J. Microbiol. Biotechnol. (2013), 23(8), 1055–1059
http://dx.doi.org/10.4014/jmb.1303.03064

jmb

Cloning and mRNA Expression Analysis of the Gene Encoding Phenylalanine Ammonia-Lyase of the Ectomycorrhizal Fungus *Tricholoma matsutake*

Hyeokjun Yoon[1], Young-Hyun You[1], Ye-Eun Kim[1], Young Ja Kim[2], Won-Sik Kong[3], and Jong-Guk Kim[1*]

[1]*Department of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea*
[2]*Korea Environmental Industry & Technology Institute, Seoul 122-040, Republic of Korea*
[3]*National Institute of Horticultural & Herbal Science, RDA, Suwon 440-706, Republic of Korea*

Received: March 19, 2013
Revised: June 11, 2013
Accepted: June 21, 2013

First published online
June 26, 2013

**Corresponding author*
Phone: +82-53-950-5379;
Fax: +82-53-955-5379;
E-mail: kimjg@knu.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2013 by
The Korean Society for Microbiology
and Biotechnology

The ectomycorrhizal fungus *Tricholoma matsutake* grows symbiotically with *Pinus densiflora*. Phenylalanine ammonia-lyase (E.C. 4.3.1.24) catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. The role of fungal phenylalanine ammonia-lyase, however, has not been clear until now. In this study, the gene encoding phenylalanine ammonia-lyase (PAL), which was isolated from *T. matsutake*, was cloned and characterized. The PAL gene (*tmpal*) consists of 2,160 nucleotides, coding for a polypeptide containing 719 amino acid residues. The deduced amino acid sequence of *tmpal* from *T. matsutake* shows high identity (70%) with that from *Laccaria bicolor*. Comparative analysis of the PAL genes among *T. matsutake* and other species of the class Agaricomycetes showed that both active sites and binding sites were significantly conserved among these genes. The transcriptional analysis of the PAL gene revealed a differential gene expression pattern depending on the developmental stages (mycelium, primordium, stipe, pileus, and gills) of *T. matsutake*. These results suggest that the PAL gene in *T. matsutake* plays an important role in multiple physiological functions.

Keywords: *Tricholoma matsutake*, mushroom, phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.24) catalyzes the deamination of L-phenylalanine to generate *trans*-cinnamic acid and ammonia [3]. *Trans*-cinnamate is a precursor of the phenylpropanoid pathway. Several secondary metabolites, such as cinnamaldehyde, coumarine, flavonoids, lignin, and resveratrol, are derived from the phenylpropanoid pathway (KEGG, http://www.genome.jp/kegg). Therefore, PAL is a key enzyme of the biosynthetic pathway related to the secondary metabolites derived from L-phenylalanine. PAL is distributed widely in higher plants and fungi. In plants, PAL has been reported to be involved in their growth, development, and defense system [10]. In particular, in the plant defense mechanism, PAL plays a key role in the biosynthesis of salicylic acid [12].

Although fungal PAL genes have been determined, their role in fungi is unclear. In the plant pathogenic fungus *Ustilago maydis*, there are no differences in fungal growth, development, and virulence between the wild-type strains and mutants with a null mutation in the PAL gene [5, 7]. In plant tissues inoculated with the phytopathogenic basidiomycete *Moniliophthora perniciosa*, fungal PAL accumulates during the necrotrophic phase. This suggests that the fungal PAL might potentially be associated with pathogenicity [9].

The ectomycorrhizal fungus *Tricholoma matsutake* belongs to the class Agaricomycetes in the phylum Basidiomycota. *T. matsutake* grows symbiotically with *Pinus densiflora*, and does not have any virulence with its host. Artificial cultivation of *T. matsutake* has not been possible until now, and the yield of this mushroom is limited because of its sensitivity to climatic conditions. In this study, a gene encoding PAL was identified from *T. matsutake*, and its

gene structure and protein domain were analyzed. The PAL gene of *T. matsutake* was characterized by analyzing the gene expression pattern by RT-PCR.

T. matsutake (primordia and fruiting bodies) were harvested in Gachang near Daegu, South Korea. The hyphae were isolated from the fruiting body of *T. matsutake*. The primordia and fruiting bodies were washed with distilled water to remove any soil and contaminants, and then stored at -70°C in a deep freezer. The mycelia were grown on potato dextrose broth (PDB; 4 g/l potato peptone, 20 g/l glucose, pH 5.6 ± 0.2) for 30 days at 25°C. The total RNA was extracted from the fruiting body of *T. matsutake* using a RNA isolation kit (Qiagen, Valencia, CA, USA). The full-length cDNA library of the fruiting body was constructed using the method reported by Kim *et al*. [6].

The plasmid DNA was extracted using a Plasmidprep 96 kit (MWG Biotech., Ebersberg, Germany). The sequencing reactions were examined on a GeneAmp PCR System 9700 thermal reactor (Perkin-Elmer, Boston, MA, USA) using a BigDye Terminator Sequencing kit, with an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The raw traces of the expressed sequence tags (ESTs) were subjected to base-calling by running Phred, and the vector sequences were trimmed using Cross_match [2]. The ESTs with a length of more than 100 bp after vector trimming were subjected to further analysis. The ESTs were aligned and assembled into contigs using CAP3 software [4]. The resulting ESTs were annotated using a BLAST search against the non-redundant protein (NR) database (NCBI, http://www.ncbi.nlm.nih.gov). Among a total of 4,980 ESTs, 3 ESTs revealed high similarity with the fungal PAL gene.

The total genomic DNA of the fruiting body of *T. matsutake* was extracted using a slight modification of the method reported by Kim *et al*. [8]. To determine both the upstream and downstream regions of the PAL gene of *T. matsutake*, nested PCR was performed using the Genome Walker kit (BD Biosciences Clontech, Palo Alto, CA, USA) with the following specific primers: 2 primers for amplification of the upstream region, TMPALGSP1dn (5′-AGGACAGTC GTCTAATTCTACGGAGGCA-3′) and TMPALGSP2dn (5′-CCATCAACCTTGACAGCCTTTCCACTT-3′); 6 primers for amplification of the downstream region, TMPALGSP1up (5′-AGACCTGTCACCCCTATCATATATT-3′), TMPALGSP2up (5′-CAACATTGAACCGCTTCCTCTTGCCT-3′), TMPALGSP3up (5′-ACCAAGCTGTCAAGCAAGTTCA-3′), TMPALGSP4up (5′-CTAGTTCCTTGGCTCTCATTTC-3′), TMPALGSP5up (5′-GGGTCTATGCTTGATTTTGGGG-3′), and TMPALGSP6up (5′-GGCGTTAGCCTTCATGCTTCTT-3′). The following primer set was used identify the intron/exon boundaries of the PAL gene: TMPALATG (5′-ATGTCTTTGCTTCTCAAT GG-3′) and oligo(dT) 18 primers. All protein sequences of

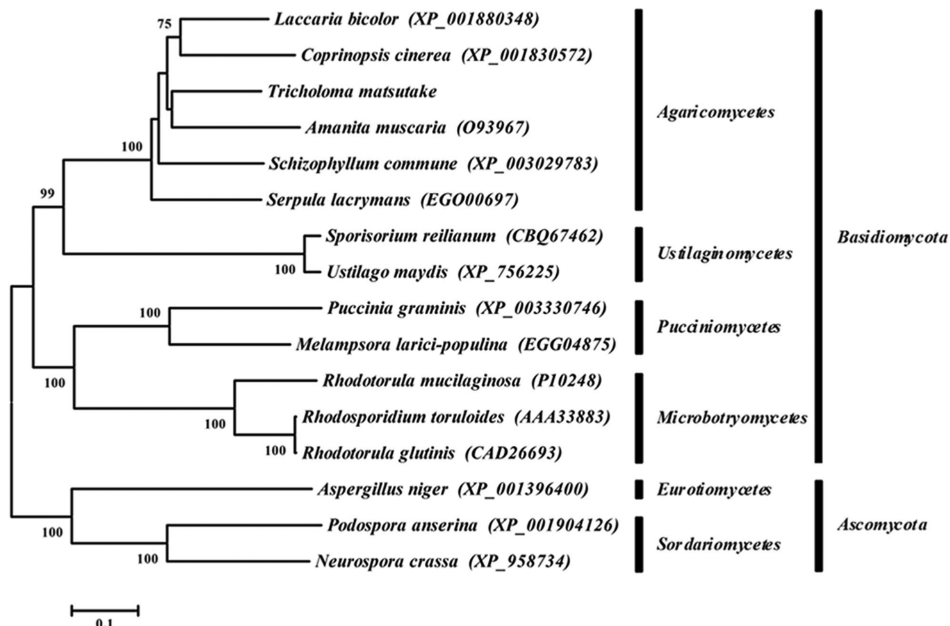

Fig. 1. Phylogenetic analysis of fungal PAL protein sequences.

The phylogenetic tree was inferred using the neighbor-joining method by bootstrapping with 1,000 replicates. Each GenBank accession number of fungal species, excluding *T. matsutake*, is shown in parentheses. Three Ascomycota species were used as outgroups.

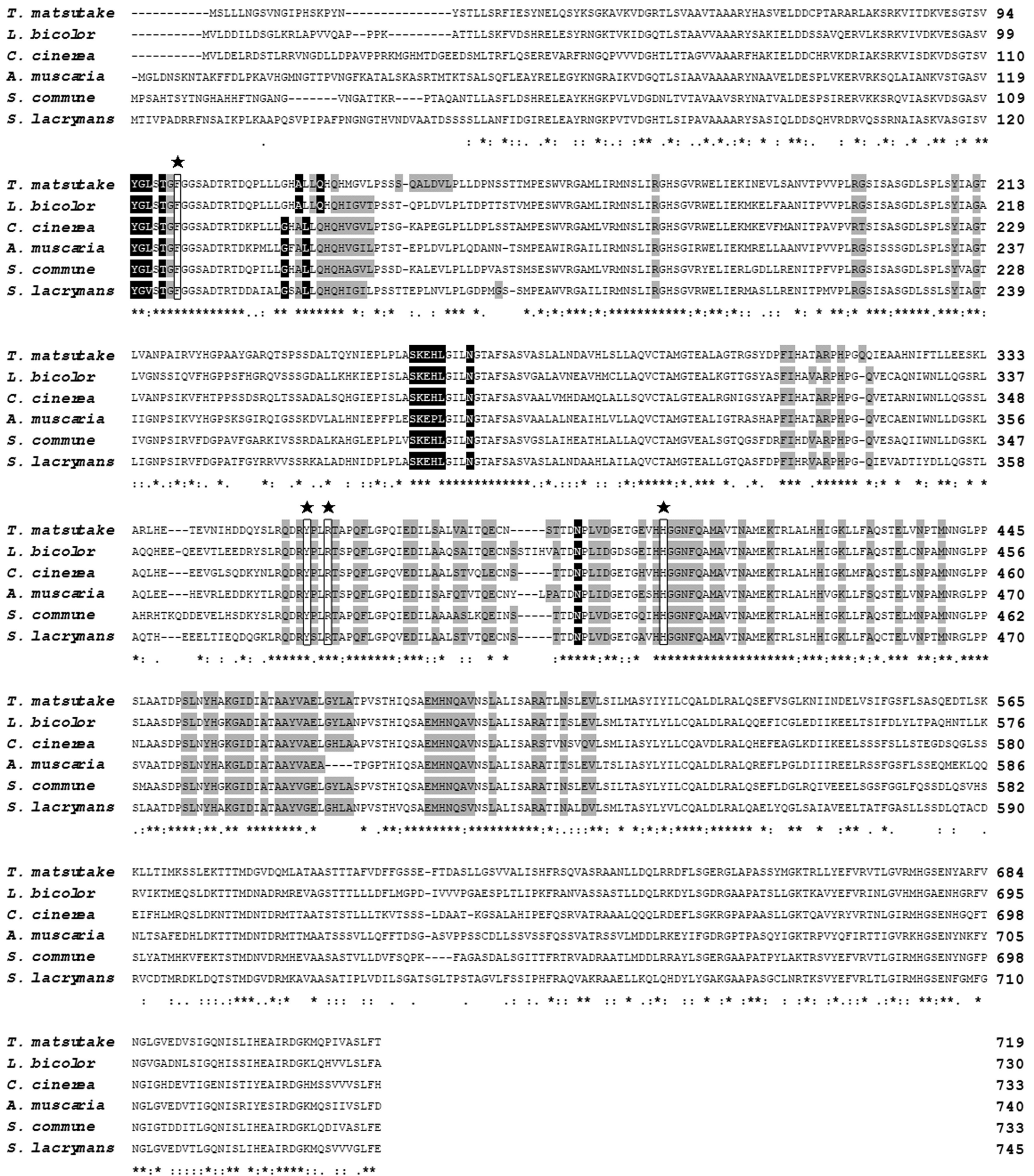

Fig. 2. Multiple alignment of PAL amino acids sequences.

The black boxes indicate a predicted active site, and the gray boxes indicate a predicted polypeptide binding site. The overlapping regions between the active site and binding site are indicated by the solid-line box with a black asterisk.

the PAL genes from 12 species of the phylum Basidiomycota were extracted from the GenBank database. The three species of the phylum Ascomycota were used as outgroups (Fig. 1). Multiple sequence alignment was performed using the Clustal X program [15]. The phylogenetic tree was constructed with MEGA4 [14]. Both active sites and binding sites of a protein sequence of the PAL gene of *T. matsutake* were analyzed using the Conserved Domain Search tool on the Conserved Domains Database (NCBI) [11]. The promoter sequences were predicted using the known promoter elements, such as GC box, CAAT box, and TATA box [1]. The polyadenylation signals were identified using the POLYAH program [13].

To analyze the expression pattern of the PAL gene of *T. matsutake*, the developmental stage of this mushroom was classified into the following five stages: mycelium, primordium, stipe, pileus, and gills. The total RNA from each developmental stage was extracted using a RNA isolation kit (Qiagen, Valencia, CA, USA). The purity of the RNA solution was measured using a NanoVue Spectrophotometer (GE Healthcare, Buckinghamshire, UK). The cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. RT-PCR was carried out using the following primer sets: a primer set for amplification of *tmpal*, TMPALrt-forward (5'-AAGTTGAAAGCGGTACCAGC-3') and TMPALrt-reverse (5'-ATGATAGGGGTGACAGGTCT-3'); and for the amplification of SSU rRNA (control), NS3 (5'-GCAAGT CTGGTGCCAGCAGCC-3') and NS6 (5'-GCATCACAG ACCTGTTATTGCCTC-3'). PCR was performed using Takara Ex Taq polymerase (Takara Bio, Otsu, Shiga, Japan) under the following conditions; initial denaturation at 95°C for 2 min followed by 25 cycles of 30 sec at 95°C, 1 min at 56°C, and 30 sec at 72°C. The RT-PCR results were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The PAL gene of *T. matsutake*, which is designated *tmpal*, consisting of 2,160 nucleotides encoding a polypeptide of 719 amino acid residues. The mRNA sequence of *tmpal* was deposited in GenBank (GU980196). As a result of phylogenetic analysis, the PAL protein sequence of *T. matsutake* formed a clade with those of five species of the class Agaricomycetes (Fig. 1). Among *T. matsutake* and the five species of the class Agaricomycetes, both active sites and binding sites of amino acids sequences of PAL were conserved significantly (Fig. 2). *tmpal* consists of seven exons, with exon 2 being the longest (835 bp). Among the six introns, the GT-AG rule was conserved only in intron 5. In the upstream region of the open reading frame (ORF) of *tmpal*, 1 GC box, 2 CAAT boxes, and 1 TATA box were identified. The two polyadenylation signals (5′-aataaa-3′) were detected in the downstream region of the ORF of *tmpal* (Fig. 3A).

An analysis of the gene expression pattern revealed *tmpal* to be expressed most strongly in the primordium

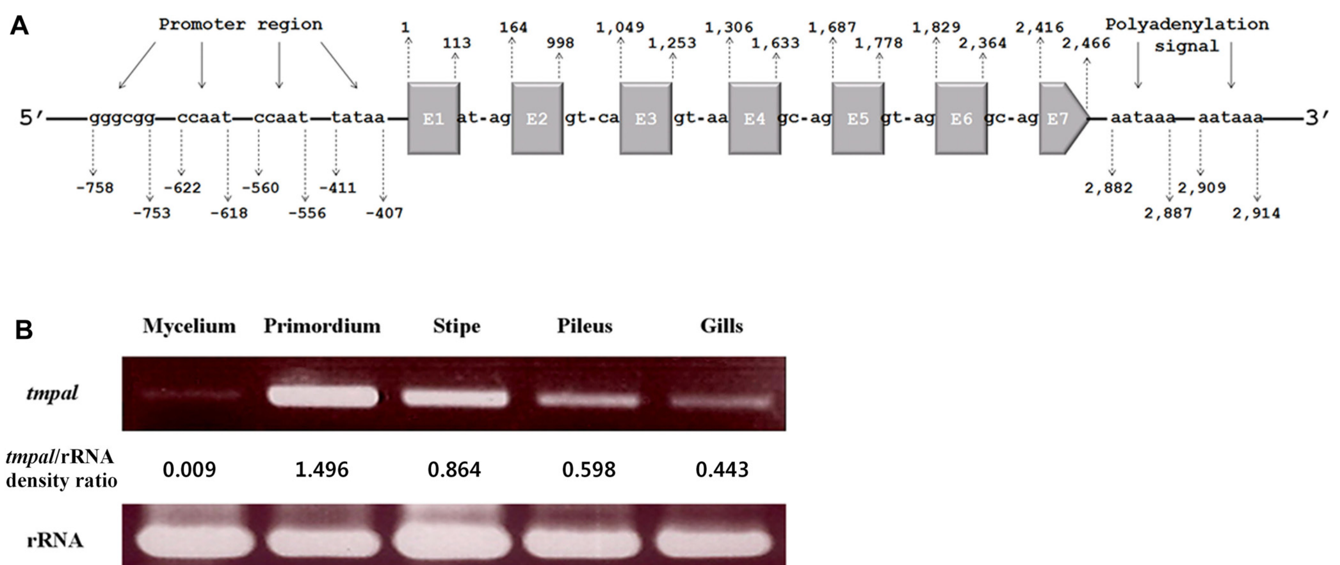

Fig. 3. Gene structure of PAL and analysis of mRNA expression.

(**A**) Gray boxes indicate the exons of the PAL gene. Each solid arrow indicates the promoter location and polyadenylation signal, respectively, and each dashed arrow indicates the nucleotide position. (**B**) RT-PCR analysis of PAL cDNA. 18S rRNA was used as a positive control. The densitometry results for the *tmpal*/rRNA density ratio are indicated.

among the developmental stages of *T. matsutake*. In the stipe of the fruiting body, *tmpal* was the second most highly expressed. In the mycelium, however, *tmpal* was expressed only slightly compared with the other developmental stages (Fig. 3B). These results indicate that the *trans*-cinnamic acid and ammonia derived from L-phenylalanine are mainly generated in the developmental stages after primordium formation. As mentioned above, *trans*-cinnamate is a precursor of secondary metabolites derived from the phenylpropanoid pathway. Thus, we inferred that the metabolites derived from L-phenylalanine are mainly produced in the primordium and fruiting body. Furthermore, it is well known that PAL plays an important role in nitrogen metabolism, as well as phenylalanine metabolism and tyrosine metabolism. Therefore, this enzyme is essential for the metabolism of carbon and nitrogen [3]. Based on these data, the differential gene expression pattern suggests that *tmpal* plays an important role in various physiological functions depending on the developmental stages of *T. matsutake*. Future studies need to investigate the exact role of *tmpal* in *T. matsutake*.

In this study, the PAL gene of *T. matsutake* was identified and its gene structure and mRNA expression pattern were analyzed. These results may provide the basic clues for understanding the correlation between PAL and the developmental stages of *T. matsutake*.

Acknowledgments

This work was supported by the Next-Generation BioGreen 21 Program (No. PJ008216), Rural Development Administration, Republic of Korea.

References

1. Dobson MJ, Tuite MF, Roberts NA, Kingsman AJ, Kingsman SM, Perkins RE, *et al*. 1982. Conservation of high efficiency promoter sequences in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. **10:** 2625-2637.
2. Ewing B, Hillier L, Wendl MC, Green P. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res*. **8:** 175-185.
3. Fritz RR, Hodgins DS, Abell CW. 1976. Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. *J. Biol. Chem.* **251:** 4646-4650.
4. Huang X, Madan A. 1999. CAP3: a DNA sequence assembly program. *Genome Res*. **9:** 868-877.
5. Hyun MW, Yun YH, Kim JY, Kim SH. 2011. Fungal and plant phenylalanine ammonia-lyase. *Mycobiology* **39:** 257-265.
6. Kim NS, Hahn Y, Oh JH, Lee JY, Oh KJ, Kim JM, *et al*. 2004. Gene cataloging and expression profiling in human gastric cancer cells by expressed sequence tags. *Genomics* **83:** 1024-1045.
7. Kim SH, Virmani D, Wake K, MacDonald K, Kronstad JW, Ellis BE. 2001. Cloning and disruption of a phenylalanine ammonia-lyase gene from *Ustilago maydis*. *Curr. Genet.* **40:** 40-48.
8. Kim WK, Mauthe W, Hausner G, Klassen GR. 1990. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. *Can. J. Bot*. **68:** 1898-1902.
9. Leal GA, Gomes LH, Albuquerque PS, Tavares FC, Figueira A. 2010. Searching for *Moniliophthora perniciosa* pathogenicity genes. *Fungal Biol.* 114**:** 842-854.
10. MacDonald MJ, D'Cunha GB. 2007. A modern view of phenylalanine ammonia lyase. *Biochem. Cell Biol.* **85:** 273-282.
11. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, *et al*. 2011. CDD: a conserved domain database for the functional annotation of proteins. *Nucleic Acids Res*. **39:** 225-229.
12. Mauch-Mani B, Slusarenko AJ. 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the Resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* **8:** 203-212.
13. Solovyev V, Salamov A. 1997. The Gene-Finder computer tools for analysis of human and model organisms genome sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol*. **5:** 294-302.
14. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24:** 1596-1599.
15. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. **24:** 4876-4882.