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 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, 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
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 immunomodulatory 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 translated amino acid sequences of
*Flammulina velutipes* (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
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
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

<table>
<thead>
<tr>
<th>Fungi</th>
<th>PAL signature motif</th>
<th>Length of amino acid</th>
<th>Identity</th>
<th>No. of introns</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>GSISASGDLTPLAYIAG</td>
<td>687</td>
<td>39</td>
<td>1</td>
<td>CBF75146</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>GTISASGDLMPLAYVTG</td>
<td>696</td>
<td>40</td>
<td>2</td>
<td>XP001822016</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>GSISASGDLPSYIAG</td>
<td>1,131</td>
<td>36</td>
<td>6</td>
<td>XP001556257</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>GTISASGDLMPSYIAG</td>
<td>750</td>
<td>35</td>
<td>0</td>
<td>EKX33006</td>
</tr>
<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>GSISASGDLALWIAA</td>
<td>627</td>
<td>41</td>
<td>0</td>
<td>XP003717468</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Coprinopsis cinerea</em></td>
<td>TSISASGDSLPSYIAG</td>
<td>734</td>
<td>62</td>
<td>9</td>
<td>XP001830572</td>
</tr>
<tr>
<td><em>Flammulina velutipes</em></td>
<td>SSISASGDSLPSYVAG</td>
<td>724</td>
<td>100</td>
<td>11</td>
<td>KF737393</td>
</tr>
<tr>
<td><em>Laccaria bicolor</em></td>
<td>GTISASGDLMPSYIAG</td>
<td>688</td>
<td>63</td>
<td>5</td>
<td>XP001880348</td>
</tr>
<tr>
<td><em>Puccinia graminis</em></td>
<td>GSISASGDSLPSYVAA</td>
<td>691</td>
<td>40</td>
<td>13</td>
<td>XP003330746</td>
</tr>
<tr>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>GTISASGDSLPSYIAA</td>
<td>720</td>
<td>42</td>
<td>5</td>
<td>P10248.2</td>
</tr>
<tr>
<td><em>Rhodospirillum toruloides</em></td>
<td>GTISASGDSLPSYIAC</td>
<td>716</td>
<td>42</td>
<td>6</td>
<td>P11544.2</td>
</tr>
<tr>
<td><em>Tricholoma matsutake</em></td>
<td>SSISASGDSLPSYIAG</td>
<td>719</td>
<td>66</td>
<td>6</td>
<td>GL980196</td>
</tr>
<tr>
<td><em>Ustilago maydis</em></td>
<td>SSISASGDSLPSYVAG</td>
<td>724</td>
<td>43</td>
<td>0</td>
<td>XP756225</td>
</tr>
</tbody>
</table>

1Phenylalanine ammonia-lyase (PAL) active site serine residue was bolded.

2The percentage of PAL protein sequence identity between *F. velutipes* 4146 PAL and other fungal PALs.