Journal of Bioscience and Bioengineering
VOL. 115 No. 4, 360–365, 2013

www.elsevier.com/locate/jbiosc

Characterization of a gene coding for a putative adenosine deaminase-related growth factor by RNA interference in the Basidiomycete *Flammulina velutipes*

Shuichi Sekiya,[1] Masato Yamada,[2] Kou Shibata,[1] Toru Okuhara,[1] Masumi Yoshida,[1] Satoshi Inatomi,[3] Goro Taguchi,[1,*] and Makoto Shimosaka[1]

Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan,[1] Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan,[2] and Mushroom Research Laboratory, Hokuto Corporation, 800-8 Shimokomazawa, Nagano 381-0008, Japan[3]

Received 19 August 2012; accepted 24 October 2012
Available online 22 November 2012

A full-length cDNA coding for a putative adenosine deaminase (*Fv-ada*) was isolated from the basidiomycete *Flammulina velutipes*. *Fv-ada* encodes a polypeptide consisting of 537 amino acid residues, which has a consensus sequence conserved among adenosine deaminase-related growth factors (ADGF) found in several metazoa, including chordates and insects. *Fv-ada* transcript was detected at all stages of growth in dikaryotic *F. velutipes* cells, with a peak at the primordial stage. Heterologous expression of *Fv-ada* in the yeast *Pichia pastoris* produced recombinant Fv-ADA that catalyzed the conversion of adenosine to inosine. Dikaryotic mycelia from *F. velutipes* were transformed with the binary plasmid pFungiway-*Fv-ada*, which was designed to suppress the expression of *Fv-ada* through RNA interference. The growth rates of the resulting transformants were retarded in response to the degree of suppression, indicating that *Fv-ada* plays an important role in the mycelial growth of *F. velutipes*. These results suggested that ADGF could function as growth factors in fungi, as is seen in other eukaryotes.

[**Key words:** Adenosine deaminase; Basidiomycete; *Flammulina velutipes*; Growth factor; RNA interference]

Edible mushrooms (fruiting bodies of the Basidiomycetes) garner much attention as nutritious and healthy foods (1,2). A limited group of edible mushrooms is produced commercially by artificial cultivation, where fruiting is induced by controlling various environmental stimuli, such as light, moisture, and temperature. Because of insufficient knowledge about the molecular mechanism of fruiting, suitable conditions for induction of fruiting must be determined for each species of the commercially cultivated mushrooms by repeated trial and error.

The winter mushroom, *Flammulina velutipes*, is a well-known edible mushroom and is cultivated commercially throughout the world. The well-synchronized formation of fruiting bodies, after induction by controlling environmental stimuli, makes this mushroom suitable to study the process of fruiting at the molecular level. We previously isolated 75 genes specifically expressed at the primordial stage (immature pin mushrooms) by differential display (3) and have previously characterized the two genes coding for hydrophobin and chitin deacetylase (4,5).

In this study, we focused on the gene coding for a putative adenosine deaminase (*Fv-ada*), which was selected from the genes preferentially expressed at the primordial stage (3). Interestingly, the *Fv-ada* open reading frame (ORF) shows sequence similarity to the proteins belonging to a group of growth factors (6), including insect-derived growth factor (IDGF) from flesh fly (*Sarcophaga peregrina*), cat eye syndrome responsible factor (CECR1) from humans, and mollusk-derived growth factor (MDGF) from sea hare (*Aplysia californica*). IDGF was originally isolated from the conditioned medium of the NIH-Sape-4 cell line derived from *S. peregrina*, and was characterized as a proteinous growth factor capable of promoting cell proliferation (7,8). CECR1 is responsible for normal human developmental processes, because its malfunction causes a cat eye syndrome characterized by abnormal development of embryo and heart (9). MDGF is expressed in the central nervous system of *A. californica* embryos at the initial stage of development (10). Interestingly, MDGF promoted cell proliferation in the NIH-Sape-4 cell line, similar to IDGF. The primary structures of these three proteins contain a conserved amino acid sequence similar to that of the catalytic centers of adenosine deaminases. Furthermore, they exhibited adenosine deaminase activity *in vitro*, and this activity was essential to fulfill their function as a growth

* Corresponding author. Tel./fax: +81 (0) 268 21 5342.
E-mail address: gtagtag@shinshu-u.ac.jp (G. Taguchi).

Abbreviations: ADA, adenosine deaminase; ADGF, adenosine deaminase-related growth factors; ATMT, *A. tumefaciens*-mediated transformation; CECR, cat eye syndrome responsible factor; EGFP, enhanced green fluorescent protein; gpd, glyceraldehyde-3-phosphate dehydrogenase; hph, hygromycin phosphotransferase; IDGF, insect-derived growth factor; MDGF, mollusk-derived growth factor; ORF, open reading frame; PDA, potato dextrose agar; RNAi, RNA interference; RT-PCR, reverse transcriptase PCR.

1389-1723/$ – see front matter © 2012, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2012.10.020

factor. Hence, these proteins are grouped into the family of adenosine deaminase-related growth factors (ADGF). ADGFs have also been found in other eukaryotic organisms, including fruit fly (*Drosophila melanogaster*) (11,12), tsetse fly (*Glossina morsitans*) (13), cabbage armyworm (*Mamestra brassicae*) (14), and African clawed flog (*Xenopus laevis*) (15). Recent analyses of whole genomic sequences have revealed that ADGF-like proteins also exist in many fungi, including Ascomycetes and Basidiomycetes (6), although their functions have not been reported to date. The genes coding for ADGF are specifically expressed at the stage of cell differentiation or proliferation, and thus considered to be involved in the process of organogenesis. Hence, it is of much interest to study the ADGF-like gene, *Fv-ada*, because it may provide a novel function of ADGFs that could be related to cell differentiation of the Basidiomycetes, as represented by fruiting. In this study, we examined recombinant Fv-ADA expressed in the yeast *Pichia pastoris* and investigated the change in the phenotype of *F. velutipes* mycelia in which the expression of *Fv-ada* was suppressed by RNA interference (RNAi). Based on these findings, we were able to determine the physiological function of Fv-ADA in *F. velutipes*.

MATERIALS AND METHODS

Strain and culture *F. velutipes* dikaryotic strain MH092086 (3), which is used for commercial cultivation, was used in this study. Culture conditions for fruiting were described previously (5). Oligonucleotides used for polymerase chain reaction (PCR) amplifications are shown in Table 1.

Isolation of *Fv-ada* The partial *Fv-ada* cDNA fragment (named 44b) was originally isolated by differential display targeting genes specifically expressed at the primordial stage (3). The corresponding genomic fragment was amplified by PCR with primer set 44b-F and 44b-R, using genomic DNA as template. The amplified PCR fragment (approximately 1100 bp) was labeled with [^{32}P]-dCTP using a Random Primer DNA Labeling Kit (Takara Bio, Otsu, Japan), and used as a probe to screen positive clones from a *F. velutipes* genomic library (comprising ~20,000 clones) constructed in the lambda Dash II vector (Agilent Technologies, Santa Clara, CA, USA) (4). The full-length *Fv-ada* cDNA was amplified from total RNA prepared from cells at primordial stage (3) using an RNA PCR kit (Takara Bio). First strand cDNA was synthesized with a 5′-(oligo dT$_{13-16}$)CA-3′ primer (Takara Bio), and then the cDNA was amplified by PCR with primer set ada-F1 and ada-R1 under the following conditions: 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified PCR fragment was cloned into the pT7Blue vector (Merck, Darmstadt, Germany). Nucleotide sequence was determined using an ABI PRISM 310 genetic analyzer or PRISM 3100 genetic analyzer (Life Technologies, Grand Island, NY, USA).

Semi-quantitative RT-PCR The amount of *Fv-ada* transcript was estimated by semi-quantitative reverse transcriptase PCR (RT-PCR) using total RNA prepared from cells at different stages, as previously described (3). Amplification was carried out with primer set ada-F2 and ada-R1, using Ex Taq (Takara Bio) under the following conditions: 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The cDNA fragment (450 bp) of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*), amplified using primer set gpd2415 and gpd2873, was used as an internal control for quantitative standardization (4).

TABLE 1. Oligonucleotides used for PCR amplification in this work.

Oligonucleotides	Sequences (5′−3′)	Restriction cleavage sites
44b-F	TGTCAACGAAATAAAGGCCGAG	
44b-R	AGTTAGTTCATGCGAGGCTGTA	
ada-Fl	GCAATTACCCCCCATCTTTG	
ada-F2	GGCACAGAGGCAGACATGAA	
ada-F3	caccCGAAGACCTAGTGTGG	
ada-Rl	CTGGTGTGATGGTGTAAAGCC	
ada-R2	GGCAGACCTCCAACGCGATA	
ada-Cla-F	ATCGATGTCTGCAGAACAATTGGC	*Cla*I
ada-Nhe-R	GCTAGCCGTTTCTTCGAATCGTTC	*Nhe*I
gpd2415	GCCACCCAAAAGACCGTTGA	
gpd2873	CCCCACTCGTTGTCGTACCA	
eGFP-Hind-F	AAGCTTATGGTGAGCAAGGGCGAG	*Hind*III
eGFP-Pst-R	CTCGAGCTTGTACAGCTCGTCCAT	*Pst*I

Underlined sequences are cleavage sites by restriction endonucleases. Lower case sequence is an additional sequence for pENTR directional cloning.

Expression of recombinant Fv-ADA in *P. pastoris* *Fv-ada* cDNA corresponding to the entire ORF was amplified by PCR using the primer set ada-Cla-F and ada-Nhe-R. The amplified fragment was digested with *Cla*I and *Nhe*I, and then ligated into expression vector pPICZαA (Life Technologies) digested with *Cla*I and *Xba*I. The resulting plasmid pPICZα/*Fv-ada* was introduced into *P. pastoris* X-33 (Life Technologies) by electroporation in accordance with the manufacturer's instructions. *Fv-ada* expression was induced with methanol, and the recombinant protein Fv-ADA secreted in the culture fluid was collected by ammonium sulfate precipitation (85% saturation). The protein was purified by affinity chromatography targeted to a poly-histidine tag using a His-Trap kit (GE Healthcare, Little Chalfont, UK). The recombinant protein (5 μg) was separated by SDS-PAGE on a 10% polyacrylamide gel, and then blotted onto a polyvinylidene fluoride membrane. Glycosylated proteins were stained with periodic acid-Schiff reagent (Wako Pure Chemical Industries, Osaka, Japan).

Enzyme assay Adenosine deaminase was assayed in reaction mixture (750 μl) containing 50 mM potassium phosphate buffer (pH 7.5), 60 μM adenosine, and appropriate amounts of recombinant Fv-ADA. The reaction was initiated by addition of Fv-ADA, and a reduction in the absorbance at 265 nm was monitored at 25°C with a DU7400 spectrophotometer (Beckman-Coulter, Brea, CA, USA). The amount of adenosine converted into inosine was calculated using a molar extinction coefficient of 8100 M^{-1} cm^{-1}. One unit of activity was defined as the amount of enzyme required to convert 1 μmol of adenosine into inosine per minute under the described conditions. Protein concentration was determined by a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. To detect the reaction product, inosine, a reaction mixture (200 μl) containing 50 mM potassium phosphate buffer (pH 7.5), 5 mM adenosine, and 2.3 μg of the recombinant Fv-ADA protein was incubated at 25°C for 0, 5, 10 and 30 min. The reaction was terminated by boiling and an aliquot (10 μl) was spotted onto a silica gel plate (Silica gel 60 F_{254}, Merck), then developed with a mixture of 1-butanol, acetone, acetic acid, 5% ammonia water (9:3:2:2:4, v/v). The product was detected by UV exposure (254 nm).

Suppression of *Fv-ada* expression through RNA interference (RNAi) The RNAi binary vector, pFungiway was designed to suppress *Fv-ada* expression in an easy and simple manner by adopting Gateway technology (Fig. 1). Briefly, the construct was composed of the following elements placed in order: the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*), originating from *F. velutipes*, was used to drive the constitutive transcription of the objective DNA insert designed for RNAi; reading frame cassette A of the Gateway vector conversion system (Life Technologies) was placed for introduction of the objective gene fragment in the forward direction; a gene fragment from the enhanced green fluorescent protein (EGFP) from *Aequorea victoria* was placed as a spacer of hairpin RNA structure; reading frame cassette A was placed in the inverted orientation so that the objective gene fragment could be introduced in the reverse direction; the *Aspergillus nidulans trpC* terminator, originating from pDH25 (16), was set to terminate the transcription of the entire DNA construct. The DNA fragment of EGFP was obtained by PCR amplification using the plasmid pEGFP1 (Clontech, Palo Alto, CA, USA) as template. The selection marker cassette was composed of the hygromycin phosphotransferase gene (*hph*), originating from pDH25, under the control of the *F. velutipes gpd* promoter. Finally, both RNAi construct and selection marker were placed in the T-DNA region of the binary vector, pCAMBIA1200 (Cambia, Brisbane, Australia).

A partial cDNA fragment from *Fv-ada* (348 bp), amplified by PCR with primer set ada-F3 and ada-R2 using Phusion High-Fidelity DNA polymerase (Finnzymes, Vantaa, Finland), was cloned into pENTR/D-TOPO (Life Technologies). The inserted fragment was then transferred into pFungiway by *in vitro* recombination using LR Clonase (Life Technologies). The resultant binary plasmid, pFungiway-*Fv-ada*, was introduced into *Agrobacterium tumefaciens* LBA4404 (Clontech) by electroporation. Finally, the T-DNA region of the plasmid pFungiway-*Fv-ada* was transferred into mycelial cells of *F. velutipes* dikaryotic strain MH092086 by *A. tumefaciens*-mediated transformation (ATMT) (17). Transformants in which the T-DNA region was integrated into the genome were selected on potato dextrose agar (PDA) (Becton Dickinson, Franklin Lakes, NJ, USA) plates containing hygromycin B (10 mg/l). To determine the amount of *Fv-ada* transcript, total RNA was extracted from mycelia of RNAi transformants using TRI reagents (Molecular Research Center, Cincinnati, OH, USA). First strand cDNA was synthesized from 100 ng of total RNA in a 20 μl reaction mixture using RNA PCR Kit (Takara Bio) and dT$_{30}$ primer. PCR was performed with 0.5 μl of cDNA reaction mixture using Ex-Taq (Takara Bio), under the following conditions: 95°C for 4 min, 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by an incubation at 72°C for 4 min. Primer set ada-F3 and ada-R2 was used for the amplification.

Growth test of *F. velutipes* mycelia Mycelial colonies from *F. velutipes* transformants were sliced into 5 mm × 5 mm pieces and put on the center of PDA plates containing hygromycin B (10 mg/l). The plates were incubated at 22°C for 10 days, and the diameter of the resultant mycelial colony was measured. The transformant containing pFHC-*cob* (17) was used as a control as it expressed the *hph* marker gene alone. Multiple comparisons of colony diameters were performed using Dunnett's method.

Nucleotide sequence accession number The sequence of *Fv-ada* has been submitted to the DDBJ/EMBL/GenBank database under accession no. AB715331.