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Aspergillus oryzae atfB encodes a transcription factor required for stress tolerance in conidia

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ABSTRACT

Using an *Aspergillus oryzae* EST database, we identified a gene encoding a transcription factor (*atfB*), which is a member of the ATF/CREB family. Expression of *atfB* was barely detectable during vegetative growth, but was readily detected during conidiation in solid-state culture. Microarray analyses showed that expression of many other genes, including catalase (*catA*), were downregulated in an *atfB*-disruptant. The expression of most of these genes was upregulated in the wild-type strain during the conidiation phase in solid-state culture, and the expression pattern was similar to that of *atfB* itself. In the absence of stress, e.g. heat-shock or hydrogen peroxide, the conidial germination ratios for the *ΔatfB* strain and the wild-type strain were similar, but the stress tolerance of conidia carrying the *ΔatfB* deletion was less than that of the wild-type conidia. CRE-like DNA motifs, which are bound by ATF/CREB proteins, were found in the promoters of most of the downregulated genes in the *ΔatfB* strain. Thus, *atfB* appears to encode a transcription factor required for stress tolerance in conidia.

1. Introduction

Aspergillus oryzae is an industrially-important filamentous fungus that has been used for over a thousand years in traditional Japanese fermentations to produce sake, soy sauce and *miso* (soybean paste). When *A. oryzae* is used in a fermentation process, conidiospores (conidia) are generally obtained from seed suppliers. It is essential to maintain viable conidia during their distribution, thereby allowing most conidia to germinate during the preparation of koji. Thus, fungal conidia must be tolerant of a number of environmental stress factors.

Stress tolerance mechanisms in conidia have been partly elucidated in *Aspergilli*. A conidia-specific catalase gene (*catA*) has been shown to be involved in tolerance against oxidative and thermal stress in *Aspergillus nidulans* and *Aspergillus fumigatus* (Navarro et al., 1996; Paris et al., 2003). A *catA* ortholog was also isolated from *A. oryzae* (Akao et al., 2002) and its expression pattern was shown to be developmentally regulated (Hisada et al., 2005) in a similar way to that of *A. nidulans*, where *catA* is expressed specifically in conidia (Navarro and Aguirre, 1998). Although a gene encoding a transcriptional regulator, *brlA*, is known to act as a master regulator of gene expression during conidiation in *A. nidulans* (Adams et al., 1998; Yu and Keller, 2005), *brlA*-independent mechanisms appear to operate for *catA* expression (Navarro and Aguirre, 1998). SakA/HogA MAP kinase is involved in regulating *catA* expression in *A. nidulans* (Kawasaki et al., 2002), but a transcriptional regulator of *catA* has not yet been isolated. Other than *catA*, accumulation of sugars, such as mannitol and trehalose, in conidia is also important for viability under various stress conditions, including thermal, oxidative and osmotic stress in *Aspergilli* (Fillinger et al., 2001; Ruijter et al., 2003). Although expression profiles of genes for mannitol synthesis (Ruijter et al., 2003) and trehalose synthesis (Fillinger et al., 2001; Wolschek and Kubicek, 1997) have been analyzed, the mechanisms controlling their regulation have not yet been elucidated.

Molecular mechanisms underlying stress responses have been intensively analyzed in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In both species, ATF/CREB transcription factors play important roles in the expression of osmotic stress-responsive genes that operate downstream of the MAP kinase pathway (Garcia-Gimeno and Struhl, 2000; Takeda et al., 1995). Among the ATF/CREB proteins, *S. pombe* Atf1 regulates

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expression of catalase (*ctt1*) and trehalose-6-phosphate synthase (*tps1*) under conditions of osmotic and oxidative stress (Degols and Russell, 1997; Paredes et al., 2003). ATF/CREB proteins bind as homodimers or heterodimers to a specific DNA sequence, T(G/T)ACGT(C/A)A, through a bZIP structural motif, which consists of a leucine zipper that mediates dimerization and an adjacent basic region that binds to DNA (Hai and Curran, 1991).

We are interested in the regulatory mechanisms that control expression of genes involved in stress tolerance in conidia in *A. oryzae*, and we found two genes belonging to the ATF/CREB transcription factor family, designated *atfA* and *atfB*, by screening an *A. oryzae* EST database (Akao et al., 2007; http://www.nrib.go.jp/ken/EST/db/index.html). Preliminary analysis of the expression profiles of these genes showed that *atfB* was expressed in the late phase of the solid-state culture, where conidiation occurs vigorously, while *atfA* was expressed throughout the culture period. In the present study, *atfB* function was analyzed and found to be involved in stress tolerance in conidia.

2. Materials and methods

2.1. Strains and culture conditions

Aspergillus oryzae strains used in this study included the wild-type RIB40 strain and its descendants: niaD300 (*niaD*−) and NS4 (*niaD*−, *sC*−). RIB40, niaD300 and NS4 have been described (Yamada et al., 1997). *A. oryzae* was grown in solid-state culture on wheat bran (5 g of wheat bran with 4 ml of H_2O starting with an inoculum of 5.0×10^6 conidia) at 30 °C as previously described (Akao et al., 2002). Czapek-Dox medium containing 0.3% $NaNO_2$, 0.15% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 1% glucose (pH 6.0) was used as a minimal medium.

2.2. RNA preparation

Aspergillus oryzae mycelia were immediately frozen in liquid N_2 and ground to a fine powder. Total RNA was isolated with Isogen (Nippon Gene, Tokyo, Japan), using an acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987).

2.3. Northern hybridization

Northern hybridization analyses were performed using DIG Easy Hyb (Roche, Tokyo, Japan) according to the manufacturer's instructions. Probes were synthesized with DIG PCR probe synthesis kit (Roche) using EST cDNA clones (Akao et al., 2007) as templates and primer sets as follows: *atfA* PCR F and *atfA* PCR R for *atfA*, 2829F and 2829R for *atfB* (Table 1), M13 Forward primer and M13 Reverse primer (Invitrogen, Tokyo, Japan) for AoEST2948 and AoEST4839.

2.4. Construction of *ΔatfB* strain

ΔatfB strain was constructed as shown in Supplemental Fig. 1. The 5′ and 3′ regions of the *atfB* ORF were independently amplified by PCR from genomic DNA using primers listed in Table 1 and inserted into pCRbluntII (Invitrogen). The 5′ region in pCRBluntII was excised with PstI and AatII and inserted into pUSC (Yamada et al.,

Table 1
Primers

Name	Sequence (5′ → 3′)	description
atfA PCR F	GCACCTAGTCCGAACTCCCA	Probe synthesis for *atfA* detection
atfA PCR R	CCTGTTCACCTTGCACGTCA	Probe synthesis for *atfA* detection
2829F	TATGTCGGTGGACCAAACCC	Probe synthesis for *atfB* ORF detection
2829R	TTCACTAAGGCTGCTGCGATG	Probe synthesis for *atfB* ORF detection
AtfBdisruptPF	CTCGATCTCCTCCAGCGGCCGCATCTCACTTGGCACCAAC	Construction of *atfB* disruptant
AtfBdisruptPR	AGACGTCGGACCATGTGTCCAGACCTT	Construction of *atfB* disruptant
AtfBdisruptTF	AGACGTCGGCAAATCCTCCTGACGGTG	Construction of *atfB* disruptant and probe synthesis for detection of *atfB* 3′ region
AtfBdisruptTR	GCCAAGTGAGATGCGGCCGCTGGAGGAGATCGAGGTGCTG	Construction of *atfB* disruptant and probe synthesis for detection of *atfB* 3′ region
XynG2_PF	AGAGCTCGAGTTCTTCATTGTCTCAGAT	Amplifying *xynG2* promoter
XynG2_PR	ACGAACTCTAGCACAGTCGGTACCCGGGGTGGATTCGTTGAAATATGGGGG	Amplifying *xynG2* promoter
XynG2_TF	CCCCCATATTTCAACGAATCCACCCCGGGTACCGACTGTGCTAGAGTTCGT	Amplifying *xynG2* terminator
XynG2_TR	ATAAACGGCTATTGTAACCGTTTAA	Amplifying *xynG2* terminator
*atfB*_ORF_F	ATGTCGGTGGACCAAACCCTCTA	Amplifying *atfB* ORF
*atfB*_ORF_R	CTAAACATTAATCAGCTCTTCAA	Amplifying *atfB* ORF
*atfB*_genome_amp_PF	GTTGACTCCTCGTTTTCGACACGTT	Amplifying *atfB* genomic DNA to re-complement a mutant strain
*atfB*_genome_amp_R	GTGTGCGAGAACCGGGCATT	Amplifying *atfB* genomic DNA to re-complement a mutant strain
AoEST5429F	TCAGCGACGCCGACTCCTTC	For probe amplification by PCR
AoEST5429R	CTCAACGGCGCATGTCCACC	For probe amplification by PCR
AoEST2678F	ATTGGCCTGTGGCATGGAAG	For probe amplification by PCR
AoEST2678R	TGGGTAACACCCCAAGCAAG	For probe amplification by PCR
AoEST0727F	TAACTCCCAGCCGCTCACAG	For probe amplification by PCR
AoEST0727R	GCTTCATCGCGCTCAAGTCC	For probe amplification by PCR
AoEST1871F	TTGAATGGGAAGGGTTGCGG	For probe amplification by PCR
AoEST1871R	CGCCGGAGATGTAAATGCGA	For probe amplification by PCR
AoEST1733F	CGAGGGTGGCATGCTTTCGG	For probe amplification by PCR
AoEST1733R	GCGGAAAATCGCCGGGATGT	For probe amplification by PCR
AoEST6362F	GCTCGTGAGAACCTCGACAG	For probe amplification by PCR
AoEST6362R	TGTCGACACCCTTTTGCTCC	For probe amplification by PCR
AoEST5877F	TGTGGTCGCATAGCAGCCAG	For probe amplification by PCR
AoEST5877R	TCGTCCAAGCAACTCGCCAA	For probe amplification by PCR
AoEST6857F	CTGTCCTTCGCCATCTCCAC	For probe amplification by PCR
AoEST6857R	CCCGTCGAAACATAAACGCC	For probe amplification by PCR
AoEST0598F	TCCGAACCTCAAAAGCGCAA	For probe amplification by PCR
AoEST0598R	AACCATCGCCAGCAGTGTTC	For probe amplification by PCR