA*) in *A. nidulans* showed moderate down-regulation (at relative expression ratios of 1: 1.17 and 1: 1.21) under the light condition. Among the five homologues of the up-regulated gene in *A. nidulans*, four genes were also up-regulated in *A. oryzae* and the most down-regulated gene (*veA*) in *A. nidulans* was also down-regulated in RIB 1187. In previous our study, we showed that the most up-regulated genes were expressed quickly in 5 to 10 minutes under the light condition (Suzuki et al. 2013). These results showed that a common quick light response system may exist in RIB 1187 and *A. nidulans*. Further, we investigated the light regulation of the orthologous genes involved in the initiation of conidiation in *A. nidulans* (Fig. 4). FluG is considered to be at the start of the developmental program, FlbA is an RGS domain protein that controls the GTPase

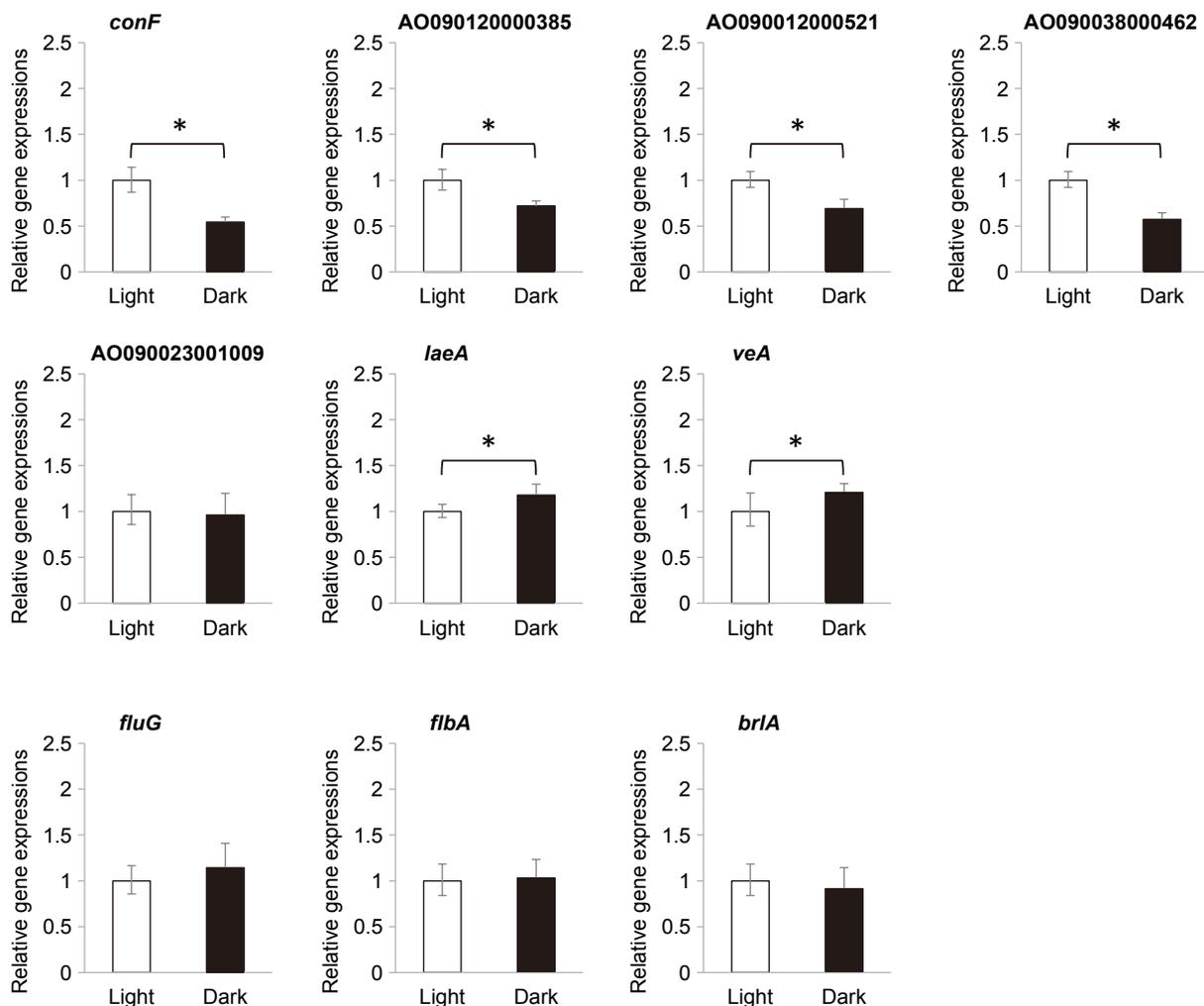


Fig. 4. Comparison of relative gene expressions under light and dark conditions by real-time PCR.

White bars: gene expression in light was considered to be 1.0. Solid bars: gene expression in the dark. Error bars represent standard deviation. The difference between the two culture conditions was evaluated in terms of statistical significance by the t-test. * denotes $0.05 > P$ value.

activity of FadA G α , and BrlA is the first component of the central developmental pathway. The relative gene expression ratio of *brlA* was 1: 0.89, while that of *flbA* was 1: 1.03, and that of *fluG* was 1: 1.14 under both light and dark conditions. No significant difference was observed in gene expression levels of these three genes between light and dark conditions. In the previous report of Hatakeyama et al., the expression level of *brlA* between light and dark conditions showed no significant differences (Hatakeyama et al. 2007). From these results, we could not explain why RIB 1187 showed a different light response to RIB40.

In conclusion, we found a strain (RIB 1187) that showed the opposite manner of light response to RIB 40. Now we have two different types of model strains to elucidate the conidiation regulation mechanism of *A. oryzae* by light — RIB 40 and RIB 1187. In a future study, we will compare the comprehensive gene expression of RIB 1187 with that of RIB40 under light and dark conditions, in order to elucidate why both strains showed the opposite manner of light response. Studies on the environmental factors that affect the growth and metabolism of filamentous fungi are necessary because such fungi can contribute to the development of artificial control technology for cellular metabolism (Velmurugan et al. 2010).

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