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Research Note

Cloning and Expression Analysis of Phenylalanine Ammonia-Lyase Gene in the Mycelium and Fruit Body of the Edible Mushroom *Flammulina velutipes*

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Abstract Phenylalanine ammonia-lyase (PAL) gene is known to be expressed in plants, and is involved in the differentiation, growth and synthesis of secondary metabolites. However, its expression in fungi remains to be explored. To understand its expression in mushroom fungi, the PAL gene of the edible mushroom *Flammulina velutipes* (*Fvpal*) was cloned and characterized. The cloned *Fvpal* consists of 2,175 bp, coding for a polypeptide containing 724 amino acids and having 11 introns. The translated amino acid sequence of *Fvpal* shares a high identity (66%) with that of ectomycorrhizal fungus *Tricholoma matsutake*. Distinctively, the *Fvpal* expression in the mycelium was higher in minimal medium supplemented with L-tyrosine than with other aromatic amino acids. During cultivation of the mushroom on sawdust medium, *Fvpal* expression in the fruit body correspondingly increased as the mushroom grew. In the fruiting body, *Fvpal* was expressed more in the stipe than in the pileus. These results suggest that *F. velutipes* PAL activity differs in the different organs of the mushroom. Overall, this is first report to show that the PAL gene expression is associated with mushroom growth in fungi.

Keywords *Flammulina velutipes*, Fruit body, mRNA expression, PAL, Phenylalanine ammonia lyase

Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.24) is the enzyme that dissociates ammonia from L-phenylalanine and produces *trans*-cinnamic acid. The conversion of the amino acid phenylalanine to *trans*-cinnamic acid is the entry step for the channeling of carbon from primary metabolism into the phenylpropanoid secondary metabolism in plants. PAL performs an important role in deriving secondary metabolites, being involved in development, differentiation and growth of plants. Though there have been a lot of studies for the role of PAL in plants, its function in fungi remains unknown. The discovery of a PAL enzyme in fungi [1] and the detection of $^{14}CO_2$ production from ^{14}C-ring-labeled phenylalanine, cinnamic acid, and benzoic acid [2], have demonstrated that fungal PAL can degrade phenylalanine by a pathway involving an initial deamination to cinnamic acid, similar to what happens in plants. Consequently, a metabolic role for the metabolism of phenylalanine via cinnamic, benzoic, *p*-hydroxybenzoic, and protocatechuic acids has been assumed in several basidiomycete fungi, including *Rhodotorula glutinis* [3], *Schizophyllum commune* [2], and *Sporobolomyces roseus* [4]. In the phytopathogenic fungus *Moniliophthora perniciosa*, PAL accumulated during the necrotrophic phase of infection in plant tissues, implying the enzyme might be involved in pathogenicity [5]. Recently, diverse physiological roles were also inferred in the ectomycorrhizal fungus *Tricholoma matsutake*, from the observation that PAL mRNA expression was dependent on the developmental stage [6].

The white-rot fungus *Flammulina velutipes* belongs to the order Agaricales in the phylum Basidiomycota. It is known as the winter mushroom, and is one of the six most actively cultivated mushrooms in the world; over 300,000

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tons of this mushroom are produced per year [7]. Its distribution is limited to the temperate zones of the world because a cold period is required for fruiting [8]. The wild *F. velutipes* mushroom has a dark brown fruit body. But through breeding, long and thin mushroom cultivars with white fruit body have been developed. Korea exports this edible mushroom to 27 countries, including the USA, Vietnam, Hong Kong, and Australia. With the findings that *F. velutipes* has strong immuno-modulatory and anti-tumoral activities [9, 10], the benefits of this mushroom have received more attention.

In an effort to understand the function of PAL in *F. velutipes*, this study was done to clone the PAL gene and characterize its expression in the fruiting body at major development stages during cultivation, and in the mycelium grown in media supplemented with aromatic amino acids.

For PAL gene cloning, RNA sequence data of *F. velutipes* was collected from our previous studies [11, 12]. By the analysis of the RNA pool derived from the *F. velutipes* mycelium and comparison of the RNA pool data to the genome sequence of *Coprinopsis cinerea*, we found a sequence of 2,172 bp PAL gene candidate. We named this candidate gene as a potent *F. velutipes* PAL gene (*Fvpal*), and planned to verify whether this potent *Fvpal* truly existed in the cell of *F. velutipes*. Thus, we cloned and re-sequenced the open reading frame sequences of *Fvpal* mRNA from *F. velutipes* 4146 strain. For this purpose, mycelia of the fungal strain grown on malt extract agar was ground to a fine powder under liquid nitrogen, and total RNA was prepared from the resulting mycelia powder using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), in accordance with the manufacturer's instructions. The prepared RNA was reverse transcribed with oligo(dT) primers using the SuperScript First-Strand Synthesis-System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The resulting cDNA (100 ng for each reaction) was used for reverse transcription polymerase chain reaction (RT-PCR) to amplify the potent *Fvpal* of *F. velutipes*. A primer set of FvPAL-F (5′-ATG CCT TCA GAA CTC TTC GAC CTC-3′) and FvPAL-R (5′-CTA GAG CTT GCT GCG AGG CA-3′) was designed based on the potent *Fvpal* sequence from the previous RNA sequence data, and used for RT-PCR. Amplifying reaction was performed using FastStart High Fidelity PCR System (Roche, Basel, Swiss) under the following conditions: initial denaturation at 95°C for 5 min followed by 30 cycles of 45 sec at 95°C, 45 sec at 58°C, and 2 min at 72°C. The amplified RT-PCR product was sequenced at Macrogen (Seoul, Korea).

A nucleotide sequence of 2,175 bp with termination codon sequence was determined. The determined sequence contained a protein coding sequence that matched 100% with the potent *Fvpal* nucleotide sequence (data not shown). This result confirmed that the potent *Fvpal* is truly present in the cell of *F. velutipes*. Consequently, we further analyzed the potent *Fvpal* sequence using Expasy bioinformatics resource portal (http://web.expasy.org). A protein coding sequence of 724 amino acids was inferred from the potent 2,172 bp *Fvpal* sequence. The PAL signature motif containing the enzyme active site, a serine residue that is unique in eukaryotic PAL [13], was present in the protein sequence of the potent *Fvpal* (Table 1). A BLASTP search of the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the translated amino acid sequences of *Fvpal* revealed that it had 35% to 66% sequence identity with those of known fungal species (Table 1). Thus, we concluded that the potent *Fvpal* is a true PAL gene that codes for *F. velutipes* PAL protein (FvPAL).

The FvPAL shared the highest sequence identity (66%) with *Tricholoma matsutake* PAL. The presence of intron in

Table 1. Comparison of *Flammulina velutipes* PAL sequence with other fungal PAL sequence properties

Fungi	PAL signature motif[a]	Length of amino acid	Identity[b]	No. of introns	GenBank accession No.
Ascomycota					
Aspergillus nidulans	GSISASGDLTPLAYIAG	687	39	1	CBF75146
Aspergillus oryzae	GTISASGDLMPLAYVTG	696	40	2	XP001822016
Botrytis cinerea	GSISASGDLSPLSYIGG	1,131	36	6	XP001556527
Fusarium oxysporum	GTISASGDLMPLSYIAG	750	35	0	EXK33006
Magnaporthe oryzae	GSISASGDLSALAWIAA	627	41	0	XP003717468
Basidiomycota					
Coprinopsis cinerea	TSISASGDLSPLSYIAG	734	62	9	XP001830572
Flammulina velutipes 4146	SSISASGDLSPLSYVAG	724	100	11	KF737393
Laccaria bicolor	GTISASGDLAPLSYIAG	688	63	5	XP001880348
Puccinia graminis	GSISASGDLMPLSYVAA	691	40	13	XP003330746
Rhodotorula mucilaginosa	GTISASGDLSPLSYIAA	720	42	5	P10248.2
Rhodosporium toruloides	GTISASGDLSPLSYIAA	716	42	6	P11544.2
Tricholoma matsutake	GSISASGDLSPLSYIAG	719	66	6	GU980196
Ustilago maydis	SSISASGDLSPLSYVAG	724	43	0	XP756225

[a]Phenylalanine ammonia-lyase (PAL) active site serine residue was bolded.

[b]The percentage of PAL protein sequence identity between *F. velutipes* 4146 PAL and other fungal PALs.

PAL genes is known in some fungi [14]. Thus, we investigated for the presence of intron in *Fvpal*. For this purpose, genomic DNA was extracted from the mycelium of *F. velutipes* using the method described by Kim *et al*. [15], and used as the template DNA for PCR amplification of *Fvpal*. Using the same amplification condition for RT-PCR and FvPAL-F and FvPAL-R primer set, a PCR product of 2,746 bp was obtained, cloned into pGEM T-easy vector (Promega, Madison, WI, USA), and sequenced. Nested primer FvPALns-F (5′-AGC ATC TCA GCC TCC GGA GA-3′) and FvPALns-R (5′-GAG GAG GTA GAG GTA CGA GGA-3′) was used for sequencing.

The determined nucleotide sequence contained a coding sequence (2,172 bp) and eleven interrupting sequences (totally 571 bp). The coding sequence was the same as the sequence of *Fvpal*. Thus, we discovered that *Fvpal* contains eleven introns (Table 1). Because only the PAL gene of the rust pathogen *Puccinia graminis* has more than eleven introns [14], *Fvpal* was considered as one of exons with a high number of introns in fungi. We deposited the sequence of *Fvpal* to GenBank with the accession number KF737393 (Table 1).

To understand the phylogenetic relationship between FvPAL and other fungal PALs, phylogenetic analysis was performed. All reference PAL protein sequences of twelve other fungal species were obtained from the GenBank database. The plant PAL sequence of *Arabidopsis thaliana* was used as an outgroup. Phylogenetic tree, based on fungal PAL protein sequences, was constructed using the maximum likelihood method by MEGA6 [16, 17]. 1,000 bootstrapping was performed to support the tree branch. The FvPAL formed a clad with those of Basidiomycota (Fig. 1). Among Basidiomycota, the FvPAL formed a group with those of *Coprinopsis cinerea*, *Laconia bicolor*, and *T. matsutake*, that typically produce mushroom as their fruit body. This result of phylogenetic relationships agreed with the result of PAL protein sequence identity between *F. velutipes* and the three mushroom species (Table 1).

Gene transformation with a gene knock vector is a useful approach to explore the function of genes in fungi, but it is still not feasible in the edible mushroom *F. velutipes*. Therefore, to understand FvPAL properties, we examined the expression properties of the FvPAL gene in both the mycelium and fruit body, using real time reverse transcription quantitative polymerase chain reaction (RT-qPCR) method. With regards to the mycelium, the effect of aromatic amino acid on *Fvpal* expression was examined because there were reports that PAL induction is regulated by aromatic amino acid or nitrogen sources [14]. Thus, *F. velutipes* 4146 strain was cultured in mushroom minimal media (MMM: 20 g/L dextrose, 0.5 g/L $MgSO_4$, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, 2 g/L DL-asparagine, 120 μg/L thiamine HCl, 20 g/L agar) amended with each different amino acid (organic nitrogen source) or inorganic nitrogen source. For the amendment, the amino acid DL-asparagine in MMM was replaced with either L-phenylalanine (0.3 mM), L-tryptophan (0.3 mM), L-tyrosine (0.3 mM), or L-histidine (0.3 mM) as the amino acid, or with ammonium nitrate as an inorganic nitrogen (20 mM), respectively. The fungal mycelia were grown in each culture medium for 7 days at 25°C. All experiments were performed in triplicate. For the fruiting body, *Fvpal* expression was examined at different stages of mushroom development during artificial cultivation in sawdust media in bottles (Fig. 2). The mushroom development of *F. velutipes* was classified into five stages: mycelium, primordium, fruit body 1, fruit body 2, and fruit

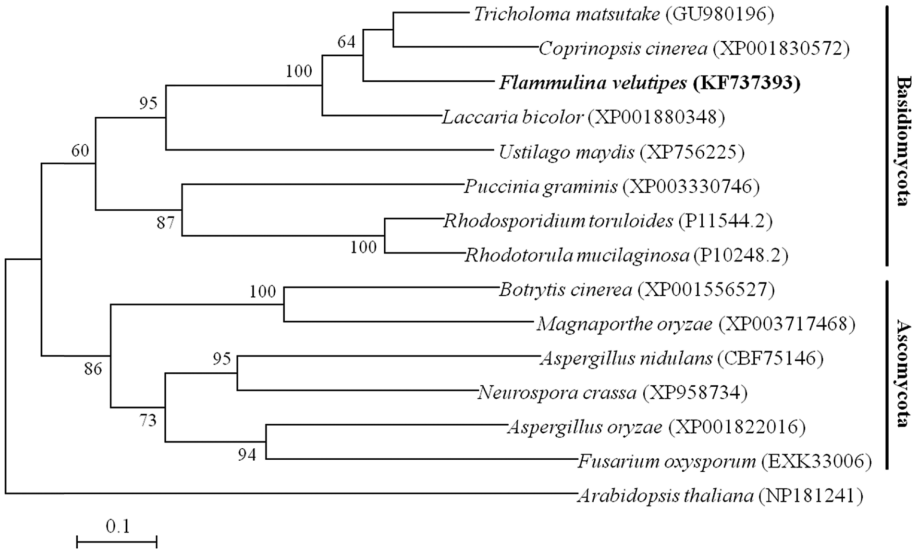

Fig. 1. Phylogenetic tree based on phenylalanine ammonia-lyase (PAL) protein sequences of 14 fungal species. Tree was constructed by the maximum likelihood method using MEGA6. Bootstrap values above 50% are shown at the nodes of the tree. An *Arabidopsis thaliana* PAL sequence was used as an outgroup. GenBank accession number of the PAL gene sequences compared is indicated in parentheses.

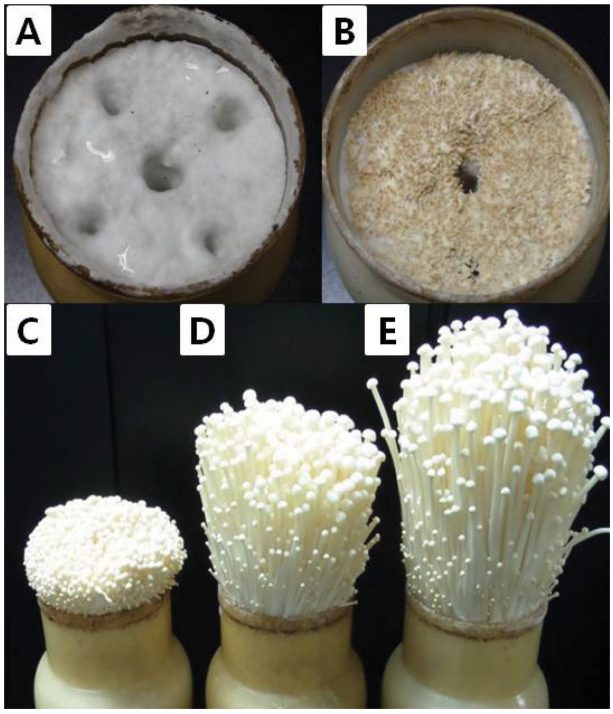

Fig. 2. Fruit body developmental stage of *Flammulina velutipes* grown on sawdust media. Developmental stage of this mushroom was classified into mycelium (A), primordium (B), fruit body stage 1 (C), fruit body stage 2 (D), and fruit body stage 3 (E).

body 3 (Fig. 2). For expression analysis, the mushroom samples of fruit body stage 2 and 3 were divided into pileus and stipe, respectively.

For RT-qPCR analysis, total RNA and cDNA were prepared from both the mycelia and fruit body samples of *F. velutipes*, as described before. RT-qPCR reaction consisted of 12.5 μL of SYBR Premix Extaq (Takara, Tokyo, Japan), 100 ng cDNA and FvPALRT-F (5′-CTC GTT GAT ACG GGG GCA TTC-3′) and FvPALRT-R (5′-GAA GGC AGA AGG TCC ATC GAA GA-3′) primers. The threshold cycle (Ct) values, which represents the PCR cycle at which fluorescence passes the threshold, were determined using the software accompanying the TP800 [18]. For normalization of the real time RT-qPCR data, actin gene expression was used as the control at each time point. FvActin-F (5′-CCA TAG GTT TCT CTC TTC CTC AC-3′) and FvActin-R (5′-CCA CGT TCC ATC AGG TTC TT-3′) were used as *F. velutipes* actin gene specific primers. Data were subjected to one-way analysis of variance (ANOVA) in SPSS ver. 21.0 (IBM Co., Armonk, NY, USA). The significant differences between the group means were compared using Duncan's multiple range test. Differences at $p < 0.05$ were considered significant.

No significant difference was found in the growth *F. velutipes* mycelia among MMM amended with different amino acids (organic nitrogen source) or inorganic nitrogen source. However, *Fvpal* expression in mycelium was increased in the growth medium supplemented with ammonium nitrate, L-phenylalanine, L-tryptophan and L-tyrosine, than in media supplemented with L-histidine and control (Fig. 3A). Interestingly, the maximum increase of *Fvpal* expression was found in medium supplemented with L-tyrosine, despite the fact that L-phenylalanine is the main substrate of PAL. Thus, it seems that *Fvpal* is not likely to be induced by a substrate-depended manner. Similarly, in the corn smut pathogen *Ustilago maydis*, PAL activity was also more induced in minimal medium amended with L-tryptophan [19]. In addition, it is noticeable that when it is compared to L-phenylalanine supplement, *Fvpal* expression enhanced

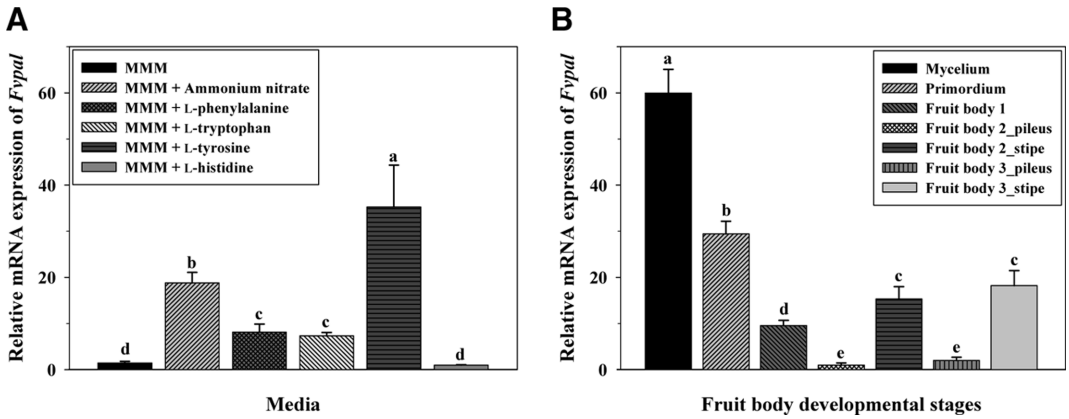

Fig. 3. Analysis of the *pal* mRNA expression in *Flammulina velutipes* using real time reverse transcription polymerase chain reaction. A, Effect of aromatic amino acid supplement in mushroom minimal medium (MMM) on the level of the *Fvpal* expression the mycelium; the DL-asparagine in the MMM was replaced with either 20 mM ammonium nitrate, 0.3 mM L-phenylalanine, 0.3 mM L-tryptophan, 0.3 mM L-tyrosine, or 0.3 mM L-histidine; B, Expression level of the *F. velutipes* PAL gene (*Fvpal*) in fruit body during the developmental stages of *F. velutipes* grown on sawdust media; the data of the *Fvpal* expression level are represented as bars with standard deviation, and values denoted by the same letters are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

more than 2-fold with ammonium nitrate supplement (Fig. 3A). This result indicates that the inorganic nitrogen source could induce the *Flammulina* mushroom PAL. Consequently, the induced PAL is expected to enhance the metabolic degradation of phenylalanine into *t*-cinnamic acid by its enzymatic function. Considering that ammonium nitrate differently affects the substrate digestibility and degradation in mushroom species [20], we cannot rule out that it could also be metabolically involved in the L-phenylalanine degradation process operated by PAL. If this case is proven, we could conclude that PAL has a metabolic role in the mycelium of *F. velutipes*. Further work is needed to explore the mechanism by which ammonium nitrate and L-tyrosine metabolically regulate the PAL gene expression in the mycelium of *F. velutipes*. So far, this is first demonstration in fungi that PAL gene expression is increased maximally with L-tyrosine supplement in the minimal medium.

The results of *pal* mRNA expression analysis during the fruiting body development of *F. velutipes* showed that the *Fvpal* was expressed most at the mycelium stage of fully covered mycelia, on the top of the cultivation bottle (Fig. 3B). The *Fvpal* expression gradually decreased as the fruit body developed from the mycelium stage to stage 1 (Fig. 3B). With the development of the fruit body from stage 1 (where the formation of mushroom shape was completed) (Fig. 2C) to stage 2 (where the mushroom elongated around 5 cm) (Fig. 2D), the *Fvpal* expression increased again (Fig. 3B). However, no more increase was found at stage 3, where the mushroom was elongated enough to be harvested for market sales (Figs. 2E and 3B). Interestingly, in the fruiting body of stage 2 and 3, the *Fvpal* expression was more in the stipe than in the pileus (Fig. 3B). These results imply that the deamination activity of ammonia from L-phenylalanine occurs in the stipe during the mushroom elongation. From Fig. 2C and 2D, we could consider the fruit body elongation as mushroom growth, which is similar to the growth of plants which is accompanied with elongation of the stems and leaves. In the context that PAL is involved in plant growth [21], we may state that PAL gene expression is associated with mushroom growth in *F. velutipes*. PAL gene expression in mushroom has been reported in button mushroom (*Agaricus bisporus*) in response to stress [22], and in *Tricholoma* mushroom (*T. matsutake*) in different structural parts [6]. However, there has been no report on the comparative analysis of PAL gene expression in fungi in relation to mushroom growth. Thus, to our knowledge, this is the first report of PAL gene expression during mushroom growth.

To summarize, the PAL gene of *F. velutipes* was cloned and its structural properties were characterized in this study. With real time RT-qPCR, we found that the *Fvpal* expression in the mycelium is greatly influenced by L-tyrosine supplement in the minimal medium, which is a distinct property that has not been reported in other fungi. These results suggest that the metabolic role(s) of the *F. velutipes* PAL could be different from those of other fungal PALs. In addition, we are the first to provide evidence that PAL gene expression is associated with fruit body growth and development in fungi. Considering that a wide range of phenolic compounds which have diverse functions in plants are synthesized by PAL function through the phenylpropanoid pathway, there is a possibility that the *F. velutipes* PAL might also be involved in the production of phenolic compounds which perform some functions in the mushroom stipe during the mushroom growth. Thus, further studies are needed to analyze phenolic compounds produced in the fruit body during the development of *F. velutipes* mushroom.

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