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Title: Characterization of a Gene Coding for a Putative Adenosine Deaminase-Related Growth Factor by RNA Interference in the Basidiomycete *Flammulina velutipes*

Running title: ADA-RELATED GROWTH FACTOR OF F. VELUTIPES

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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

Abstract

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.

Introduction

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 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.

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 1,100 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 $dT_{13\text{-}16}$)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 gdp2873, was used as an internal control for quantitative standardization (4).

Expression of recombinant Fv-ADA in *Pichia pastoris*

Fv-ada cDNA corresponding to the entire ORF was amplified by PCR using the primer set ada-F-Cla and ada-R-Nhe. 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, England). The recombinant protein (5 μg) was separated by SDS-PAGE on a 10% acrylamide gel, and then blotted onto a polyvinylidene fluoride membrane. Glycosylated proteins were stained with periodic acid-Schiff reagent (Wako Pure Chemistry 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

Fig. 1

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.

Results

Gene isolation of *Fv-ada*

We previously isolated a 900-bp cDNA fragment (named 44b), showing sequence similarity to genes coding for adenosine deaminases, from cDNA clones specifically expressed at the *F. velutipes* primordial stage (3). Using the 44b cDNA as a probe, we screened a *F. velutipes* genomic library and obtained a clone containing the full-length genomic *Fv-ada* gene. cDNA corresponding to the whole ORF was also isolated by RT-PCR, and from this we deduced the number and position of the *Fv-ada* introns. The nucleotide and deduced amino acid sequences of *Fv-ada* are shown in Fig. S1. The *Fv-ada* ORF (1,614 bp) encodes a polypeptide consisting of 537 amino acid residues, with a calculated molecular mass of 60.3 kDa. The ORF was interrupted by 10 small introns (45–54 bp in size). Nine of the introns contained a consensus sequence in both 5' and 3' splicing sites (GTPuNGPy-PyAG) (18), but the second intron did not contain the 5' splicing site. A TATA box-like sequence (TAAATT; from −122 to −117) and a CAAT box-like sequence (TTCAAT; from −204 to −199) were found upstream of the start codon.

The amino acid sequence of the *Fv-ada* ORF showed approximately 70% similarity to those of putative ADGF-like proteins from Basidiomycetes, such as *Laccaria bicolor* (78%, XP_001874953), *Coprinopsis cinerea* (73%, XP_001833082), *Schizophyllum commune* (78%, XP_003036104), and *Serpula lacrymans* (74%, EGN95556). Likewise, it shared approximately 50% sequence similarity with those ADGF family proteins that have been functionally characterized. The multiple alignment of the deduced amino acid sequences of Fv-ADA and other characterized ADGF family proteins is shown in Fig. 2. Amino acid residues that are highly conserved among ADGF proteins (6) were also found in Fv-ADA. A notable example is an MPKG motif, which is considered to be essential for zinc binding as well as adenosine deaminase activity. Hydrophobicity analysis, SignalP analysis (19) and psort analysis (20) suggested that Fv-ADA has no signal sequence in the *N*-terminus.

Fig. 2

Expression of *Fv-ada* in *F. velutipes*

The amount of *Fv-ada* transcript during fruiting body development was measured by semi-quantitative RT-PCR. Total RNA extracted from cells at three different stages was used as the template: dikaryotic mycelia before induction for fruiting, primordia, and mature fruiting body. *Fv-ada* transcripts were observed at all stages tested, and were most abundant at the primordial stage (Fig. 3A). To localize *Fv-ada* transcripts in the fruiting body, total RNA was extracted from pileus, stipes, and roots of the fruiting body. RT-PCR analysis revealed that the amount of *Fv-ada* was much higher in the pileus than in the stipes and roots (Fig. 3B).

Fig. 3

Heterologous expression of recombinant Fv-ADA in *P. pastoris*

Fv-ada was expressed in the yeast *P. pastoris* to characterize adenosine deaminase

activity of the recombinant Fv-ADA. The full-length *Fv-ada* cDNA was inserted into pPICZαA, and the resulting plasmid was introduced into *P. pastoris*. The protein band corresponding to Fv-ADA was detected in the culture fluid of the transformant, while the corresponding band did not appear in culture fluid of *P. pastoris* cells carrying the vector alone (data not shown). The molecular mass of the protein corresponding to the major band was estimated to be 62 kDa, consistent with the expected size of recombinant Fv-ADA (62.5 kDa). The yield of recombinant Fv-ADA was 0.46 mg of protein per liter of culture. After purification with nickel-chelate affinity chromatography, recombinant Fv-ADA produced a smeared band on SDS-PAGE (Fig. 4A). Similar smeared bands were also observed after periodic acid-Schiff base staining. The deduced amino acid sequence of Fv-ADA has four potential *N*-glycosylation sites (Fig. S1), indicating that the recombinant Fv-ADA was glycosylated to some extent.

Fig. 4

When purified recombinant Fv-ADA was incubated with adenosine, a complete conversion into inosine was observed by TLC analysis (Fig. 4B). This clearly demonstrated that Fv-ADA possesses adenosine deaminase activity. By spectroscopic assay, specific activity was determined to be 2.62 units/mg-protein. Instability of the purified enzyme precluded further characterization.

RNAi-mediated suppression of *Fv-ada* in *F. velutipes*

Conventional methods to disrupt the target gene by homologous gene recombination are not applicable for dikaryotic mycelium because the homologous gene at another nucleus can complement the disrupted gene. To analyze the function of Fv-ADA in *F. velutipes*, we used RNAi to specifically suppress the expression of *Fv-ada*. The partial *Fv-ada* cDNA fragment (346 bp), which included the putative active center of the adenosine deaminase, was inserted into the binary vector pFungiway (Fig. 1). ATMT

yielded 10 independent mycelial colonies resistant to hygromycin B. PCR analysis revealed that all of the 10 clones contained both the RNAi construct and the *hph* selection marker, which were conferred by the transfer of the T-DNA region of the binary vector and by integration into the genome (data not shown).

The effect of RNAi on the transcription level of *Fv-ada* was investigated in each of the transformants by semi-quantitative RT-PCR. The amounts of *Fv-ada* transcripts were considerably reduced in all of the transformants tested, as compared with that of wild type (Fig. 5). The growth rate of each RNAi transformant was examined on PDA plates containing hygromycin B (Fig. 5A). A transformant carrying the *hph* selectable marker alone was used as a control. This control colony grew on the PDA plate containing hygromycin B at a similar growth rate to a wild-type colony growing on a PDA plate without hygromycin B, indicating that selective pressure caused by hygromycin B did not have any effect on the growth rate of *F. velutipes*. The transformants with a decreased amount of *Fv-ada* transcript showed a slower growth rate in proportion to the decreased level of *Fv-ada* transcript (Fig. 5).

Fig. 5

Discussion

The molecular mechanism of fruiting body formation in Basidiomycetes is still poorly understood, making it difficult to develop commercial cultivation methods for valuable mushroom crops. We isolated a group of genes that were specifically expressed at the fruiting stage in *F. velutipes*, with the aim of clarifying the mechanism of fruiting in Basidiomycetes (3). In the present work, we studied the function of *Fv-ada*, which encoded a polypeptide showing sequence similarity to ADGF family proteins.

Growth factors have been found in a wide range of both vertebrate and invertebrate animals, and regulate cell growth and differentiation. Among them, ADGF proteins are

presumed to be a novel type of cell proliferation factor, and are distinguished by their endogenous adenosine deaminase activity. A growth-regulating function of ADGF proteins was reported to be tightly linked to their adenosine deaminase activity through the regulation of extracellular concentrations of adenosine (8). Most ADGF proteins have been found in animals, including vertebrates and insects. ADGF-like proteins are also found in fungi, including Basidiomycetes, but their actual functions have not been clarified to date.

The deduced amino acid sequence of the *Fv-ada* ORF contained a motif for zinc binding, which is highly conserved in the active center of ADGF proteins (6). We demonstrated that the recombinant Fv-ADA heterologously expressed in *P. pastoris* exhibited adenosine deaminase activity (Fig. 4). It should be noted that the specific activity of recombinant Fv-ADA was as low as 2.6 units/mg-protein, whereas the corresponding values for IDGF (from *S. peregrine*) and ADGF-A (from *D. melanogaster*) were 5,500 and 157 units/mg-protein, respectively (8, 21). We failed to detect adenosine deaminase activity in the crude extract of mature fruiting bodies from *F. velutipes*. This was not surprising because cellular amounts of growth factors are generally low. In fact, the expression level of *Fv-ada* appeared to be relatively low, as the *Fv-ada* transcript signal was barely detected by northern analysis even at the primordial stage, which was where the highest amount of transcript was detected by RT-PCR (3). These results led us to surmise that Fv-ADA may play a regulatory role in cell differentiation processes such as fruiting.

Most ADGF proteins originating from metazoa have a hydrophobic signal peptide (6). IDGF was found to be secreted into the culture medium of *S. peregrina* NIH-Sape-4 cells (7), implying that metazoan ADGF proteins may fulfill their function at the intercellular space. Intercellular concentrations of adenosine, which is thought to be

regulated through the action of ADGF (21), could regulate cell proliferation, larval development, and the inflammatory response of immune cells via the adenosine receptor in *Drosophila* (21-23). This kind of signaling pathway was also reported in the embryogenesis of *Xenopus* (15). Conversely, fungal ADGF-like proteins reported from *Neurospora crassa* and *Gibberella zeae*, as well as Fv-ADA, are devoid of signal peptides (6), suggesting that they are cytoplasmic proteins. Accordingly, the function of fungal ADGF-like proteins would be different from those of animal ADGF proteins, even if they still play a role as growth factors.

To examine the physiological function of Fv-ADA in *F. velutipes*, we suppressed the expression of *Fv-ada* by RNAi. Because our purpose was to elucidate the molecular mechanism of fruiting, we needed to use a dikaryotic mycelial strain that is capable of fruiting. Gene disruption is an effective tool of reverse genetics; however, it cannot be applied to dikaryotic mycelia because it is very difficult to knock out the two homologous genes in individual nuclei at the same time. Accordingly, RNAi appears to be an effective alternative, as it can suppress the accumulation of transcripts originating from both nuclei. Successful examples of gene silencing through RNAi have been reported in dikaryotic strains of Basidiomycetes (24-27).

F. velutipes mycelial cells were successfully transformed with the binary plasmid pFungiway-*Fv-ada* (RNAi construct for Fv-ada) via ATMT (Fig. 1). We observed a retardation of cell growth in mycelia in proportion to the suppression level of *Fv-ada* expression. This clearly demonstrated that Fv-ADA functions to regulate the growth of mycelia. Taking into account endogenous adenosine deaminase activity, Fv-ADA has a similar function to animal ADGF proteins, and thus could be defined as a fungal growth factor. The amount of *Fv-ada* transcript was significantly higher at the primordial stage, although it was detected at all differential stages tested (Fig. 3A). It should be noted

that *Fv-ada* transcript was mostly detected in pileus in which meiosis and basidiospore formation occur, suggesting that Fv-PDA is involved in the process of fruiting body formation as well as mycelial growth (Fig 3B). Unfortunately, the slow growth rate of *Fv-ada*-suppressed transformants made it difficult to grow them in sawdust medium in order to induce fruiting. Construction of updated pFungiway vectors, in which RNAi constructs can be transcribed at the fruiting stage but not in vegetative mycelia, is now being undertaken using a promoter of genes specifically expressed at the fruiting stage. The use of this vector will make it possible to analyze the physiological role of Fv-ADA at the particular stage of fruiting.

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Figure legends

FIG. 1. Maps of T-DNA region in RNAi binary vector pFungiway and pFungiway-*Fv-ada*. (A) pFungiway was constructed on binary vector pCAMBIA1200, and designed to be applicable to versatile genes in an easy and simple manner by adopting Gateway technology. (B) pFungiway-*Fv-ada*, an RNAi vector for the suppression of *Fv-ada* expression in *F. velutipes*, was constructed by Gateway recombination using LR clonase. LB and RB, left and right border sequences of the Ti plasmid; Pgpd, glyceraldehyde-3-phosphate dehydrogenase promoter; TtrpC, TrpC terminator; *hph*, hygromycin phosphotransferse gene; Gateway RfA, Gateway reading frame A. A *gfp* gene was used as a spacer of hairpin structure. Black arrows indicate the direction of transcription.

FIG. 2. Multiple alignment of the deduced amino acid sequence of Fv-ADA with other ADGF family proteins. The phylogenetic analysis of the selected ADGF family proteins was performed in ClustalW (28) using a Blosum matrix, and illustrated by Genetyx-SV/RC (ver. 15.0.5) software (Genetyx, Tokyo, Japan). AsMDGF, mollusk-derived growth factor from *A. californica* (accession no. AAD13112); DmADGFA, ADGFA protein from *Drosophila melanogaster* (AAL40912); SpIDGF, an insect-derived growth factor from *Sarcophaga peregrine* (BAA11812); HsCECR1, cat eye syndrome responsible protein from human (NP059120); XlCECR1, cat eye syndrome responsible protein from *Xenopus laevis* (AY986978). Asterisks (*) indicate the conserved amino acid residues engaged in the binding with Zn^{2+}. Open circles (°) indicate amino acid residues conserved in the catalytic center of adenosine deaminase.

FIG. 3. Semi-quantitative RT-PCR analysis of *Fv-ada* transcripts. (A) Stage specific accumulation. M, mycelia; P, primordia; F, matured fruiting body. (B) Accumulation in parts of fruiting body. Pi, pileus; S, stipes; R, roots. (C) Schematic diagram of *F. velutipes* fruiting body. The glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was amplified as an internal control for quantitative standardization.

FIG. 4. Detection of adenosine deaminase activity in recombinant Fv-ADA. (A) SDS-PAGE analysis of recombinant Fv-ADA expressed in *P. pastoris*. Crude proteins in culture fluid of the transformant were concentrated by ammonium sulfate precipitation, and then recombinant Fv-ADA was purified by nickel-chelate affinity chromatography. M, size marker; S, the purified recombinant Fv-ADA; P, the recombinant Fv-ADA stained with periodic acid-Schiff reagent. (B) TLC analysis of reaction products with the purified Fv-ADA. Numerals show incubation periods (min). ST, standard compounds; Ade, adenosine (substrate); Ino, inosine (reaction product).

FIG. 5. Effect of RNAi on the amount of *Fv-ada* transcript and mycelial growth. (A) Comparison of mycelial growth on PDA plates containing hygromycin B. Mycelial colonies (5 mm × 5 mm square) were put on the center of plates, and were incubated at 22°C for 10 days. A hygromycin-resistant (hyg^r) strain was used as a positive control. (B) Effect of RNAi on the amount of *Fv-ada* transcript. The amount of transcript was evaluated by semi-quantitative RT-PCR using the *gpd* gene as an internal control for quantitative standardization. Wt, wild-type strain. hyg^r, transformant strain possessing hygromycin-resistant gene alone. (C) Diameters of mycelial colonies were measured at 10 days post-inoculation. The data represents mean ± SD (n=5). The symbols '*' and '**' indicate a significant difference at $p<0.05$ and $p<0.01$ versus control (hyg^r),

respectively. WT, wild-type strain grown on PDA without hygromycin B; hyg^r, transformant strain possessing hygromycin-resistant gene alone on PDA with hygromycin B (10 mg/l).

Table 1. Oligonucleotides used for PCR amplification in this work

Oligo-nucleotides	Sequences (5' -3')	Restriction cleavage sites
44b-F	TGTCAACGAAATAAAGGCCGAG	
44b-R	AGTTAGTTCATGCGAGGCTGTA	
ada-F1	GCAATTACCCCCCATCTTTG	
ada-F2	GGCACAGAGGCAGACATGAA	
ada-F3	caccCGAAGACCTAGTGTGG	
ada-R1	CTGGTGTGATGGTGTAAAGCC	
ada-R2	GGCAGACCTCCAACGCGATA	
ada-Cla-F	ATCGATGTCTGCAGAACAATTGGC	*Cla*I
ada-Nhe-R	GCTAGCCGTTTCTTCGAATCGTTC	*Nhe*I
GPD2415	GCCACCCAAAAGACCGTTGA	
GPD2873	CCCCACTCGTTGTCGTACCA	
eGFP-Hind-F	AAGCTTATGGTGAGCAAGGGCGAG	*Hin*dIII
eGFP-Pst-R	CTGCAGCTTGTACAGCTCGTCCAT	*Pst* I

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

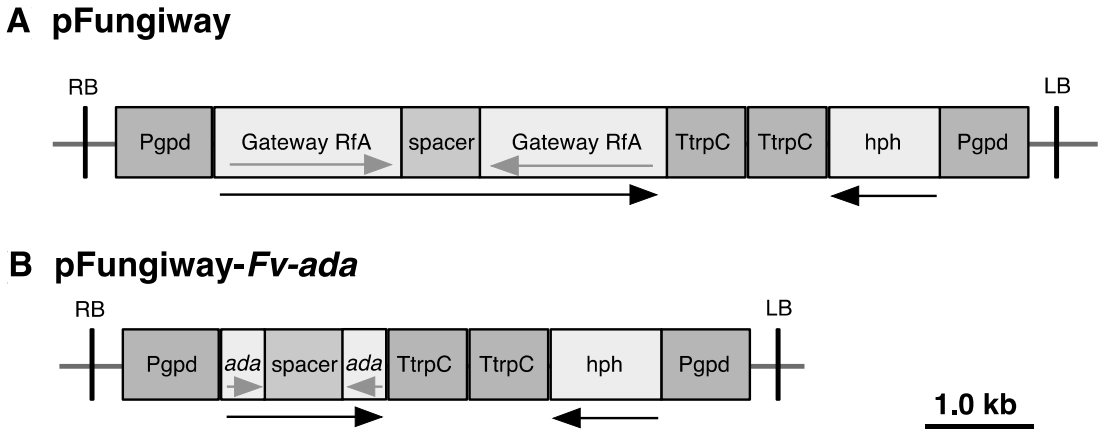


FIG. 1. Sekiya *et al.*

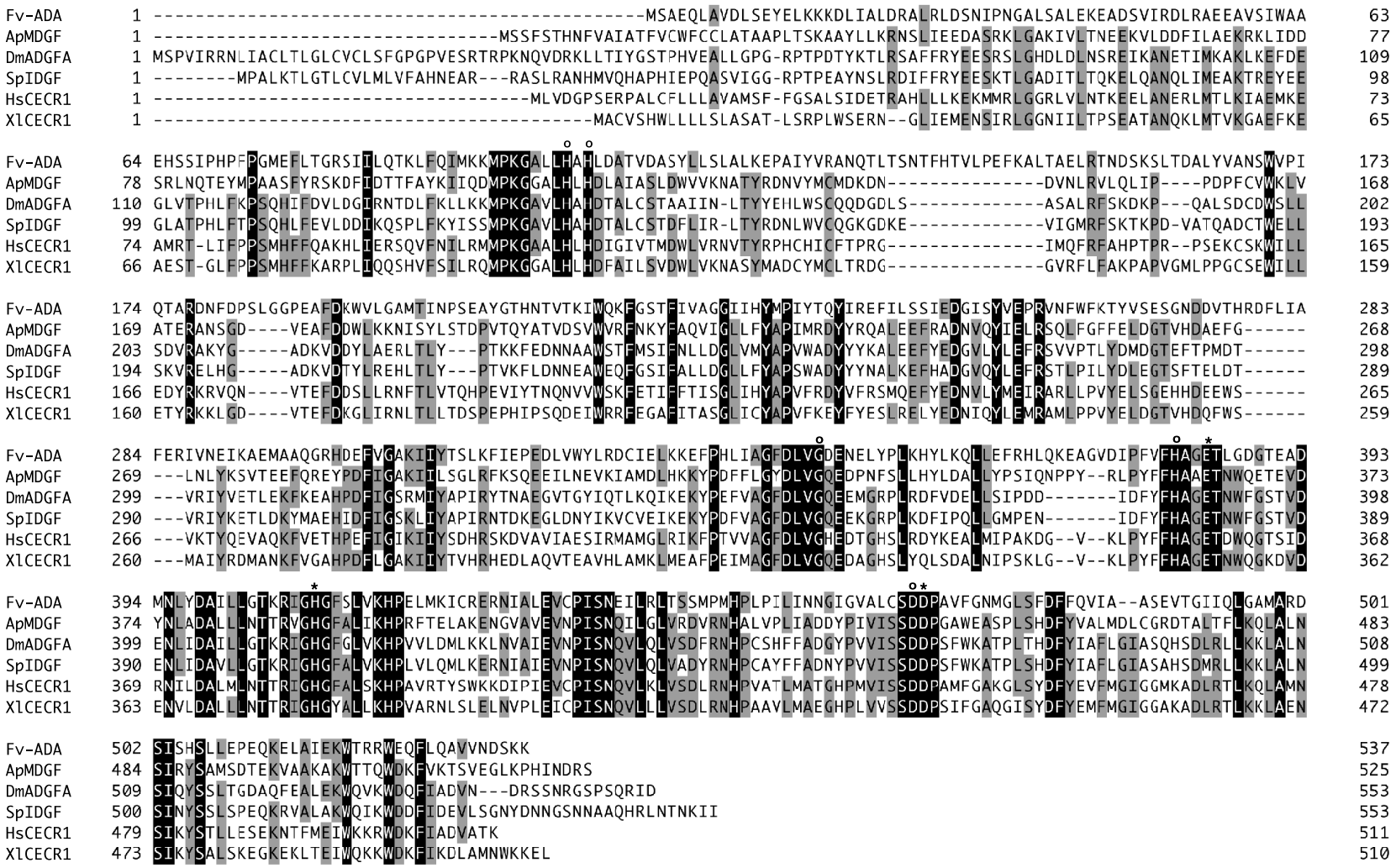


FIG. 2. Sekiya *et al.*

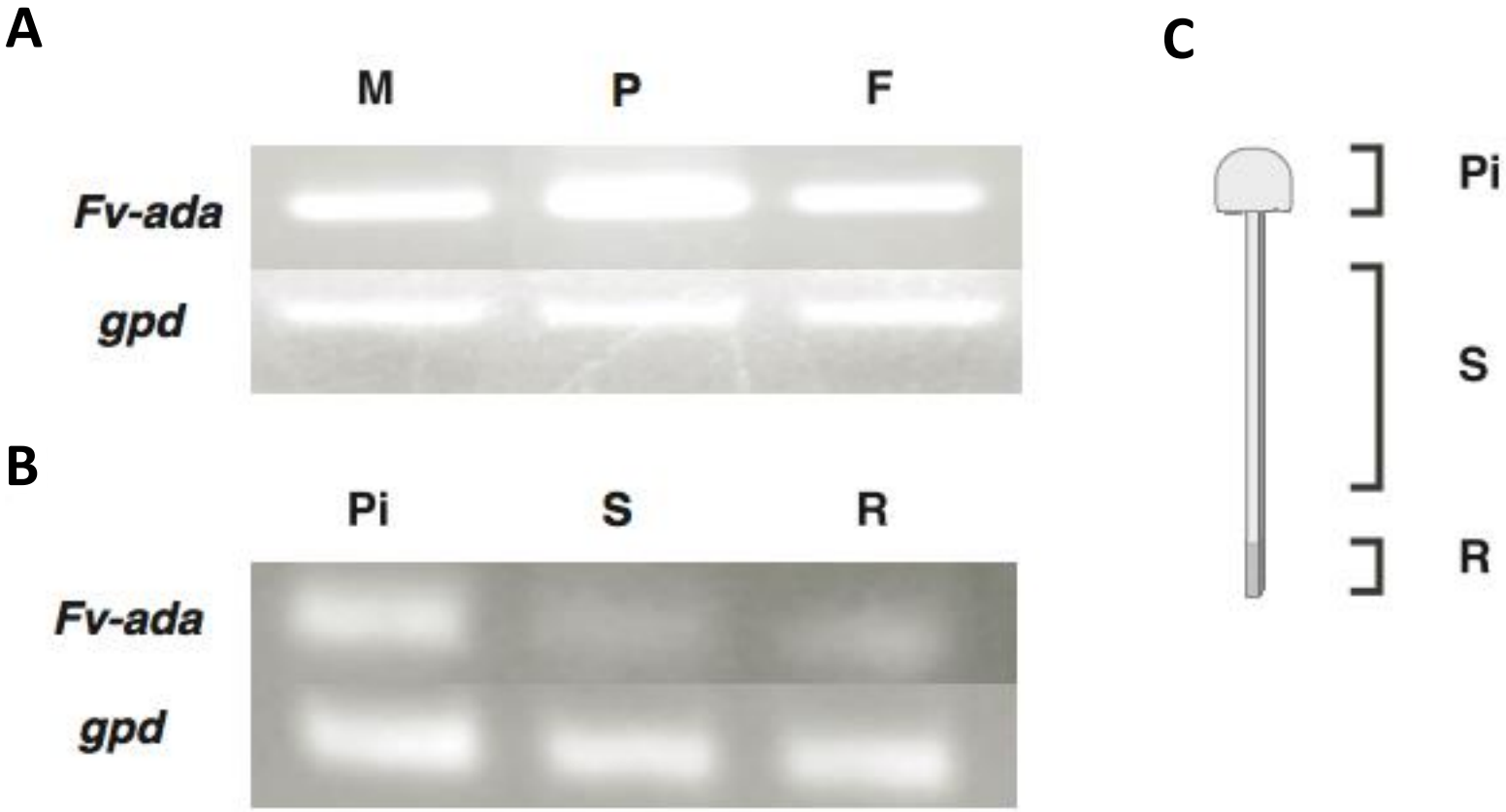
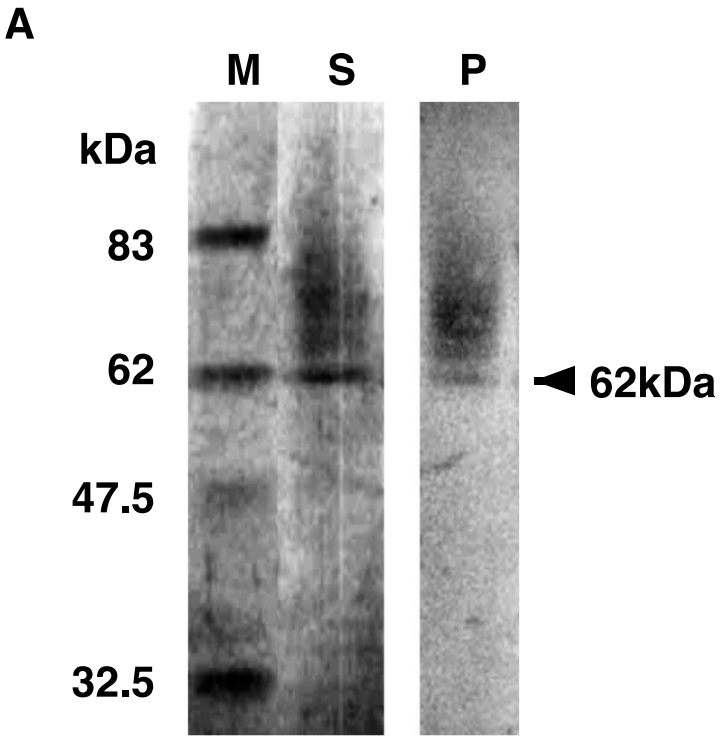


FIG. 3. Sekiya *et al.*



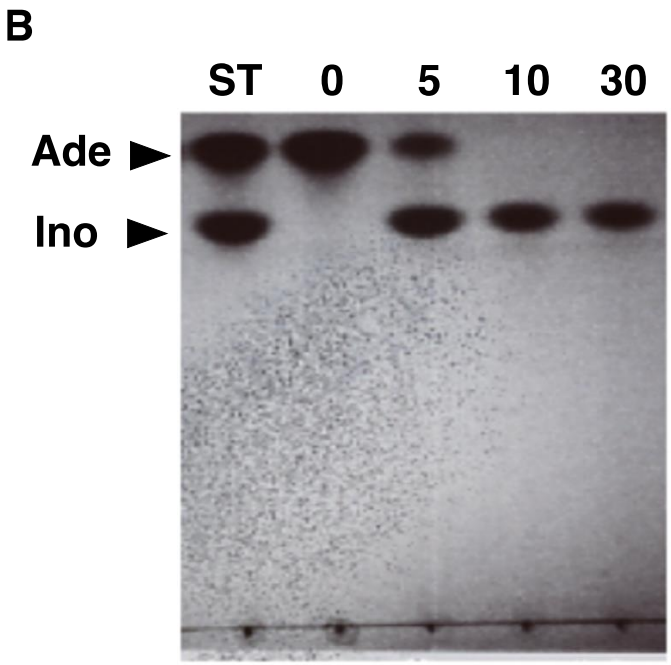


FIG. 4. Sekiya *et al.*

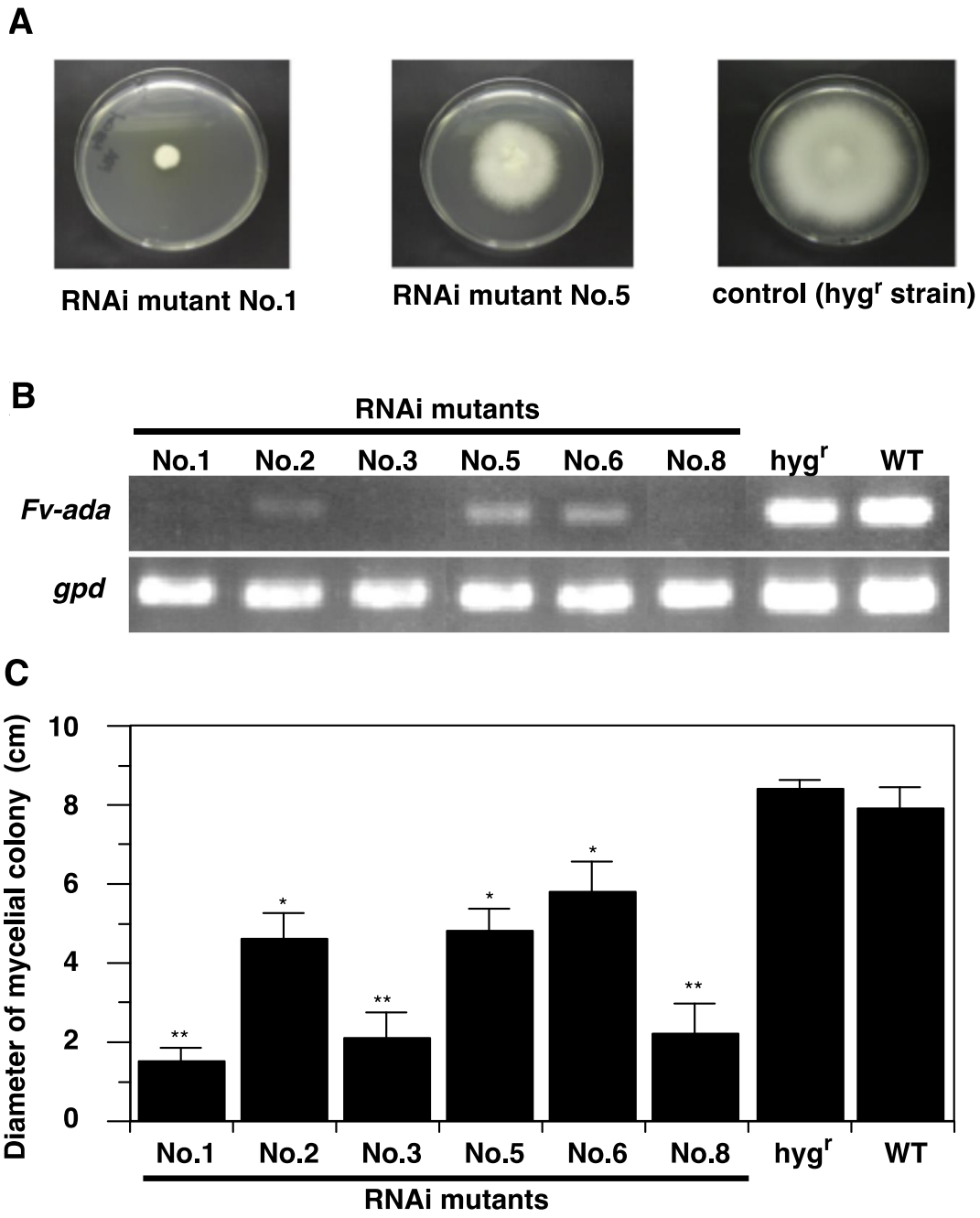


FIG. 5. Sekiya *et al.*

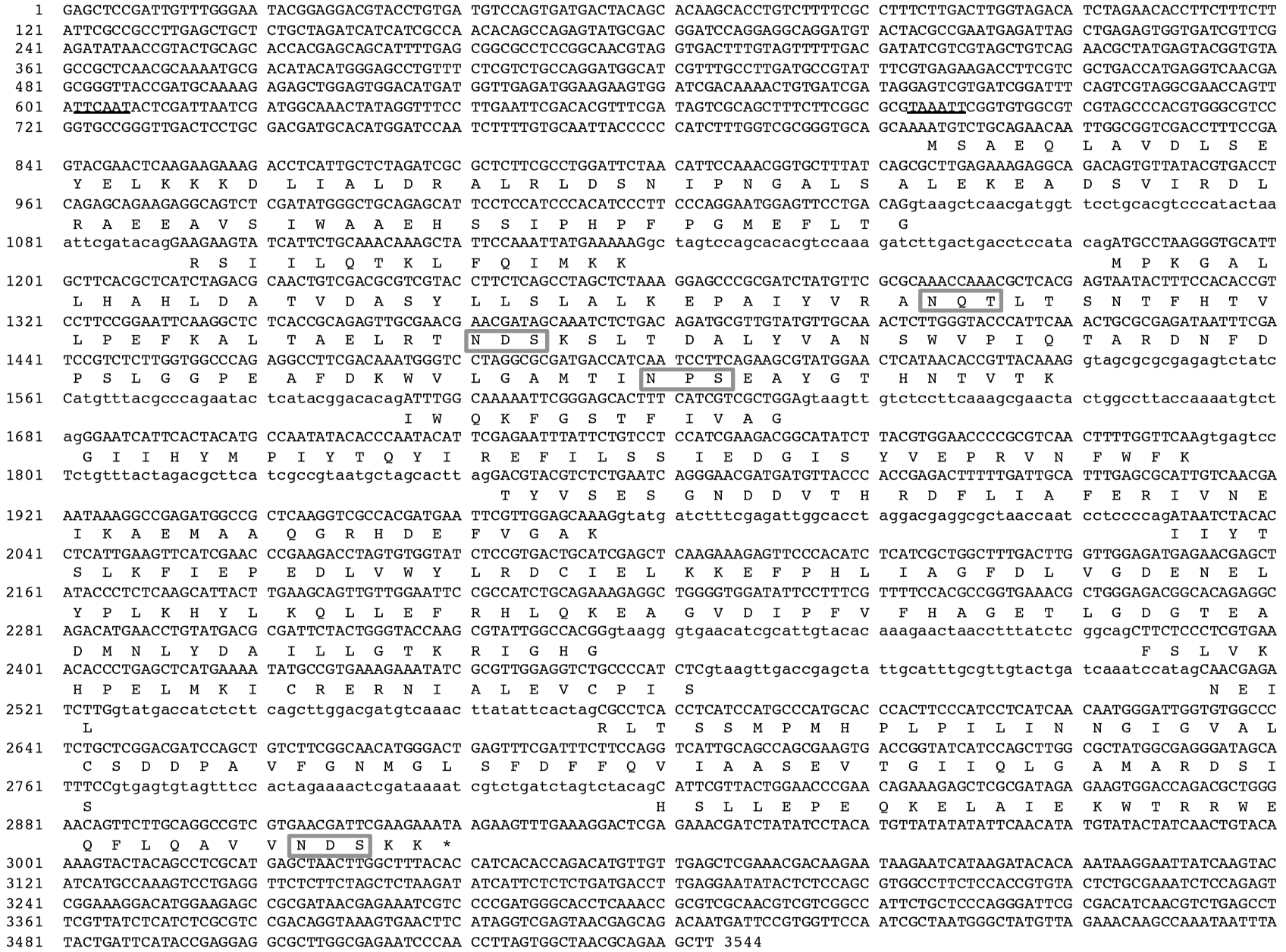


Figure S1 Nucleotide and deduced amino acid sequence of *Fv-ada*.

The nucleotide sequences shown in lowercase indicate introns. Gray boxes indicate putative TATA box-like and CAAT box-like sequences. Open boxes indicate the potential *N*-glycosylation sites.