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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Running title: Cloning and expression of Fvpal

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Abstract

Phenylalanine ammonia lyase (PAL) gene is known to express in plants to differentiate, grow and synthesize 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 has 11 introns. The translated amino acid sequence of Fvpal shares high identity (66%) with that of ectomycorrhizal fungus Tricholoma matsutake. Distinctively, the Fvpal expression in the mycelium was higher in the 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 increased as mushroom grew. In the fruiting body Fvpal 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 that 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 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 of PAL’s roles in plants, it still remains which role the enzyme plays in fungi. The discovery of a PAL enzyme in fungi [1] and the detection of 14CO2 production from 14C-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, as 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]. While, 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 depended on the developmental stage [6].

The white-rot fungus Flammulina velutipes belongs to the order Agaricales in the phylum Basidiomycota. It is known as winter mushroom and is one of the six most actively cultivated mushrooms in the world; over 300,000 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]. In the wild F. velutipes mushroom has dark brown fruit body but through
breeding long and thin mushroom cultivars with white fruit body have been developed. Korea is exporting this edible mushroom to twenty seven countries including USA, Vietnam, Hong Kong, and Australia. With the findings that *F. velutipes* has strong immuno-modulatory and anti-tumoral activities [9,10], the values of this mushroom have been received more attention.

In an effort to understand the function of PAL in *F. velutipes*, this study was carried to clone PAL gene and characterize its expression in fruiting body at major developing stages during cultivation and in mycelium grown at media supplement with aromatic amino acids. For PAL gene cloning, RNA seq data of *F. velutipes* was collected from our previous study [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 2172 bp PAL gene candidate. We named this candidate gene as potent *F. velutipes* PAL gene (*Fv*pal) and planed to verify whether this potent *Fv*pal really existed in the cell of *F. velutipes*. Thus, we cloned and re-sequenced the open reading frame (ORF) sequences of *Fv*pal mRNA from *F. velutipes* 4146 strain. For this aim, mycelia of the fungal strain grown on MEA was ground to a fine powder under liquid nitrogen and total RNA was prepared using TRIzol reagent (Life Technologies, USA) and RNaseasy plant mini kits (Qiagen, USA) in accordance with the manufacturer's instructions. Purified RNA was reverse transcribed using the SuperScript First-Strand Synthesis-System (Invitrogen, USA) according to the manufacturer’s recommendations with oligo (dT) primers. The resulting cDNA (100 ng for each reaction) was used for RT-PCR to amplify the potent *Fv*pal 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 *Fv*pal sequence from
the previous RNA seq data and used for RT-PCR. Amplifying reaction was performed using
FastStart High Fidelity PCR System (Roche, 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 company (Seoul,
Korea). Nucleotide sequence of 2,175 bp with termination codon sequence was determined.
The determined sequence contained a protein coding sequence that 100% matched 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 enzyme active site serine residue that is
unique in eukaryotic phenylalanine ammonia-lyase [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 PAL genes is known in some fungi [14]. Thus, we
investigated the presence of intron in Fvpal. For this work, 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, USA), and sequenced. Nested primer FvPALns-
F (5'-AGC ATC 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 same with the sequence of Fvpal. Thus, we found Fvpal contains eleven introns (Table 1). Because only the PAL gene of rust pathogen Puccinia graminis has more than eleven introns [14], Fvpal was considered as one of exons with 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 outgroup. Phylogenetic tree based on fungal PAL protein sequences was constructed using the maximum likelihood method by MEGA6 [16, 17]. 1000 bootstrapping was performed to support the tree branch. The FvPAL formed a clad with those of Basidiomycota (Fig. 2). Among Basidiomycota, the FvPAL formed a group with those of Coprinopsis cinerea, Laconia bicolor, and T. matsutake that could produce typically mushroom as their fruit body (Fig. 2). This result of phylogenetic relationships agreed with the result of PAL protein sequence identity between F. velutipes and the three mushroom species, C. cinerea, L. bicolor, and T. matsutake (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 both in mycelium and fruit body using real time RT-per method. For 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 4166 strain was cultured in mushroom minimal media (MMM; 20 g/l dextrose, 0.5 g/l MgSO4, 0.46 g/l KH2PO4, 1 g/l K2HPO4, 2 g/l DL-asparagines, 120 uGu/l thiamine HCL, 20 g/l agar) amended with each different amino acid or nitrogen source. The amino acid DL-asparagine was replaced with either L-phenylalanine (0.3 mM) or, L-tryptophan (0.3 mM) or, L-tyrosine (0.3 mM) or, L-histidine (0.3 mM) or, ammonium nitrate (20 mM). Mycelia were grown in each culture mycelium for 7 days at 25°C. All experiments performed in triplicate.

Meanwhile, to analyze the expression properties of the FvPAL gene in fruit body, Fvpal expression was examined at different mushroom development stage 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 body 3 (Fig. 2). The mushroom samples of fruit body stage 2 and 3 were divided into pileus and stipe for expression analysis. Total RNA and cDNA from F. velutipes samples that were from the mycelia grown in MMM with different amino acid supplement or nitrogen source and from the fruit body sampled at each mushroom developmental stage, were prepared as described before and subjected to real time RT-qPCR reaction. The reaction consisted of 12.5 μl of SYBR Premix Extaq (Takara), 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 represent 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 version 21.0. The significant differences between group means were compared using Duncan’s multiple range test. Differences at $p<0.05$ were considered significant.

The results from real time RT-qPCR of mRNA expression of *F. velutipes pal* revealed that *Fvpal* expression in mycelium increased in the growth medium supplemented with ammonium nitrate, L-phenylalanine, L-tryptophan, L-tyrosine, and L-tyrosine, but not in control and with L-histidine (Fig. 3A). Interestingly, the most increase of *Fvpal* expression was found in the medium supplemented with L-tyrosine despite the fact that L-phenylalanine is the main substrate of PAL. Thus, it looks in *F. velutipes* PAL gene is not likely to be induced in the substrate-depended manner. Similarly, in *Ustilago maydis*, PAL activity was also more induced in minimal medium amended with L-tryptophan [19]. It is noticeable that when it is compared to L-phenylalanine supplement, *Fvpal* expression enhanced more than ten times with ammonium nitrate supplement (Fig. 3A). Considering that ammonium nitrate effect differently on the substrate digestibility and degradation in mushroom species [20], we cannot rule out that it could also be metabolically involved in L-phenylalanine degradation process operated by PAL. If this case is proven, we could think PAL does a metabolic role in the mycelium of *F. velutipes*. Further work is needed to explore the way that ammonium nitrate and L-tyrosine regulate metabolically PAL gene expression in the mycelium of *F. velutipes*. So far, this is first demonstration in fungi that PAL gene expression is most increased with L-tyrosine supplement in the minimal medium.

The results of *pal* mRNA expression analysis during the fruit body development of *F. velutipes* showed that the *Fvpal* was most expressed at the stage 1 of fully covered mycelia
The Fvpal expression gradually decreased as the fruit body developed from the stage 1 to the stage 3. With the fruit body development from the stage 3 where the formation of mushroom shape was completed (Fig. 2C) to the stage 4 where the mushroom elongated around 5 cm (Fig. 2D), the Fvpal expression did increase again but no more increase was found at the stage 5 where the mushroom elongated enough to harvest for market sale (Fig. 2E). Interestingly, in the fruiting body of the stage 4 and 5, the Fvpal expression was more revealed in the stipe than in the pileus (Fig. 3B). These results imply that the deamination activity of ammonia from L-phenylalanine is occurring during the mushroom elongation in the stipe part. From Fig. 2C-D, we could consider the fruit body elongation as mushroom growth, which is similar to the growth of plants which accompany with elongation of 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.

Overall, the PAL gene of F. velutipes was cloned and its structural properties were characterized in this study. With the 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 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 first provided an 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 phenylpropanoid pathway, there is possibility the *F. velutipes* PAL might also be involved in the production of phenolic compounds which do some functions in the mushroom stipe during the mushroom growth. Thus, further studies are expected to analyze phenolic compounds produced in the fruit body during the development of *F. velutipes* mushroom.

**Acknowledgements**

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**References**


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13. Pilbak S, Tomin A, Retey J, Poppe L. The essential tyrosine-containing loop


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

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<th>Fungi</th>
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*aPAL active site serine residue was bolded. bThe percentage of PAL protein sequence identity between *F. velutipes* 4146 PAL and other fungal PALs.*
**Figure legends**

**Fig. 1.** Phylogenetic tree based on 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 was indicated in parentheses.

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

**Fig. 3.** Analysis of the pal mRNA expression in *F. velutipes* using real time RT-qPCR. (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 or, 0.3 mM l-phenylalanine or, 0.3 mM l-tryptophan or, 0.3 mM l-tyrosine or, 0.3 mM l-histidine. (B) Expression level of the *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.
Fig. 1.
Fig. 2.
Relative mRNA expression of FvPal

A

![Graph with relative expression of FvPal in different media conditions.](image)

B

![Graph with relative expression of FvPal in different fruit body developmental stages.](image)