Cloning and Characterization of a Novel Laccase Gene, \textit{fvlac7}, Based on the Genomic Sequence of \textit{Flammulina velutipes}

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\textbf{Abstract}

Laccases (EC 1.10.3.2) are copper-containing polyphenol oxidases found in white-rot fungi. Here, we report the cloning and analysis of the nucleotide sequence of a new laccase gene, \textit{fvlac7}, based on the genomic sequence of \textit{Flammulina velutipes}. A primer set was designed from the putative mRNA that was aligned to the genomic DNA of \textit{F. velutipes}. A cDNA fragment approximately 1.6-kb long was then amplified by reverse transcriptase-PCR using total RNA, which was subsequently cloned and sequenced. The cDNA sequence of \textit{fvlac7} was then compared to that of the genomic DNA, and 16 introns were found in the genomic DNA sequence. The \textit{fvlac7} protein, which consists of 538 amino acids, showed only 42~51\% identity with 12 different mushroom species containing two laccases of \textit{F. velutipes}, suggesting the \textit{fvlac7} is a novel laccase gene. The first 25 amino acids of Fvlac7 correspond to a predicted signal sequence, four copper-binding sites, and four N-glycosylation sites. \textit{Fvlac7} cDNA was heterologously overexpressed in an \textit{Escherichia coli} system with an approximate expected molecular weight of 60 kDa.

\textbf{Keywords} \textit{Flammulina velutipes}, Molecular characterization, Novel laccase gene \textit{fvlac7}

Laccases are widely distributed in higher plants and fungi, and have been found in insects and bacteria [1]. Fungal laccases have been found in Ascomycetes, Deuteromycetes, and Basidiomycetes, and are particularly abundant in many white-rot fungi involved in lignin metabolism [2]. White-rot basidiomycetes are the most efficient degraders of lignin and the most widely studied [3]. Laccase is catalyzed by the reduction of one molecule of oxygen to water, accompanied with one-electron oxidation of a wide range of aromatic compounds including polyphenols, methoxy-substituted monophenols, and aromatic amines [2]. This oxidation results in the generation of an oxygen-centered free radical that can be converted to quinone in a second enzyme-catalyzed reaction. Laccases have widespread applications ranging from effluent discoloration and detoxification to pulp bleaching, removal of phenolics from wines, organic synthesis, biosensors, synthesis of complex medical compounds, and dye transfer blocking functions in detergents and washing powders [2].

\textit{Flammulina velutipes}, a winter white-rot fungus, is a well-known edible mushroom cultivated worldwide, especially in Asia; however, relatively little information is available regarding its genome. The genome size of \textit{F. velutipes} is approximately 33 Mb and it is organized into eight chromosomes [4]. Comparative genome analysis based on sequence data provides a large amount of information that enables exploration of gene function on a genome-wide scale. The entire genome for the monokaryotic strain 4019-20 of \textit{F. velutipes} has been sequenced by a research group in Korea [5]. In a previous report, a laccase gene, \textit{lccFv}, was isolated from \textit{F. velutipes} by rapid amplification of cDNA ends and expressed in \textit{Pichia pastoris} [6]; however, additional \textit{F. velutipes} laccase genes have not yet been reported. In a previous study, cDNA sequences were aligned with the whole genome sequence of \textit{F. velutipes} using bioinformatic tools [5, 7], revealing eight putative laccase genes that exist in the \textit{F. velutipes} genome (data not shown).

This study was conducted to investigate the molecular characteristics and expression of a novel laccase gene, \textit{fvlac7}, belonging to \textit{F. velutipes} strain 4019-20 found in Korea using genomic information.
MATERIALS AND METHODS

Fungal strain and culture conditions. *F. velutipes* monokaryotic strain 4019-20 was obtained from the Mushroom Research Division of the Rural Development Administration and grown at 25°C on potato dextrose agar (Difco, Detroit, MI, USA) medium. The strain was maintained on 1.5% agar slants containing 1% malt extract (Difco), 1.5% yeast extract (Difco), and 0.5% glucose. For preparation of genomic DNA, the mycelia of *F. velutipes* monokaryotic strain 4019-20 were grown in 200 mL of potato dextrose (PD) broth (Duchefa, Haarlem, The Netherlands) with gentle shaking at 25°C for 7 days.

Total RNA isolation and cDNA synthesis. For isolation of total RNA, *F. velutipes* monokaryotic strain 4019-20 was grown in 100 mL of PD broth (Duchefa) with shaking at 28°C for 7 days. Total RNA was then extracted from the mycelium using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA was treated with DNase (Promega, Madison, WI, USA) for 20 min at 37°C. For synthesis of the laccase gene cDNA, an open reading frame (ORF) was found within the mRNA when aligned with the genomic sequence of *F. velutipes* 4019-20 strain, and denoted *fvlc7*. The FvLac7-F (5'-ATGCTTAGTCTCAAGGGTCTCAGGC-3') and FvLac7-R (5'-CTACTGGAACTCGGGTGAGAGG-3') primer set was designed from sequences containing the start and stop codons of the ORF. Reverse transcriptase (RT)-PCR was performed using the standard protocols recommended by the manufacturer (TRT-101, ReverTra Ace; Toyobo, Osaka, Japan). Total RNA was added to a 20-µL reaction mixture, including primer (FvLac7-F and FvLac7-R; 5 pmol each), 4 µL of 5 × RT buffer, 2 µL of dNTPs (10 mM), 10 units of RNase inhibitor, and 10 units of reverse transcriptase, and the samples were then incubated in a PTC-225TM thermocycler (MJ Research, Inc., Waltham, MA, USA) at 42°C for 30 min. After synthesis of the cDNA, 10 µL of the cDNA product were used for PCR in a 50-µL reaction mixture according to the manufacturer’s instructions under the following conditions: 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min. A fragment of approximately 1.6 kb was recovered from the RT-PCR reaction and cloned into the pGEM-T Easy vector (Promega).

DNA sequencing analysis. DNA was sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Sequences were analyzed on an ABI Prism 3730 genetic analyzer (Applied Biosystems), after which the sequence data were analyzed using the Lasergene software (DNASTar Inc., Madison, WI, USA). MegAlign software was used for ClustalW2 multiple sequence alignment of amino acids from different laccases. DNA sequences and the deduced protein sequences obtained in this study were compared using the National Center for Biotechnology Information (NCBI) BLAST network sources (http://www.ncbi.nlm.nih.gov/BLAST). SignalP was used for theoretical signal peptide determination (www.cbs.dtu.dk/services/SignalP).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article was deposited into the GenBank nucleotide sequence database under accession number JN627444.

Overexpression of *fvlc7*. The *fvlc7* gene was PCR-amplified using a forward primer (5'-CAGCCATGCAATGCTTAGTCTCACGGTT-3') containing a SpI I restriction site (underlined) at the start codon of the ORF and a reverse primer (5'-CTAGAAGCTTCTACTGGAACCTCGGGTGAGG-3') containing a Hind III restriction site (underlined) after the stop codon. The pQE80-2 expression vector was a gift from Dr. Suh (Auburn University, Auburn, AL, USA). The PCR amplicon was double-digested with the restriction enzymes SpI I and Hind III, ligated into pQE80L containing a 6 × His tag upstream of the multiple cloning site, and transformed into *Escherichia coli* DH10B, yielding the recombinant clone pfvlc7. *E. coli* harboring pfvlc7 was grown in L broth containing ampicillin (50 mg/L) until an optical density (at 600 nm) of 0.5 at 37°C was attained. Overexpression was induced by adding 0.5 mM isopropyl-1-thio-β-galactopyranoside for 3 hr. Bacterial cells were pelleted and then resuspended in loading buffer, boiled, and centrifuged. The supernatant was analyzed by SDS-PAGE and protein bands were visualized by Coomassie brilliant blue staining.

RESULTS AND DISCUSSION

Molecular description of the *F. velutipes* laccase gene *fvlc7*. The whole genome sequence of *F. velutipes* was previously determined and annotated [5]. We found eight laccase genes in the genome sequence data. Of them, a putative laccase gene, *fvlc7*, was spliced with the mRNA from genomic DNA using an online analysis tools program (http://molbiol-tools.ca/). An ORF containing the start and stop codons was found in the mRNA sequencing data. RT-PCR primers containing both codon sequences were designed from the mRNA sequence information. RT-PCR was performed using the total RNA of *F. velutipes*. A 1.6-kb cDNA amplicon was produced by RT-PCR and confirmed by sequencing. Sequence analysis revealed an ORF of 1,608 bp (538 amino acids) that included the mature laccase protein with a molecular mass of 60 kDa. Translation of the first 74 bp after the initiation codon ATG generated a 25-amino acid-long putative signal peptide typically involved in the sorting of secreted proteins in eukaryotes (Fig. 1). The promoter region of this gene contains a TATA element that is an important sequence for initiation of transcription. The putative TATA element was localized at nucleotide positions -46 and -26. Comparison of the cDNA fragment
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obtained by RT-PCR with the genomic DNA sequence of fvlac7 confirmed the presence of 16 introns with sizes ranging from 49 to 59 bp (Fig. 1). The number of introns vary in the laccases from mushroom species, such as F. velutipes ggcFv (9 introns), P. ostreatus pox1 (19 introns), pox2 (19 introns), P. eryngii (19 introns), Laccaria bicolor lcc1 (13 introns), Agaricus bisporus lcc1 (14 introns), and Coprinopsis cinerea lac3 (13 introns) [8]. The fvlac7 laccase gene has fewer introns than laccase genes from Pleurotus species, while F. velutipes laccase has more introns relative to the laccase genes from other basidiomycetes. The variation in the number of introns indicates a mutual vestige of divergence.

The deduced amino acid sequence from fvlac7 was compared with that of other laccases (Fig. 2A). The Fvlac7 laccase amino acid sequence shows similarity to lac2 (45.5% identity) and lacFv (48.1% identity) from F. velutipes, P. ostreatus pox1 (42% identity), C. cinerea lac17 (50% identity), P. sajor-caju lac3 (44% identity), P. pulmonarius lac6 (45.5% identity), P. eryngii (46% identity), Lentinula edodes lac1 (43.8% identity), and Neurospora crassa lac1 (50% identity). Consequently, comparison of Fvlac7 laccase with mushroom laccases containing F. velutipes laccases [6] in Fig. 2 revealed distinct differences, indicating that fvlac7 is a novel laccase gene belonging to F. velutipes. Analysis of the phylogenetic relationship among the amino acids of laccases (Fig. 2B) showed that the F. velutipes laccases were clustered in one group and closely linked to laccases from Pleurotus species, L. edodes, and T. versicolor, but significantly distinct from other laccases.

The deduced amino acid sequence of fvlac7 was also found to contain four potential copper-binding histidine-rich sites, similar to the other fungal laccases mentioned, as well as three potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) (Fig. 3). Laccase is part of a broad group of enzymes known as polyphenol oxidases that contain copper atoms in the catalytic center and are usually referred to as multicopper oxidases [9, 10]. Typically, laccase-mediated catalysis occurs with the reduction of oxygen to water, accompanied by oxidation of the substrate [11, 12]. Laccases contain three types of copper atoms, one of which is responsible for their characteristic blue color. Enzymes lacking the blue copper atom are called yellow or white laccases [2]. Like most fungal extracellular enzymes, laccases are glycoproteins and the extent of glycosylation usually ranges from 10% to 25%. Conversely, low levels of glycosylation were detected in P. eryngii, where laccase I contained 7% and laccase II contained only 1% of bound sugars [3]. It is well known that glycosylation of fungal laccases plays important roles in secretion, susceptibility to proteolytic degradation, copper retention, and thermal stability [13, 14].

Overexpression of fvlac7. The fvlac7 gene was overexpressed in an E. coli system using the pQE80L plasmid. As shown in Fig. 4, a band corresponding to an approximate expected molecular weight of 60 kDa of Fvlac7 was identified as the fusion protein with the 6× His tag on SDS-PAGE. However,
the overexpressed Fvlac7 laccase could not be purified as a soluble protein with enzymatic activity. Generally, expression in an *E. coli* system offers a means for the rapid and economic production of recombinant proteins compared to fungal expression systems that are much more difficult to work with. Unfortunately, overexpression of heterologous
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proteins in the cytoplasm of *E. coli* often results in the formation of insoluble and biologically inactive aggregates known as inclusion bodies [15]. The formation of inclusion bodies is a major obstacle to large-scale production. It is well known that, in the case of fungal laccases, it is difficult to overexpress the recombinant forms in *E. coli* systems [16], although bacterial laccase genes have been successfully overexpressed in *E. coli* cells. Therefore, heterologous expression of fungal laccase genes has also been studied in *Saccharomyces cerevisiae*, *Trichoderma reesei*, *Aspergillus oryzae*, *Pichia pastoris*, and *Aspergillus niger* [1, 3, 17]. These data suggest that heterologous expression studies of *fvlac7* using different hosts should be conducted to elucidate the enzymatic characteristics of this laccase.

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