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Aspergillus oryzae atfA controls conidial germination and stress tolerance

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ABSTRACT

We compared *atfA* and *atfB*, the genes encoding the respective ATF/CREB-type transcription factors in *Aspergillus oryzae*. The germination ratio of Δ*atfA* conidia was low without any stress, unlike that of Δ*atfB* conidia. The Δ*atfA* conidia were more sensitive to oxidative stress than the Δ*atfB* conidia, which are also sensitive to oxidative stress. We compared the gene expressions of these strains by using a DNA microarray, GeneChip. Almost all the genes regulated by *atfB* were also regulated by *atfA*, but *atfA* also regulated many genes that were not regulated by *atfB*, including some genes putatively involved in oxidative stress resistance. The level of glutamate, the major amino acid in *A. oryzae* conidia, was significantly low only in the Δ*atfA* conidia, and the glycerol accumulation during germination was not observed only in the Δ*atfA* strain. We therefore concluded that *atfA* is involved in germination via carbon and nitrogen source metabolism.

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1. Introduction

Aspergillus oryzae is an industrially important fungus, and has been used in the production of traditional Japanese foods such as sake, soy sauce, and miso. Conidial stress tolerance is important because *A. oryzae* is distributed as conidia from the conidia-producing company to the sake-making company. Furthermore, the availability in industries has increased because *A. oryzae* has recently been used in producing enzymes or recombinant proteins. Because recombinant organisms must not proliferate beyond the controlled area, it is important for such strains to be susceptible to stresses.

The molecular mechanisms of stress tolerance have been vigorously investigated in the yeast *Schizosaccharomyces pombe*. In this organism, two transcription factors, Atf1 and Pcr1, activate gene expressions upon environmental stresses such as oxidative stress or osmotic stress (Takeda et al., 1995; Sanso et al., 2008). Both Atf1 and Pcr1 are included in the ATF/CREB family, a family of bZIP transcription factors, and upregulate many genes, such as the catalase gene *ctt1* or the trehalose-6-phosphate synthase gene *tps1* (Degols and Russell, 1997; Bell et al., 1998; Paredes et al., 2003). Atf1 and Pcr1 have been shown to bind to the CRE sequence (T[G/T]ACGT[C/A]A) of some target genes by forming a heterodimer with each other (Hai and Curran, 1991).

From *A. oryzae*, we had previously isolated *atfB*, encoding the corresponding ATF/CREB transcription factor (Sakamoto et al., 2008). This gene upregulates some other genes including the conidia-specific catalase gene, *catA*, at the late phase of solid-state culture when *A. oryzae* produces conidia vigorously. Our previous result shows that *atfB* is involved in conidial stress tolerance. In the *A. oryzae* genome, there is a homologous gene, *atfA*, putatively encoding the ATF/CREB protein whose DNA-binding domain is almost identical to that of AtfB, and might bind to the same DNA sequence motifs. Therefore, AtfA might control the same genes as AtfB and might also have similar functions. Recently, Hagiwara et al. (2008) reported that the conidia of the *Aspergillus nidulans* Δ*atfA* strain were sensitive to stress, similar to the *A. oryzae* Δ*atfB* strain. In this study, we compared *atfA* and *atfB* to determine their functional differences in *A. oryzae*.

2. Materials and methods

2.1. Strains and culture conditions

The *A. oryzae* strains used in this study included the descendants of wild-type RIB40: NS4 (*niaD*, *sC*) and dsC299 (*niaD*,

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sC:*An_sC*) (Yamada et al., 1997). The medium containing 0.3% $NaNO_2$, 0.15% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 1% glucose (pH 6.0) was used as a minimal medium. Agar plates were incubated in a constant temperature and humidity chamber (ECONAS LH-20 or LH-21: Nagano Science; Osaka, Japan) at 30 °C with 95% relative humidity.

2.2. RNA preparation

After giant colonies were cultured on the agar plates for 7 days at 30 °C and 95% relative humidity, whole agar plates were frozen in liquid nitrogen, and aerial hyphae and conidiospores on the agar surface were gathered with a spatula. Total RNA was isolated with Isogen (Nippon Gene) by using the acid-guanidium thiocyanate-phenol–chloroform extraction method (Chomczynski and Sacchi, 1987).

2.3. Construction of the *ΔatfA* strain

The *ΔatfA* strain was constructed as shown in Supplemental Fig. 1. The 5′ and 3′ regions of *atfA* were independently amplified by PCR from genomic DNA using primers listed in Supplemental Table 1 and inserted into pCRBluntII (ZERO Blunt TOPO PCR Cloning Kit; Invitrogen).

The 3′region in pCRBluntII was excised with *Xba*I and *Not*I. The resulting 3′ fragment was inserted into pCRBluntII in which the 5′ region was previously digested with the same enzymes. This new plasmid containing 3′ and 5′ regions digested with *Xba*I was blunted and digested with *Kpn*I. This resulting DNA fragment, containing 3′ and 5′ regions, was inserted into pUSC (Yamada et al., 1997), which was previously digested with *Xba*I, blunted, and then digested with *Kpn*I to generate the plasmid for *atfA* disruption. Furthermore, an *oliC* fragment was inserted into the plasmid to facilitate the desired recombination (Ward et al., 1986; Takahashi et al., 2004), and this plasmid was then digested with *Not*I before transformation of *A. oryzae* NS4 to generate the *ΔatfA* strain. Southern hybridizations were performed (Supplemental Fig. 2) using DIG Easy Hyb (Roche) according to the manufacturer's instructions. Probes were synthesized with a DIG PCR probe synthesis kit (Roche) by using the following primer sets: atfA PCR F and atfA PCR R for detecting the *atfA* ORF fragment and *atfA* disruption TF2 and *atfA* disruption TR2 for detecting the 3′ fragment of *atfA*.

2.4. Recomplementation of the *ΔatfA* strain with a wild-type clone of *atfA*

The *atfA* ORF was amplified by PCR from genomic DNA by using primers listed in Supplemental Table 1 (*atfA*_GFP_PF and *atfA*_GFP_TR) and inserted into pUN1 (Yamada et al., 1997) digested with *Sma*I. The resultant plasmid was digested with *Mlu*I before transformation of the *ΔatfA* strain to generate a recomplemented strain. Southern hybridizations were performed (Supplemental Fig. 3) using DIG Easy Hyb (Roche) according to the manufacturer's instructions. Probes were synthesized with a DIG PCR probe synthesis kit (Roche) by using primer sets *atfA* PCR F and *atfA* PCR R (Supplemental Table 1).

2.5. Long-term viability of conidia on plate culture

The conidial suspension (2×10^5 conidia/2 μl) was inoculated at the center of the minimal medium to form a giant colony and incubated for the indicated number of days. Conidia were recovered from the plate, and 10^5 conidia/10 μl were spread on another minimal medium plate. The germinating conidia on the plates were counted microscopically after incubation for 20–24 h at 30 °C.

2.6. Conidial oxidative stress tolerance

After 7 days of growth on the minimal medium plate, the conidia were harvested, washed, and resuspended in a 0.05% (w/v) Tween-80 solution. Freshly prepared conidial suspensions were then subjected to stress treatment. Conidia (10^6 conidia/ml) were incubated with various concentrations of H_2O_2 at 30 °C for 30 min, followed by washing twice with centrifugation for 15 s at 1500*g*. The obtained conidial pellets were suspended in 50 μl of sterilized water, and 10-μl aliquots were spread on minimal agar plates.

2.7. GFP expression by the catA promoter

The *catA* promoter was amplified by PCR with primers (*catA* promoter F and *catA* promoter R) listed in Supplemental Table 1. The amplified fragment was digested with *Pst*I and *Sal*I in primer sequences and inserted into pNEF1-GFP (Utashima et al., in preparation), previously digested with the same restriction enzymes, to express *GFP*. To generate the *catA* promoter with mutations in three CRE sequences, three fragments from the *catA* promoter were independently amplified with three sets of primers (*catA* promoter F and *catA*Cre1subR, *catA*Cre1subF and *catA*Cre23subR, and *catA*-Cre23subF and *catA* promoter R). The obtained fragments were mixed and used as templates for the second PCR with primer set *catA* promoter F and *catA* promoter R. This amplified mutated promoter was also inserted into pNEF1-GFP. Both plasmids were digested with *Apa*I for integration into the *niaD* locus of the host genome. Transformants possessing one copy of the plasmid were selected by Southern blot analysis and observed by microscopy.

2.8. GeneChip analysis, labeling, and array hybridization

RNAs were extracted by Isogen from the hyphae and conidia of the giant colonies cultured for 7 days. Extracted total RNAs were purified by using an RNeasy mini kit (Qiagen). The experiment was performed by using three replicates from the fungal culture. The RNAs were labeled by using a GeneChip One-cycle Target Labeling and Control Reagent Kit (Affymetrix) according to manufacturer's instructions. GeneChip was designed by Affymetrix using publicized ORFs at the NRIB Web site (http://nribf2.nrib.go.jp). Hybridization and washing were performed according to manufacturer's instructions.

2.9. Data analysis of GeneChip results

Data were quantified from a scanned CEL-data file (scaling factor = 500) by using the GCOS software (Affymetrix). The calculated data were analyzed by GeneSpring (Agilent Technologies) using per-chip normalization. The genes with a detection call of "present" in all the three replicate measurements were accepted as expressed and used for further analysis. Raw signal values were normalized per chip to the 50th percentile before comparison of the strains. Genes expressed differentially in three strains (dsC299 as control, *ΔatfA*, and *ΔatfB*) were identified by a fold change less than 0.25 or more than 4 against the signal intensity of the control strain from genes identified by one-way ANOVA with a cutoff *p*-value of 0.01.

2.10. In silico analysis by MEME software

Common sequences were extracted from the promoters of the top 40 genes whose expression signals were decreased in the Δ*atfA* strain or the Δ*atfB* strain versus the control strain. A 500-bp sequence of each promoter was obtained from the NRIB Web site and compared by using MEME (Bailey and Elkan, 1995; http://meme.sdsc.edu/meme/cgi-bin/meme.cgi?) with the following parameters: "any number of repetitions," 8 for "minimum width,"

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