Visualization of Nuclei in Aspergillus oryzae with EGFP and Analysis of the Number of Nuclei in Each Conidium by FACS

Jun-ichi Maruyama, Harushi Nakajima, and Katsuhiko Kitamoto*

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Received December 12, 2000; Accepted February 15, 2001

Aspergillus oryzae has been reported to form multinuclei. In order to analyze nuclei in living cells, we developed an expression system of the A. nidulans histone H2B protein tagged by EGFP (H2B::EGFP). In both A. oryzae niaD300 and A. nidulans FGSC89 transformants expressing H2B::EGFP, fluorescence was detected in nuclear regions of hyphae and conidia. While a conidium contained only one fluorescent spot in the A. nidulans transformant, approximately 66% of conidia had two, 24% had one, and 10% had three or more in the A. oryzae transformant. The conidia expressing H2B::EGFP were put through FACS (fluorescence-activated cell sorting) analysis and two sharp peaks, corresponding to one and two nuclei in each conidium, were noted in the A. oryzae transformant. In addition, the A. oryzae uninucleate conidia that were successfully isolated by FACS reproduced conidia with almost the same number distribution of nuclei as that of the original. Conidia of five A. oryzae strains used in sake brewing were scored for the number of nuclei, showing that a varied number of nuclei existed in each conidium and some strains had a small number of uninucleate conidia.

Key words: Aspergillus oryzae; nuclei; conidia; EGFP; FACS

Aspergillus oryzae has been important in the Japanese traditional food industry such as in sake, soy sauce and miso manufacture, as well as production of commercial enzymes. Recently, molecular genetic approaches in A. oryzae have facilitated the production of valuable heterologous proteins and fundamental investigations. In spite of its fermentative and industrial use, its molecular mechanism of cellular organization has been poorly understood because A. oryzae was difficult to study with classical genetics as well as molecular genetics. In the fermentative industry, strain breeding of A. oryzae has been attempted for many years in order to enhance the productivity of hydrolytic enzymes that increase the utilization efficiency of the raw materials and improve the quality of final products.1) Mutational treatment has been tried; however, this is effective in some cases but tends to result in deleterious effects on the strain, such as poor growth and defective conidiation. It has been reported that A. oryzae forms conidia with multinuclei, which are considered to attenuate the effects of mutagenesis and to result in genetic stability during fermentative processes and problems in classical genetic application and industrial strain breeding. Although this characteristic of A. oryzae is of great significance, to date few studies on the number of nuclei in each conidium have been reported.

Green fluorescent protein (GFP) of the jellyfish Aequorea victoria is a bioluminescent substance and has been frequently used as a genetic reporter for monitoring gene expression and protein localization in many organisms.2) Red-shifted variants of GFP have been developed, resulting in much brighter fluorescence than wild-type. Among them, GFP(S65T) has the Ser-65 to Thr mutation and a single, shifted peak at 490 nm3) and EGFP (enhanced GFP) contains the double-amino-acid substitutions Phe-64 to Leu and Ser-65 to Thr, the excitation maximum peak of which, 488 nm, is appropriate for FACS (fluorescence-activated cell sorting) analysis.4) A synthetic version of GFP(S65T), sfGFP(S65T), was developed for use in plants5) and has been expressed successfully in a number of fungi, including Aspergillus nidulans.6) In A. nidulans the molecular mechanisms of nuclear migration have been largely investigated7,8) and visualization of nuclei has been performed by expression of the GFP-fused proteins.5,9-11) On the contrary, the use of GFP expression in A. oryzae has not been reported.

In this study we focused on the number of nuclei in each conidium of A. oryzae. Expression of EGFP resulted in successfully visualizing nuclei in hyphae and conidia of A. oryzae. FACS analysis was used for the first time in research on living filamentous fungi expressing GFP and this enabled us to isolate and analyze uninucleate conidia of A. oryzae.

1) To whom correspondence should be addressed. FAX: +81-3-5841-8033; E-mail: akitamo@mail.ecc.u-tokyo.ac.jp
Materials and Methods

Strains, plasmids, and media. A. oryzae niaD300 (niaD·)2) and A. nidulans FGSC89 (blA1; argB2, Fungal Genetics Stock Center) were used as recipient strains for expression of H2B:EGFP. A. oryzae RIB40, RIB128, RIB177, RIB430, RIB609, and RIB6473 (gifts from National Research Institute of Brewing, Higashi-Hiroshima, Japan) were used to score the number of nuclei. Escherichia coli DH5α was used for DNA manipulations. pNR10 and pBARG2 containing A. oryzae niaD and A. nidulans argB, respectively, were used as transformation marker plasmids. Czapek-Dox medium (CD: 0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, 2% glucose, pH 5.5) and minimal medium with supplements (MM: 1% glucose, nitrate salts, trace elements, pH 6.5) were used as selective media in transformation of A. oryzae and A. nidulans, respectively. The trace elements, vitamins, and nitrate salts were prepared as described.10 CD or koji-extract media and MM were used for the conidiation of A. oryzae and A. nidulans, respectively.

Transformation experiment. E. coli transformation was done by the method of Hanahan.15) The transformation of A. oryzae and A. nidulans was done as described by Gomi et al.16)

Construction of gene fusion. For conversion of sGFP(S65T) to sGFP(F64L, S65T), EGFP, four primers were designed: GFP-primer 1,5′-CCATGGTGAGCAAGGCGAGGAC-3′; GFP-primer 2,5′-CCGCGAGCCCGGGGCGGCCGTTCAGCA-3′; GFP-primer 3,5′-CCCGGGTCGATGGTGACGCA-3′; and GFP-primer 4,5′-CCTGCAGCCCGGGGCGGCCGCTTTACTGTACAGC-3′. Annealing and extension were repeated 3 times by mixing the 3′- and 5′-fragments. The last reaction was done with the annealed 5′- and 3′-fragment and GFP-primers 1 and 2 as a template and primers, respectively. The site-directed mutagenized sGFP(S65T) (egfp) gene was digested with NcoI and BsrGI and inserted back into pBlue-sGFP(S65T)nos3-SK. The resultant plasmid was designated pEGFP-F.

Two primers, H2B-S 5′-AATCCCGGTTGAC-TGTACACGGACAGGTG-3′, H2B-A 5′-GCT CCGGG TTTGCGATAGGAATACCTCGTA-3′, were designed on the basis of A. nidulans histone H2B (h2b) gene sequence17) and introduced with the SmaI site (underlined letters). The PCR-amplified h2b fragment from its own promoter to the codon of the last amino acid was digested with SmaI and ligated into the EcoRV site of pEGFP-F. The BamHI-HindIII fragment of the resultant plasmid pH2BG was blunted with a DNA Blunting Kit (Takara Shuzo, Kyoto, Japan) and ligated into SmaI sites of pNR10 and pBARG2 containing A. oryzae niaD and A. nidulans argB, respectively (Fig. 1).

Observation of EGFP or DAPI stained nuclei. Conidia were treated with 70% ethanol for 30 min at room temperature and rinsed twice in distilled water. They were incubated in a solution containing 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St. Louis, MO) and mounted in mounting solution (90% glycerol, 1% n-propyl-gallate).

For observation of EGFP and DAPI staining at the same time (Figs. 3(A)(B)), conidia of H2B:EGFP expressing transformants were treated with 70% ethanol for 1 min at room temperature and incubated in a solution containing 1 µg/ml DAPI.

Microscopic observation was done using an Olympus AX80 microscope with a UPlanApo 100X objective lens (Olympus, Tokyo, Japan). Fluorescence of EGFP and DAPI was seen with U-MWIBA/GFP blue excitation and BH-DMU ultraviolet excitation cubes (Olympus, respectively).

FACS analysis. The conidia were analyzed on a FACStar fluorescence-activated cell sorter (Becton Dickinson & Co., Mountain View, CA).

Results

Observation of nuclei in A. oryzae hyphae by expressing H2B:EGFP

sGFP(S65T) has been successfully expressed in filamentous fungi, including A. nidulans6,9-11) and A. niger.18,19) We also observed the fluorescence of sGFP(S65T) when expressed in cytoplasm of A. oryzae (data not shown). In addition, to use FACS for quantitative analysis of the GFP-fluorescence in conidia of A. oryzae, we replaced Phe-64 of sGFP(S65T) with Leu by site-directed mutagenesis (Materials and Methods), resulting in construction of EGFP, the excitation wavelength of which shifted to 488 nm. Expression of EGFP in A. oryzae visualized organelles such as vacuole and endoplasmic reticulum (ER) (manuscript in preparation).

Expression of GFP in A. nidulans facilitated observation of nuclear dynamics in living cells6,8,10) and isolation of mutants with a defect in nuclear migration.20) In order to analyze nuclei in living cells we tried to visualize nuclei in A. oryzae by expressing EGFP. A. nidulans histone H2B,21) one of the principal structural proteins of eukaryotic chromosomes, was used for visualization of nuclei since it was reported that a fusion protein of GFP and histone

NII-Electronic Library Service
H2B was localized in nuclei and incorporated into nucleosomes.\textsuperscript{21-23} The chimeric construct, h2b-egfp was connected with marker plasmids containing A. oryzae niaD and A. nidulans argB (Fig. 1) and introduced into A. oryzae niaD300 (niaD\textsuperscript{−}) and A. nidulans FGSC89 (argB\textsuperscript{−}), respectively. EGFP-fluorescence in the resultant transformants was examined with fluorescence microscopy. Most of the transformants contained the EGFP-fluorescence and had normal hyphal growth and conidiation compared with the control transformants with only the marker genes. In both A. oryzae and A. nidulans transformants expressing H2B::EGFP, the fluorescence was detected in the nuclear regions of hyphae (Fig. 2). The fluorescence of H2B::EGFP was associated with nuclei throughout the cell cycle, enabling us to follow the dynamics of nuclear division such as chromatin condensation and segregation of sister chromatids as observed by expressing the H2B-GFP fusion protein in mammalian cells.\textsuperscript{23} By time-lapse microscopy it was observed that apical nuclei migrated toward tips in growing hyphae. These results suggested that the fluorescence of H2B::EGFP localized in nuclei and behaved properly throughout the cell cycle.

\textbf{Observation of nuclei in A oryzae conidia by expressing H2B::EGFP}

Although it is written in a textbook that A. oryzae forms conidia with multinuclei, there has been no reliable report on the distribution of the number of nuclei in each conidium. For verification of this, the conidia in the A. oryzae and A. nidulans transformants expressing H2B::EGFP were observed with fluorescence microscopy. EGFP-fluorescence in nuclei was detected in conidia of both A. oryzae and A. nidulans transformants. Each conidium contained only one fluorescent spot in the A. nidulans transformant, which coincides with the report that A. nidulans has uninnucleate conidia (Fig. 3(B)).\textsuperscript{28} Contrarily, the number of EGFP-fluorescent spots in each conidium was mainly from one to three in the A. oryzae transformant (Fig. 3(A)). A. oryzae conidia containing only one fluorescent spot were smaller. Two hundred conidia of the A. oryzae transformant expressing H2B::EGFP were scored based on the number of EGFP-fluorescent spots that they contained. Approximately 66\% conidia had two fluorescent spots, 24\% had one, and 10\% had three or more (Fig. 4(A)). In order to confirm whether the number of EGFP-fluorescent spots represents that of nuclei, we stained the A. oryzae transformant with DAPI and scored for the number of nuclei in each conidium. The spots of DAPI-stained nuclei in conidia co-localized with those of EGFP-fluorescence (Figs. 3(A)(B)) and conidia of the A. oryzae transformant showed almost the same pattern of the number of nuclei in each conidium (Fig. 4(B)) as that of EGFP-fluorescence in Fig. 4(A). This indicated that the fluorescence of H2B::EGFP corresponded to...
Visualization of Nuclei with EGFP in Conidia of *A. oryzae* and *A. nidulans.*

Conidia of the *A. oryzae* (A) and *A. nidulans* (B) transformants expressing H2B::EGFP were collected from CD medium and MM, respectively, and observed with fluorescence and DIC microscopy. Conidia of the transformants expressing H2B::EGFP (AB) and the *A. oryzae* control transformant with only the marker gene (C) were stained with DAPI. Note that conidia of *A. oryzae* have one to three nuclei and those of *A. nidulans* have one. Bar, 10 μm.

![Fig. 3](image)

**Fig. 3.** Expression of H2B::EGFP in Conidia of *A. oryzae* and *A. nidulans.*

nuclei in conidia of the *A. oryzae* transformant. In order to eliminate the possibility that expression of H2B::EGFP might influence the number of nuclei in each conidium, we examined the control transformant with only the marker gene. It had conidia with mainly 1-3 nuclei (Fig. 3(C)) and had a similar distribution of the number of nuclei (Fig. 4(C)), indicating that the expression of H2B::EGFP had little, if any, effect on the number of nuclei in each conidium.

**FACS analysis with *A. oryzae* conidia expressing H2B::EGFP**

FACS analysis can analyze a large number of cells. In order to analyze many conidia quantitatively for the distribution of the number of nuclei in each, the conidia of the *A. oryzae* and *A. nidulans* transformants expressing H2B::EGFP were analyzed by FACS. Two sharp peaks, prominently the right and secondly the left one, were noted in the *A. oryzae* transformant (Fig. 5(A)), while only one peak corresponding to one nucleus in each conidium was detected in the *A. nidulans* transformant (Fig. 5(C)). There was no fluorescence in the *A. oryzae* (Fig. 5(B)) and *A. nidulans* (Fig. 5(D)) control transformants with only the marker genes. An overlaid histogram showed that the peak in Fig. 5(C) coincided with the left one in Fig. 5(A) (Fig. 5(E)), suggesting that the left and the right peaks represented one and two nuclei in each conidium, respectively. To confirm this speculation, we fractionated the conidia in the two peaks by using a cell sorter. Observation with fluorescence microscopy showed successful isolation of uninucleate and binucleate conidia from the left and right peaks, respectively (Fig. 5(F)). This indicated that the left and right peaks corresponded to one and two nuclei in each conidium of the *A. oryzae* transformant, respectively. In order to examine whether FACS-isolated uninucleate conidia stably reproduce conidia with only one nucleus, we grew them and scored reproduced conidia for the number of nuclei. We confirmed that almost all the uninucleate conidia germinated with microscopy (data not shown). Uninucleate conidia reproduced conidia with the number of nuclei ranging from one to three, which was similar to the distribution shown in Fig. 4(A) (Fig. 6). FACS analysis with reproduced conidia supported this result by showing a similar pattern as in Fig. 5(A) (data not shown). It is suggested that *A. oryzae* is genetically programmed to produce conidia with a varied number of nuclei.

**The number of nuclei in each conidium of *A. oryzae* strains used in sake brewing**

Although *A. oryzae* conidia has been reported to contain 4-6 nuclei in each conidium, conidia of the transformants derived from *A. oryzae* niaD300 had a varied number of nuclei mainly from one to three (Fig. 4). *A. oryzae* has a wide variety of strains differentiated on the basis of morphological and physiological characteristics. Conidia of wild type strain, R1B40, and five *A. oryzae* strains used in sake brewing.
Fig. 5. FACS Analysis of the Conidia Expressing H2B::EGFP.
Flow cytometry histograms of the A. oryzae (A) and A. nidulans (C) transformants expressing H2B::EGFP and the A. oryzae (B) and A. nidulans (D) control transformants. (E) Overlaid histogram (Filled: histogram (A), Line: histogram (C)). (F) FACS-isolated conidia from the two peaks in (A). Bar, 10 μm.

Fig. 6. The Number of Nuclei in Each Conidium Reproduced from FACS-Isolated Uninucleate Conidia.
Uninucleate conidia of the A. oryzae transformant expressing H2B::EGFP were isolated by a cell sorter and grown on CD medium. Two hundred reproduced conidia were scored for the number of nuclei with EGFP fluorescence. Percentages calculated on the basis of seven independent experiments are presented.

in some strains. Our results revealed, for the first time, that some A. oryzae strains had a small portion of uninucleate conidia as well as a majority of multinucleate conidia.
Discussion

Multinucleate conidia in *A. oryzae* have been one of the obstacles to classical genetic analysis and industrial strain breeding. However, little attention has been given to the number of nuclei in each conidium of *A. oryzae* as a subject. In order to elucidate the mechanism of formation of multinucleate conidia in *A. oryzae*, we visualized nuclei by expressing EGFP and used FACS analysis.

We applied GFP expression to visualize nuclei by expressing H2B::EGFP in *A. oryzae* for the first time (Figs. 2, 3). The fluorescence of H2B::EGFP in hyphae was associated with nuclei throughout the cell cycle. In mammalian cells H2B-GFP localized properly in nuclei and allowed researchers to follow nuclear behavior without perturbing cell cycle control or intracellular structures. Thus, our H2B::EGFP expression system permits us to monitor nuclear dynamics in *A. oryzae* throughout the cell cycle. We are now tracking nuclear migration and mitosis in H2B::EGFP expressing cells with time-lapse microscopy. Cytoplasmic dynein and dynactin complex are implicated in nuclear migration of filamentous fungi. The two *A. oryzae* genes (*arpA* and *dhcA*) encoding actin-related protein 1 (Arp1) in dynactin complex and cytoplasmic dynein heavy chain, respectively) have been cloned and characterized. Both *arpA* and *dhcA* disruptants showed a defect in nuclear distribution, which was supported by the observation of the disruptants expressing H2B::EGFP (data not shown). This H2B::EGFP expression system can be an intensive tool for further characterization of genes as *arpA* and *dhcA* which are involved in nuclear migration.

This report is unique on the aspect of using FACS analysis for study of filamentous fungi expressing GFP. We examined a large number of conidia for the distribution of the number of nuclei in each. Expression of H2B::EGFP and DAPI staining showed that 60–70% conidia had two nuclei and approximately 20–25% had one in the *A. oryzae* transformants (Fig. 4) whereas all of the conidia contained only one in the *A. nidulans* transformants (Fig. 3). FACS analysis with the conidia expressing H2B::EGFP (Fig. 5) supported the results obtained in Fig. 4. FACS analysis has an advantage in isolating living cells in an interesting region with some fluorescent intensity. Uninucleate conidia are considered to be useful in mutagenesis for genetic analysis and strain breeding. Therefore, by using a cell sorter we successfully isolated uninucleate conidia from *A. oryzae*. Unexpectedly, reproduced conidia from the FACS-isolated uninucleate conidia had a similar distribution of the number of nuclei to that of the original (Fig. 6). Although stable uninucleate conidia could not be isolated, FACS-isolated uninucleate conidia are, at least, expected to improve the efficiency of mutagenesis. FACS analysis was used in isolation of yeast vacuole morphological mutants. Expression of GFP in nuclei of *A. nidulans* allowed the isolation of mutants with a defect in nuclear migration. These studies prompt us to obtain mutants stably forming uninucleate conidia using FACS analysis.

*A. oryzae* has been reported to contain 4–6 nuclei in each conidium. DAPI staining showed that more than 90% conidia were multinucleate in five *A. oryzae* strains used in sake brewing (Fig. 7). The number of nuclei in each conidium of those strains was mostly from two to five, which is slightly lower than that reported previously. This result reflects the fact that the difficulty in mutagenesis of *A. oryzae*, which results in genetic stability in fermentative processes and problems in classical genetic investigation and industrial strain breeding, was attributed to formation of multinucleate conidia. In addition, we described here for the first time that some *A. oryzae* strains used in sake brewing contained uninucleate conidia (Fig. 7). Although it is generally difficult to attempt industrial strain breeding, it is possible to increase the efficiency of mutagenesis by selecting strains with a low range of the number of nuclei in each conidium. In *A. oryzae* niaD300 (data not shown) and its derivative transformants (Fig. 4) uninucleate conidia occupied a higher portion than in the parental strain of niaD300, RIB40, and the five strains used in sake brewing (Fig. 7). We suppose that during mutagenesis of RIB40 for host selection niaD300 was endowed with the property to form a higher portion of uninucleate conidia. This process might be required to obtain hosts from the parental strain forming multinucleate conidia.

Formation of multinucleate conidia in *A. oryzae* is important for genetic stability and higher germinating rate during fermentative processes. However, the mechanism remains unknown. It was reported that mutants forming multinucleate conidia in *A. nidulans* and genes involved in nuclear migration (*arpA, dhcA*), conidiation (*brlA*) in *A. oryzae* were isolated. FACS and further genetic analyses of nuclear migration and conidiation in *A. oryzae* could enable us to discover the mechanism of formation of multinucleate conidia and obtain mutants stably forming uninucleate conidia.

References

4) Cormack, B. P., Valdivia, and R., Falkow, S.,