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Research Article

Genome-Wide Identification and Characterization of Novel Laccase Genes in the White-Rot Fungus *Flammulina velutipes*

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Abstract The aim of this study was to identify and characterize new *Flammulina velutipes* laccases from its whole-genome sequence. Of the 15 putative laccase genes detected in the *F. velutipes* genome, four new laccase genes (*fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac*-4) were found to contain four complete copper-binding regions (ten histidine residues and one cysteine residue) and four cysteine residues involved in forming disulfide bridges, *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4*, encoding proteins consisting of 516, 518, 515, and 533 amino acid residues, respectively. Potential N-glycosylation sites (Asn-Xaa-Ser/Thr) were identified in the cDNA sequence of *fvLac-1* (Asn-454), *fvLac-2* (Asn-437 and Asn-455), *fvLac-3* (Asn-111 and Asn-237), and *fvLac4* (Asn-402 and Asn-457). In addition, the first 19~20 amino acid residues of these proteins were predicted to comprise signal peptides. Laccase activity assays and reverse transcription polymerase chain reaction analyses clearly reveal that $CuSO_4$ affects the induction and the transcription level of these laccase genes.

Keywords Copper sulfate, *Flammulina velutipes*, Genome, Laccase

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductases) are multicopper enzymes belonging to the "blue" oxidase group that catalyze the oxidation of a wide variety of organic and inorganic compounds, including diphenols, polyphenols, diamines, and aromatic amines [1]. Laccases are prevalent enzymes, especially among plants and fungi [2, 3], and fungal laccases are the most frequently studied. The potential for biodegradation of various pollutants by laccase-producing microorganisms or purified laccases is one of the most exciting subjects in environmental biotechnology research [4]. There has been growing interest in the use of fungal laccases for applications such as bio-bleaching, catalysis of complex chemical conversions in the paper industry, textile dye decolorization, and detoxification of environmental pollutants [5-8]. Numerous studies have focused on the molecular characterization of fungal laccases, as well as on methods for improving laccase production levels.

F. velutipes is one of the major actively cultivated mushroom species in the world; over 300,000 tons of this mushroom are produced per year [9, 10]. In a recent study, we determined the whole genome sequence of *F. velutipes* and identified 12 putative laccase genes [11]. In the whole genome sequence, it was revealed *F. velutipes* retains many genes encoding laccase compared with either *Postia placenta*, *Laccaria bicolor*, *Schizophyllum commune*, or *Phanerochaete chrysosporium*. Thus, it is reasonably assumed that *F. velutipes* has potential ability for lignin degradation. Laccase genes have been isolated from different mushroom species and their copper-ligand domain that includes one cysteine and ten histidine residues were characterized [12, 13].

The aim of this study was to identify and characterize laccase genes in the *F. velutipes* genome in order to increase the availability of these industrially useful enzymes. Using genome information from *F. velutipes*, we cloned and sequenced the cDNAs of laccase genes and defined the organization of their exon-introns, copper-binding sites, and signal peptides. In addition, we examed the expressional

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Table 1. Primers used for RT-PCR

Gene	Accession No.	Forward (5′-3′)	Reverse (5′-3′)
β-Actin	Control	tggacaagtcatcaccatcg	gaagcacttgcgatgaacaa
fvLac-1	KM276550	ctgccaacagagtcgttgaa	tgtccgtagtgaagcgaatg
fvLac-2	KM276551	cgtaatcactttggccgat	ccctggatcgagaacaaaaa
fvLac-3	KM276552	gcttcgagagagctgctgtt	ttagcagcgattggacactg
fvLac-4	KM276553	gctgatcaagcagtggacaa	gctgatcaagcagtggacaa

RT-PCR, reverse transcription polymerase chain reaction.

induction of individual laccase genes by copper.

MATERIALS AND METHODS

Strains and growth conditions. *Flammulina velutipes* monokaryotic strain KACC42780 was obtained from the Korean Agricultural Culture Collection (KACC; Rural Development Administration, Korea; http://www.genebank.go.kr/) and was grown at 26°C on mushroom complete medium (MCM) agar (0.2% peptone, 2% glucose, 0.2% yeast extract, 0.05% $MgSO_4$, 0.046% KH_2PO_4, 0.1% K_2HPO_4, and 1.5% agar) for 14 days. To induce laccase expression, mycelia were grown in MCM medium supplemented at the time of inoculation with various concentrations of copper sulfate ($CuSO_4$). For genomic DNA and total RNA isolation from mycelia, a 300-mL Erlenmeyer flask containing 50 mL MCM medium was inoculated with fresh plugs from a plate (five mycelial plugs per flask) and incubated at 26°C for 2 wk without agitation.

Laccase gene identification. The genome-wide gene identification of laccases was conducted by applying a combination of several methods, including *ab initio* gene structure prediction (Fgenesh; http://www.softberry.com), a homology-based approach (Fgenesh+; http://www.softberry.com), and transcriptome-based gene identification (Cufflinks; http://cufflinks.cbcb.umd.edu/manual.html) [11] to the *F. velutipes* whole genome sequence (AQHU00000000). Gene prediction using the AUGUSTUS tool [14] with default parameters based on *Coprinopsis cinerea* was also performed. Functional annotation of the predicted genes was conducted using BLAST ver. 2.2.17 software with a series of protein databases, including the NCBI nucleotide (nt; http://blast.ncbi.nlm.nih.gov/Blast.cgi), and nonredundant set (nr; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Total RNA preparation, cDNA synthesis, and reverse transcriptase (RT)-PCR. Mycelia samples were ground to a fine powder under liquid nitrogen using a mortar and pestle and stored at −80°C. Total RNA was prepared from tissue samples (100 mg) using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Total RNA (10 μg) was treated for 30 min at 37°C with 1 U of RQ1 RNase-free DNase (Promega, Madison, WI, USA). cDNA synthesis and RT-PCR analysis were performed using 1 μg RNA in a 20-μL reaction volume with oligo-dT18 and ImProm-II reverse transcriptase (Promega). Reactions were first incubated at 25°C for 5 min, next at 42°C for 60 min, and finally at 70°C for 10 min to inactivate the reverse transcriptase. PCRs were conducted in a 50-μL reaction mixture containing 10 mM dNTP mixture, 10 pmol of each specific primer (Table 1), one unit Taq-polymerase (TaKaRa Korea Biomedical Inc., Seoul, Korea), 10× PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, and 25 mM $MgCl_2$), and 1 μL cDNA product.

Sequence analysis. DNA was sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer instructions. Sequences were analyzed on an ABI Prism 3730 genetic analyzer (Applied Biosystems), after which the sequence data were further analyzed using the Lasergene software (DNASTAR Inc., Madison, WI, USA). The nucleotide and amino acid (aa) sequences of the laccases were aligned using the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Prediction of signal peptides for the *F. velutipes* laccases was conducted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). N-glycosylation sites (Asn-Xaa-Ser/Thr) were identified using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). The GenBank accession numbers of the sequences reported in this paper are KM276550 (*fvLac-1*), KM276551 (*fvLac-2*), KM276552 (*fvLac-3*), and KM276553 (*fvLac-4*).

Laccase activity and zymogram assays. Laccase activity was determined using a modified 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma, St. Louis, MO, USA) oxidation assay as previously reported [15]. The assay mixture contained 9 μL ABTS (1.8 mM, Sigma) and 10 μL culture supernatant in 181 μL of sodium acetate buffer (50 mM, pH 4.5). Oxidation of ABTS was monitored by determining the increase of absorbance at 420 nm (ε420, 36,000/M/cm). One unit of laccase activity was defined as the amount of substrate in micromoles transformed per minute, reported in units per volume. All experiments were performed in triplicate by using three replicates of each set of conditions at each time point. Laccase activity was determined using zymograms with a modified sodium dodecyl sulfate polyacrylamide gel electrophoresis technique [15, 16]. The separating and stacking gels were 12% and 5% acrylamide, respectively,

Table 2. The predicted laccase genes of *Flammulina velutipes* identified by BLAST analysis against the NCBI-nr database

<table>
<tr><th rowspan="2">Laccase</th><th colspan="2">Specificity for rediction tool</th><th colspan="5">Subject</th><th colspan="2">Score</th><th rowspan="2">Identities (%)</th><th rowspan="2">Gaps (%)</th></tr>
<tr><th>CA</th><th>AU</th><th>Subject ID</th><th>Description</th><th>Length</th><th>Start</th><th>End</th><th>Bit</th><th>E-Value</th></tr>
<tr><td>fvLac-1</td><td>CA_fvLac-1</td><td>AU_fvLac-1</td><td>gb|AHD24907.1|</td><td>Putative laccase 6 [Flammulina velutipes]</td><td>516</td><td>1</td><td>516</td><td>243</td><td>0</td><td>96</td><td>4</td></tr>
<tr><td>fvLac-2</td><td></td><td>CA_fvLac-2</td><td>gb|AHD24908.1|</td><td>Putative laccase 7 [Flammulina velutipes]</td><td>520</td><td>1</td><td>520</td><td>942</td><td>0</td><td>96</td><td>0</td></tr>
<tr><td></td><td></td><td></td><td>gb|AFV15793.1|</td><td>Laccase [Leucoagaricus gongylophorus]</td><td>637</td><td>21</td><td>522</td><td>511</td><td>3.00E-173</td><td>52</td><td>16</td></tr>
<tr><td></td><td></td><td></td><td>gb|AHD24909.1|</td><td>Putative laccase 8 [Flammulina velutipes]</td><td>523</td><td>1</td><td>503</td><td>931</td><td>0</td><td>92</td><td>5</td></tr>
<tr><td></td><td>CA_fvLac-3</td><td>AU_fvLac-5</td><td>gb|ADX07329.1|</td><td>Putative laccase [Flammulina velutipes]</td><td>906</td><td>1</td><td>367</td><td>744</td><td>0</td><td>99</td><td>0</td></tr>
<tr><td></td><td>CA_fvLac-4</td><td></td><td>gb|AHD24917.1|</td><td>Putative laccase 4 [Flammulina velutipes]</td><td>642</td><td>1</td><td>632</td><td>1,273</td><td>0</td><td>97</td><td>0</td></tr>
<tr><td></td><td>CA_fvLac-5</td><td>AU_fvLac-6</td><td>gb|AHD24913.1|</td><td>Putative laccase 9 [Flammulina velutipes]</td><td>535</td><td>1</td><td>535</td><td>1,065</td><td>0</td><td>97</td><td>2</td></tr>
<tr><td>fvLac-3</td><td>CA_fvLac-6</td><td>AU_fvLac-7</td><td>gb|ADX07319.1|</td><td>Putative laccase 5 [Flammulina velutipes]</td><td>598</td><td>1</td><td>515</td><td>1,060</td><td>0</td><td>100</td><td>0</td></tr>
<tr><td></td><td>CA_fvLac-7</td><td>AU_fvLac-8</td><td>gb|AHD24910.1|</td><td>Putative laccase 10 [Flammulina velutipes]</td><td>502</td><td>103</td><td>502</td><td>816</td><td>0</td><td>99</td><td>0</td></tr>
<tr><td></td><td></td><td>AU_fvLac-9</td><td>gb|ADX07303.1|</td><td>Putative laccase 1 [Flammulina velutipes]</td><td>699</td><td>526</td><td>627</td><td>125</td><td>3.00E-31</td><td>56</td><td>2</td></tr>
<tr><td>fvLac-4</td><td>CA_fvLac-8</td><td>AU_fvLac-10</td><td>gb|AHD24916.1|</td><td>Putative laccase 3 [Flammulina velutipes]</td><td>670</td><td>1</td><td>670</td><td>1231</td><td>0</td><td>90</td><td>8</td></tr>
<tr><td></td><td>AU_fvLac-11</td><td></td><td>gb|ADX07303.1|</td><td>Putative laccase 1 [Flammulina velutipes]</td><td>699</td><td>532</td><td>699</td><td>185</td><td>1.00E-51</td><td>55</td><td>5</td></tr>
<tr><td></td><td>AU_fvLac-12</td><td></td><td>gb|ADX07303.1|</td><td>Putative laccase 1 [Flammulina velutipes]</td><td>699</td><td>556</td><td>699</td><td>182</td><td>7.00E-50</td><td>64</td><td>1</td></tr>
<tr><td></td><td>CA_fvLac-9</td><td>AU_fvLac-13</td><td>gb|AFA35114.1|</td><td>Laccase [Flammulina velutipes]</td><td>535</td><td>1</td><td>535</td><td>1,107</td><td>0</td><td>100</td><td>0</td></tr>
<tr><td></td><td></td><td>AU_fvLac-14</td><td>gb|ADX07316.1|</td><td>Putative laccase 17 [Flammulina velutipes]</td><td>859</td><td>557</td><td>845</td><td>600</td><td>0</td><td>99</td><td>1</td></tr>
</table>

CA, combiined approches (Fgenesh, Fgensh+, and cufflinks); AU, AUGUSTUS tool.

and the electrode reservoir solution contained 25 mM Tris and 192 mM glycine, pH 8.4. Gels were stained for laccase activity using 5 mM ABTS as the substrate. The total extracellular protein concentration in the culture supernatants was measured using the Bradford assay with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Identification of laccase genes in the *F. velutipes* genome. The predicted amino acid sequences of *F. velutipes* genes, determined using an approach combining several techniques (Fgenesh, Fgenesh+, and cufflinks) [11], were compared against the NCBI-nr database using BLASTP. This examination showed that nine of the predicted proteins shared sequence similarity with fungal laccases (Table 2). Gene prediction using the AUGUSTUS tool uncovered 14 laccase genes in *F. velutipes*, a higher number than revealed using the combination approach (Table 2). Eight of the laccase genes were identified by both prediction approaches (Table 2, Fig. 1). Fungal laccases are secreted, glycosylated proteins with two disulfide bonds and four copper atoms

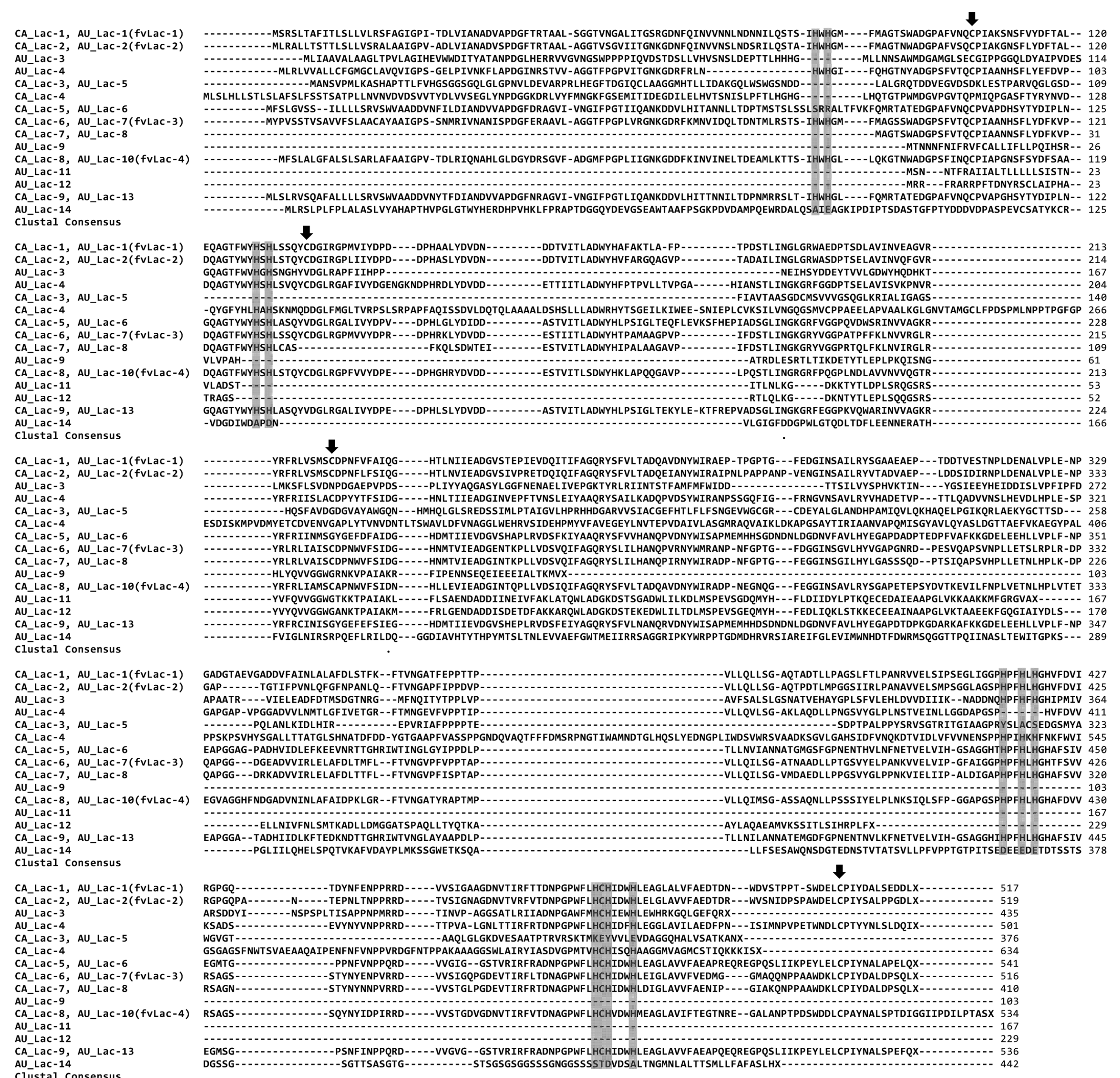


Fig. 1. Amino acid sequence alignment of *Flammulina velutipes* laccase genes identified using either the combination approach or the AUGUSTUS tool. Histidine (His) and Cysteine (Cys) residues predicted to be involved in the binding of copper are highlighted with gray boxes. Arrows indicate Cys residues involved in the formation of disulfide bridges.

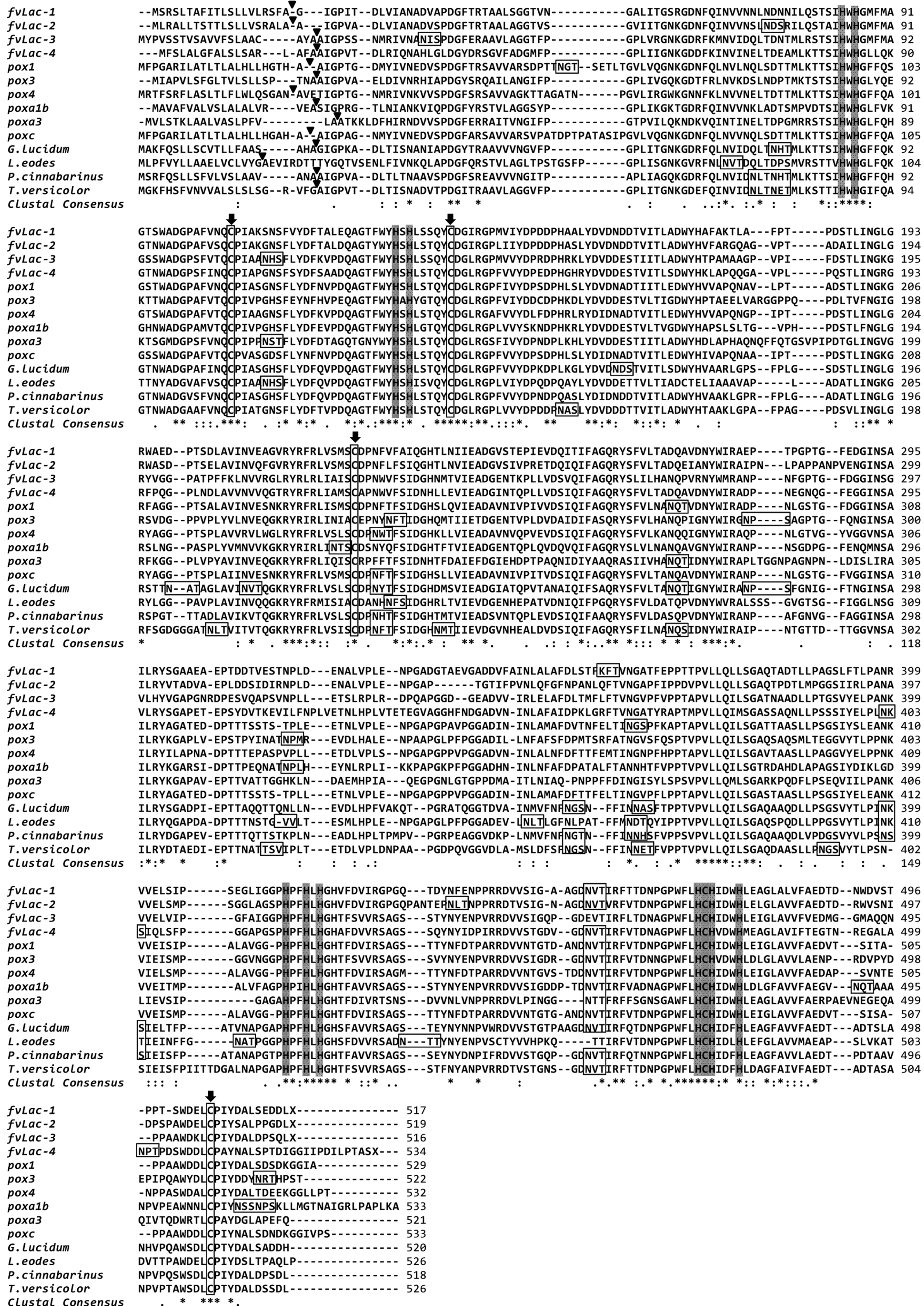
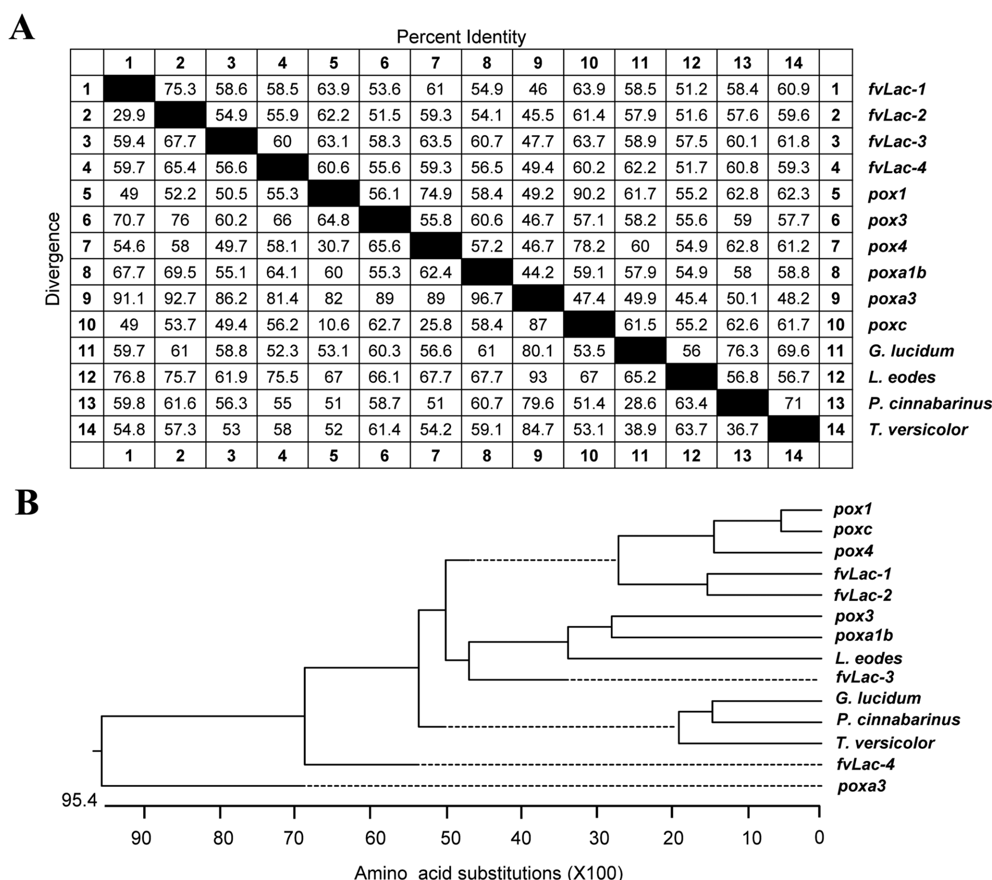


Fig. 2. Amino acid sequence alignment of laccase genes from *Flammulina velutipes* and other Basidiomycetes. His and Cys residues predicted to be involved in the binding of copper are highlighted with gray boxes. Potential N-glycosylation sites (N-X-S/T) are highlighted with boxes. Arrows indicate Cys residues involved in the formation of disulfide bridges. Triangles indicate the position of signal peptide cleavage sites predicted by SignalP V4.1. Positions of identical amino acid residues are marked with asterisks below the sequence. Colons and dots indicate the positions of amino acid residues with strong and weak similarity, respectively.

distributed between a mononuclear site (T1, where the substrate is reduced) and a trinuclear cluster (T2/T3, where oxygen is bound and reduced to H_2O) [6]. Thus, electrons are transferred from substrate molecules to the trinuclear T2/T3 center via the T1-bound copper; subsequent to the electron transfer, the dioxygen in the trinuclear center is reduced to two molecules of H_2O [17, 18]. The protein sequences of previously predicted laccase proteins indicate that all these enzymes contain four conserved copper-binding regions, as well as highly conserved copper-binding ligands consisting of ten histidine residues and one cysteine residue [13]. Of the 15 putative laccase genes identified in *F. velutipes*, only four genes (*fvLac-1*, *fvLac-2*, *fvLac-3*, and *fvLac-4*) conformed to the fungal laccase characteristics described above (Fig. 1). The amino acid sequence of the other 11 genes did not contain either the four complete copper-binding regions (ten histidine residues and one cysteine residue) or the four cysteine residues involved in the formation of the disulfide bridges (Fig. 1). Using cDNA sequence analysis, the open reading frame size of laccase genes *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac*-4 were estimated to be 1,551 bp (516 aa), 1,557 bp (518 aa), 1,548 bp (515 aa), and 1,602 bp (533 aa), respectively. The intron positions of *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4* were determined analyzed by aligning between their genomic DNA and cDNA sequences. Obtained by cDNA sequencing. These comparisons revealed that the genomic DNA of *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4* contain 17, 17, 13, and 16 introns, respectively, with an average intron size of 52.5 bp, and that all the splicing sites follow the GT-AG rule (Supplementary Figs. 1~4). Figs. 2 and 3 show the alignment of the predicted amino acid sequences of genes *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4* with those of previously reported fungal laccases. The *F. velutipes* laccases share 45.5~63.9% homology with the laccases of other fungi, including *Pleurotus ostreatus* (*poxc* [GenBank accession No. Z34848], *pox1* [GenBank accession No. Z34847], *poxa1b* [GenBank accession No. AJ005018], *pox3* [EMBL accession No. FM202671], *pox4* [EMBL accession No. FM202672], and *poxa3* [EMBL accession No. AJ344434]), *Ganoderma lucidum* (GenBank accession No. ACR24357), *Lentinula edodes* (GenBank accession No. AAF13037), *Pycnoporus cinnabarinus* (GenBank accession No. O59896), and *Trametes versicolor* (GenBank accession No. BAA23284). The predicted amino acid sequences of *F. velutipes fvLac-1* and *fvLac-2* showed the highest level of homology (75.3%) (Fig. 3). In addition, the amino acid residues required for copper-binding disulfide bridge formation were completely conserved in



A Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		75.3	58.6	58.5	63.9	53.6	61	54.9	46	63.9	58.5	51.2	58.4	60.9	1	*fvLac-1*
2	29.9		54.9	55.9	62.2	51.5	59.3	54.1	45.5	61.4	57.9	51.6	57.6	59.6	2	*fvLac-2*
3	59.4	67.7		60	63.1	58.3	63.5	60.7	47.7	63.7	58.9	57.5	60.1	61.8	3	*fvLac-3*
4	59.7	65.4	56.6		60.6	55.6	59.3	56.5	49.4	60.2	62.2	51.7	60.8	59.3	4	*fvLac-4*
5	49	52.2	50.5	55.3		56.1	74.9	58.4	49.2	90.2	61.7	55.2	62.8	62.3	5	*pox1*
6	70.7	76	60.2	66	64.8		55.8	60.6	46.7	57.1	58.2	55.6	59	57.7	6	*pox3*
7	54.6	58	49.7	58.1	30.7	65.6		57.2	46.7	78.2	60	54.9	62.8	61.2	7	*pox4*
8	67.7	69.5	55.1	64.1	60	55.3	62.4		44.2	59.1	57.9	54.9	58	58.8	8	*poxa1b*
9	91.1	92.7	86.2	81.4	82	89	89	96.7		47.4	49.9	45.4	50.1	48.2	9	*poxa3*
10	49	53.7	49.4	56.2	10.6	62.7	25.8	58.4	87		61.5	55.2	62.6	61.7	10	*poxc*
11	59.7	61	58.8	52.3	53.1	60.3	56.6	61	80.1	53.5		56	76.3	69.6	11	*G. lucidum*
12	76.8	75.7	61.9	75.5	67	66.1	67.7	67.7	93	67	65.2		56.8	56.7	12	*L. eodes*
13	59.8	61.6	56.3	55	51	58.7	51	60.7	79.6	51.4	28.6	63.4		71	13	*P. cinnabarinus*
14	54.8	57.3	53	58	52	61.4	54.2	59.1	84.7	53.1	38.9	63.7	36.7		14	*T. versicolor*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig. 3. A, Comparison of amino acids similarity of four laccase genes (*fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4*) from *Flammulina velutipes* with laccases from *Pleurotus ostreatus* (*pox1*, *pox3*, *pox4*, *poxa1b*, *poxa3*, and *poxc*), *Ganoderma lucidum*, *Lentinula edodes*, *P. cinnabarinus*, and *Trametes versicolor*; B, The phylogenetic relationship of the laccases.

the four *F. velutipes* laccases (Fig. 2). One putative N-glycosylation site (Asn-Xaa-Ser/Thr) was identified in *fvLac-1* (Asn-454) and two putative sites were identified in *fvLac-2* (Asn-437 and Asn-455), *fvLac-3* (Asn-111 and Asn-237), and *fvLac-4* (Asn-402 and Asn-457). The initial 19~20 residues of the four laccases conformed to the structure of a signal peptide typical of extracellular enzymes, i.e., a positively charged amino terminus, a hydrophobic stretch, and small amino acid residues [19]. These characteristic structures showed that the *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac*-4 genes encode mature laccases consisting of 496, 498, 496, and 497 amino acid residues, respectively (Fig. 2).

The effect of copper on laccase activity and transcription. Copper ($CuSO_4$) has been reported to be a strong inducer of laccases in several species, including *P. ostreatus* [20], *Phanerochaete chrysosporium* [16], and *T. versicolor* [21]. In addition, copper has been shown to induce both laccase transcription and activity [21]. The increase in laccase activity is proportional to the level of copper used. In order to evaluate the effect of $CuSO_4$ on laccase production in *F. velutipes*, we first tested laccase activity in response to growth with various concentrations of $CuSO_4$. Laccase activity in a medium containing 0.5 mM $CuSO_4$ drastically increased from day 3 and showed a peak activity on day 9 (3.03 U/mL) (Fig. 4A). This level of activity is approximately 450% (0.55 U/mL) higher than that in *F. velutipes* grown without $CuSO_4$ (Fig. 1B). Laccase activity in cells grown without $CuSO_4$ gradually increased from day 3 to a peak activity of 2.25 U/mL on day 9 (Fig. 1). Laccase activity in cells grown with 0.25 mM $CuSO_4$ was lower on days 6 and 9 than that in *F. velutipes* cells grown without $CuSO_4$. Interestingly, laccase activity in cells grown with 1 mM $CuSO_4$ had drastically decreased by day 9, and was lower overall than that of the other conditions tested. Several studies have indicated that although copper can induce both laccase transcription and activity, even very low concentrations of laccase are toxic to most fungi [21, 22]. To evaluate the effect of apple pomace on the production of laccase enzyme, we utilized native polyacrylamide gel electrophoresis to examine the level of laccase activity in 0.33-μg protein samples collected on different days (3, 6, and 9) from the culture supernatants of cells supplemented with various concentrations of $CuSO_4$ (0, 0.25, 0.5, 1, and 2 mM). As shown in Fig. 4B, an increased level of laccase activity was apparent in the 0.5 mM $CuSO_4$ samples on both days 6 and 9. Although the highest level of activity was observed for the 0.5 mM $CuSO_4$ day 6 sample (Fig, 4B), it was not significantly increased compared to the activity shown in Fig. 4A.

To confirm and further elucidate the effects of $CuSO_4$ on the mRNA transcription levels of the laccase genes, including *fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4*, we conducted semi-

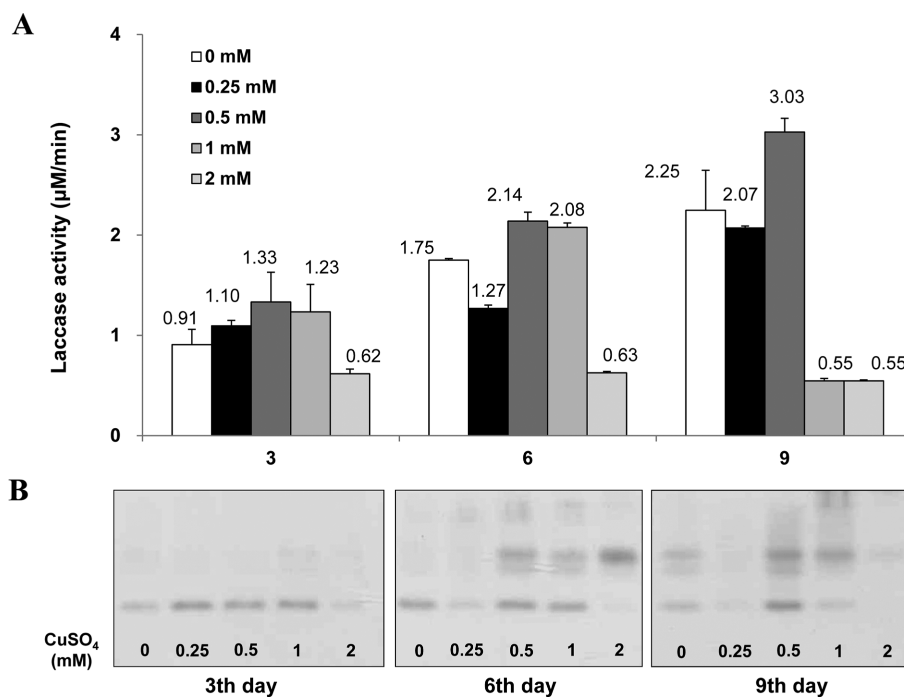


Fig. 4. Laccase activity of *Flammulina velutipes*. Time course examination of laccase activity in *F. velutipes* cells supplemented with different concentrations of $CuSO_4$ (0, 0.25, 0.5, 1, and 2 mM) (A). Zymogram of laccase isoenzymes in culture supernatants of *P. ostreatus*. Samples contained 0.33 μg protein collected from culture supernatants supplemented with different concentrations of $CuSO_4$ (0, 0.25, 0.5, 1, and 2 mM) on different days (3, 6, and 9) (B). Staining was performed with 5 mM ABTS in 50 mM sodium acetate buffer (pH 5.2).

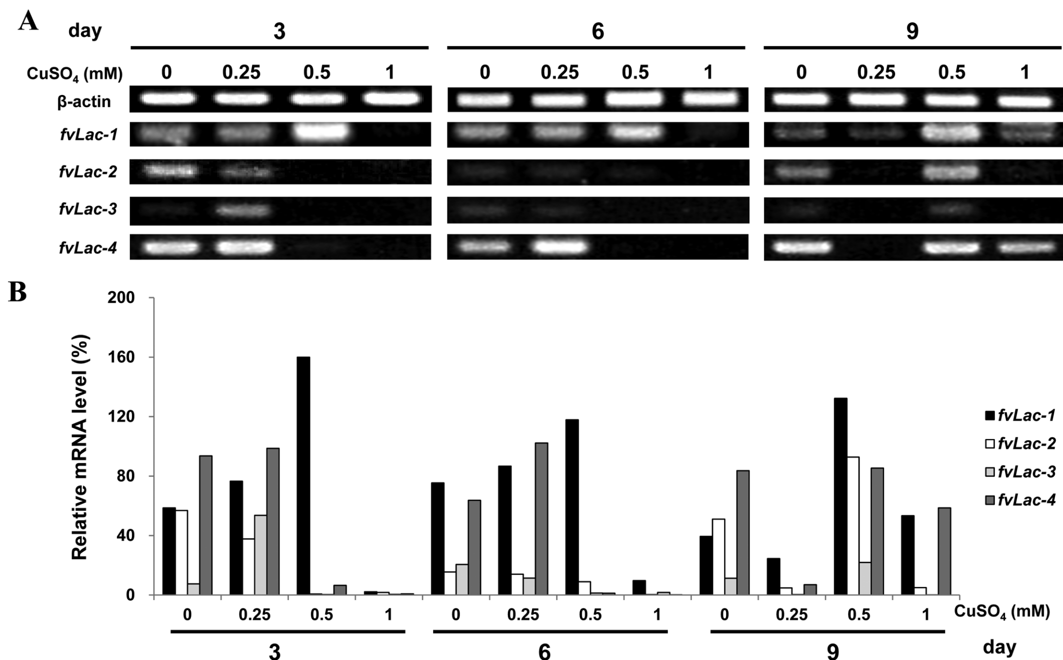


Fig. 5. Reverse trascription-PCR assays (A) and mRNA transcription levels (B) of *Flammulina velutipes* laccase genes. Total RNA was isolated from mycelia grown with different concentrations of $CuSO_4$ (0, 0.25, 0.5, and 1 mM) and collected at different time points (days 3, 6, and 9).

quantitative RT-PCR. This analysis clearly demonstrated the effect of $CuSO_4$ on the induction of transcription of these laccase genes (Fig. 5). Although laccase activity was relatively lower in cells cultured with 0.25 mM $CuSO_4$ than in cells cultured with either 0.5 or 1 mM $CuSO_4$ (Fig. 4), the transcripts of all four laccases (*fvLac-1*, *fvLac-2*, *fvLac3*, and *fvLac-4*) could be detected in day 3 samples by RT-PCR (Fig. 5A). This might be due to the rapid effect of copper on induction during the early phase of fungal growth. The *fv-Lac-1* gene exhibited the highest transcript levels in all the 0.5 mM $CuSO_4$ samples analyzed. Moreover, the transcript level of *fv-Lac-1* increased in correlation with $CuSO_4$ concentration, while *fv-Lac-3* mRNA was barely detectable even under inducing conditions except in the day 3 sample. Interestingly, the *fvLac-2*, *fvLac3*, and *fvLac-4* mRNAs were detectable in the 0.5 mM $CuSO_4$ in the sample of 9 days, but not in other days. Similarly, the *fv-Lac-1* and *fvLac-4* mRNAs were detectable only in the 1 mM $CuSO_4$ day 9 sample (Fig. 5). Fernandez-Larrea and Stahl [22] reported that free copper ions, and the production of toxic compounds, could result in oxidative stress at an advanced stage of fungal growth, which could be responsible for late transcriptional induction.

In order to identify putative response elements in the promoter regions of the laccase genes, we analyzed the nucleotide sequences extending 500 bp upstream from the start codons of the four laccase genes (Supplementary Fig. 5). This analysis allowed us to identify the unique distribution of several putative response elements. The promoter region of *fvLac-1* revealed a potential antioxidant responsive element motif known to be involved in the phenol antioxidant response in mammalian cells, and previously detected in the promoters of *P. ostreatus* laccases (*pox3*, *pox4*, and *poxa1b*) [23], as well as in the *P. sajor-caju lac4* promoter [12]. A putative stress responsive element corresponding with the consensus sequence CCCCT [24] was identified in the *fvLac-3* promoter (Supplementary Fig. 5).

The four laccase genes were first overexpressed in an *Escherichia coli* system (data not shown). However, the overexpressed laccases formed insoluble inclusion bodies [25] lacking enzymatic activity, consistent with a previous study describing the difficulty of overexpressing recombinant forms of fungal laccases in *E. coli* systems [26]. Therefore, the development of an expression system for the production of higher levels of these useful enzymes would be greatly advantageous. The results of this study indicate that further experiments are required to elucidate the enzymatic characteristics of laccases, and to obtain higher production levels of these proteins.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including five figures can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-42-322-s001.pdf.

ACKNOWLEDGEMENTS

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Rural Development Administration, Republic of Korea.

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Genome-Wide Identification and Characterization of Novel Laccase Genes in the White-Rot Fungus *Flammulina velutipes*

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http://www.mycobiology.or.kr/src/sm/mb-42-322-s001.pdf.

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fvLac-1_gDNA  ATGTCGCGGTCTCTTACCGCTTTCATAACCCTCTCTTTGCTCGTTTTGCGCTCCTTTGCTGGTATTGGGCCAATTACGGACTTGGTTATTGCCAACGCAGACGTTGCTCC 110
fvLac-1_cDNA  ATGTCGCGGTCTCTTACCGCTTTCATAACCCTCTCTTTGCTCGTTTTGCGCTCCTTTGCTGGTATTGGGCCAATTACGGACTTGGTTATTGCCAACGCAGACGTTGCTCC 110

fvLac-1_gDNA  CGATGGCTTCACGCGCACTGCAGCTCTCTCGGGCGGCACAGTGAACGGCGCGCTCATCACGGGCAGTAGGGTGAGTCCGTTTACCCATCTTACCCTCTATGTCGCTGATC 220
fvLac-1_cDNA  CGATGGCTTCACGCGCACTGCAGCTCTCTCGGGCGGCACAGTGAACGGCGCGCTCATCACGGGCAGTAGG-------------------------- Intron 1 ---- 180

fvLac-1_gDNA  GCGTTTGACTGCGCCCAGGGAGATAACTTTCAGATCAATGTTGTCAACAATTTGAACGACAATAATATCTTGCAAAGTACCTCAATCGTAAGTGGTTGATCTTGATTGCT 330
fvLac-1_cDNA  -----------------GGAGATAACTTTCAGATCAATGTTGTCAACAATTTGAACGACAATAATATCTTGCAAAGTACCTCAATC----------------------- 249

fvLac-1_gDNA  AGCGTGTATGCTGACAATCCCCATCCTAGCATTGGCATGGCATGTTCATGGCTGGCACTAGTTGGGCAGATGGTCCCGCTTTTGTTAACCAGTGCCCTATCGCAAAGAGC 440
fvLac-1_cDNA  ---- Intron 2 ---------------CATTGGCATGGCATGTTCATGGCTGGCACTAGTTGGGCAGATGGTCCCGCTTTTGTTAACCAGTGCCCTATCGCAAAGAGC 330

fvLac-1_gDNA  AACTCGTTCGTGTACGATTTCACTGCGCTCGAACAAGCAGGAACATTCTGGTATCACTCTCATTTGTGTGAGTCCATGGAATGGAACATATCATTCTTGATGGTAAATTG 550
fvLac-1_cDNA  AACTCGTTCGTGTACGATTTCACTGCGCTCGAACAAGCAGGAACATTCTGGTATCACTCTCATTTGT------------------ Intron 3 --------------- 397

fvLac-1_gDNA  ATAAACTTTGTAGCGAGTCAATACTGCGACGGAATTCGTGGTCCTATGGTGATCTATGACCCCGATGACCCCCACGCTGCCCTGTACGACGTCGACAATGGTAAGTCGAT 660
fvLac-1_cDNA  -------------CGAGTCAATACTGCGACGGAATTCGTGGTCCTATGGTGATCTATGACCCCGATGACCCCCACGCTGCCCTGTACGACGTCGACAATG---------- 484

fvLac-1_gDNA  ACCAAGCTTCTTTTTCTCAAGCACCTTGCCTAACGACGTTGCCGTTAAGACGATACTGTGATCACTTTGGCAGATTGGTACCACGCGTTTGCGAAGACTTTGGCGTTCCC 770
fvLac-1_cDNA  ------------- Intron 4 -------------------------ACGATACTGTGATCACTTTGGCAGATTGGTACCACGCGTTTGCGAAGACTTTGGCGTTCCC 545

fvLac-1_gDNA  GTGAGTACACACGCAGTCAATATTCTGCTCTATCTCATCCCCTCAGGACGCCTGATTCCACTCTCATCAATGGTCTCGGACGGTAAGGCGCTCATCATCGGTCTGATGTC 880
fvLac-1_cDNA  ------------------ Intron 5 -----------------GACGCCTGATTCCACTCTCATCAATGGTCTCGGACG----------------- Intron 6 -- 581

fvLac-1_gDNA  CAAGATATTGAATATTCGGGCATTAGGTGGGCGGAGGATCCAACTTCTGACCTGGCTGTCATAAATGTTGAGGCTGGAGTGCGGTGAGTCCTATCTTCTTCTAGATGCTT 990
fvLac-1_cDNA  -------------------------GTGGGCGGAGGATCCAACTTCTGACCTGGCTGTCATAAATGTTGAGGCTGGAGTGCG-------------------------- 638

fvLac-1_gDNA  GCTGCGGTATTGATTCATCGTGCGTGTCAGATATCGTTTCCGTCTGGTCAGCATGTCCTGCGATCCCAACTTCGTCTTTGCGATCCAGGGCCACACCTTGTAAAATTTTT 1100
fvLac-1_cDNA  -- Intron 7 ------------------ATATCGTTTCCGTCTGGTCAGCATGTCCTGCGATCCCAACTTCGTCTTTGCGATCCAGGGCCACACCTT----------- 707

fvLac-1_gDNA  CATCTCATTATCTAACAAAGTGGCTGACTTGTTTCAGAAACATTATTGAGGCGGACGGTGTGAGCACCGAGCCCATCGAAGTCGACCAGATCACGATCTTCGCCGGCCAG 1210
fvLac-1_cDNA  -- Intron 8 -------------------------AAACATTATTGAGGCGGACGGTGTGAGCACCGAGCCCATCGAAGTCGACCAGATCACGATCTTCGCCGGCCAG 780

fvLac-1_gDNA  AGGTACTCGTTCGTCGTAAGTACCATCACCGCTCGATCTTGACATATTCTGACTACTCCACTTGTAGCTTACTGCCGATCAAGCTGTCGACAACTACTGTGCGCATTAAA 1320
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fvLac-1_gDNA  ATCCACTTTGGCCTAACGTCGACTGACGATGATTGTAGGGATTCGCGCTGAGCCTACTCCCGGCCCCACAGGATTCGAGGATGGCATCAACTCTGCCATTCTTCGTTATT 1430
fvLac-1_cDNA  -- Intron 10 ------------------------GGATTCGCGCTGAGCCTACTCCCGGCCCCACAGGATTCGAGGATGGCATCAACTCTGCCATTCTTCGTTATT 898

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fvLac-1_cDNA  CCGGCGCGGCTGAGGCGGAGCCCACAGACGATACCGTTGAGAGCACCAATCCATTGGACGAGAATGCGCTCGTG------------- Intron 11 ----------- 972

fvLac-1_gDNA  GCTTACCTTTGATATAGCCTCTCGAGAACCCCGGCGCTGTATGACACCGTTTTATCCTTAGATTATGCCGTCTAAATATTTTTTAGGACGGTACTGCGGAAGTCGGAGCA 1650
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fvLac-1_gDNA  GACGACGTCTTCGCCATCAACCTTGCATTGGCCTTTGATGTAAGCAGCGGCGTTTGTATGGTGAAAAGTACTGACACGTTATACAGCTCAGCACCTTTAAATTCACCGTC 1760
fvLac-1_cDNA  GACGACGTCTTCGCCATCAACCTTGCATTGGCCTTTGAT--------------- Intron 13 --------------------CTCAGCACCTTTAAATTCACCGTC 1080

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fvLac-1_cDNA  CAACAGAGTCGTTGAACTCTCGATCCCCTCTGAGGGCCTTATCGGTGGTCCTCATCCGTTCCATCTGCACGGC------------------- Intron 14 ------- 1263

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fvLac-1_cDNA  CAACGTTACCATTCGCTTCACTACGGACAATCCTGGACCTTGGTTCCTCCACTG--------------------- Intron 15 --------------------CC 1414

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fvLac-1_cDNA  CTGGGATGTTAGCACTCCTCCTA---------------- Intron 17 ------------------------CTTCTTGGGATGAACTTTGCCCCATCTATGATGCT 1530

fvLac-1_gDNA  CTGTCTGAAGATGACCTTTGA 2441
fvLac-1_cDNA  CTGTCTGAAGATGACCTTTGA 1551
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Supplementary Fig. 1. Comparison of the nucleotide sequences of *fvLac-1* genomic DNA and cDNA. Introns are numbered based on the comparison of genomic DNA and cDNA. Asterisks indicate the splicing junctions based on the GT-AG rule.

```
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fvLac-2_cDNA  ATGCTACGGGCCCTTCTTACATCCACTACCCTCTCTTTGCTTGTTTCGCGCGCTCTCGCTGCGATAGGACCAGTTGCAGACCTGGTCATTGCCAATGCTGATGTCTCCCC 110

fvLac-2_gDNA  CGATGGATTTACGCGTACTGCAGCTCTGGCGGGGGGCACAGTGAGCGGCGTGATTATCACTGGCAACAAAGTGAGTCTAATTTTTCCTCTCTCCCTCTCTTGAATATTCG 220
fvLac-2_cDNA  CGATGGATTTACGCGTACTGCAGCTCTGGCGGGGGGCACAGTGAGCGGCGTGATTATCACTGGCAACAAA-------------------------- Intron 1 ---- 180

fvLac-2_gDNA  TTGATCATGTCTGAATACAGGGAGATAATTTCCAAATCAACGTCGTTAACAGTTTGAACGACAGTAGAATACTACAGAGTACTGCCATCGTATGTATTCGACATCTGTCA 330
fvLac-2_cDNA  -------------------GGAGATAATTTCCAAATCAACGTCGTTAACAGTTTGAACGACAGTAGAATACTACAGAGTACTGCCATC--------------------- 249

fvLac-2_gDNA  GTACGGTTCCATACTTATCGCCTTTCCTAGCATTGGCACGGAATGTTTATGGCCGGCACTAACTGGGCAGACGGCCCAGCCTTCGTCAGCCAGTGCCCCATTGCGAAAGG 440
fvLac-2_cDNA  -------- Intron 2 ------------CATTGGCACGGAATGTTTATGGCCGGCACTAACTGGGCAGACGGCCCAGCCTTCGTCAGCCAGTGCCCCATTGCGAAAGG 329

fvLac-2_gDNA  AAACTCCTTCCTCTACGACTTCACTGCCCTAGATCAGGCAGGAACTTACTGGTACCATTCTCATCTCTGTAAGTTCTTGAACTACATCGATATCAATAAATACTCACCGT 550
fvLac-2_cDNA  AAACTCCTTCCTCTACGACTTCACTGCCCTAGATCAGGCAGGAACTTACTGGTACCATTCTCATCTCT-------------------------- Intron 3 ----- 397

fvLac-2_gDNA  CTTCTTTCAGCGACTCAATACTGTGACGGTATTCGTGGGCCGTTGATAATCTACGATCCCGACGATCCACATGCTTCTTTGTACGACGTCGACAATGGTACGTTTGCCCT 660
fvLac-2_cDNA  ----------CGACTCAATACTGTGACGGTATTCGTGGGCCGTTGATAATCTACGATCCCGACGATCCACATGCTTCTTTGTACGACGTCGACAATG------------- 484

fvLac-2_gDNA  CCATATCCGTGGTTCCGTAGATCTGACATTAGTTTGAGACGATACCGTAATCACTTTGGCCGATTGGTATCATGTCTTCGCTAGGGGTCAGGCTGGAGTTCCGTAAGAGC 770
fvLac-2_cDNA  ------- Intron 4 ---------------------ACGATACCGTAATCACTTTGGCCGATTGGTATCATGTCTTCGCTAGGGGTCAGGCTGGAGTTCC-------- 548

fvLac-2_gDNA  CCAAACACTTGTGTTCCATACGCCTCGTCTTATATCCCCTCTAGAACAGCCGATGCTATCCTGATCAACGGCCTCGGGCGGTAAGTCGGTCATCAGTGGCCTCTATTTGC 880
fvLac-2_cDNA  ------------ Intron 5 ---------------------AACAGCCGATGCTATCCTGATCAACGGCCTCGGGCG------------------ Intron 6 -- 584

fvLac-2_gDNA  ATGTACTAAGTCTATGTTGTGCGCGTTAGATGGGCGTCAGATCCAACTTCTGAGCTTGCTGTTATCAATGTACAGTTTGGAGTTAGGTGGGTCAAATTCTTCCAGTCAAT 990
fvLac-2_cDNA  ----------------------------ATGGGCGTCAGATCCAACTTCTGAGCTTGCTGTTATCAATGTACAGTTTGGAGTTAG----------------------- 641

fvLac-2_gDNA  GTGGCGTTACTGATTCGTCGTATCTAGATACCGATTCCGTCTGGTTAGTATGTCCTGCGACCCAAACTTTTTGTTCTCGATCCAGGGCCACACTTTGTAAGTTTCTTCCT 1100
fvLac-2_cDNA  ---- Intron 7 -------------ATACCGATTCCGTCTGGTTAGTATGTCCTGCGACCCAAACTTTTTGTTCTCGATCCAGGGCCACACTTT------------- 710

fvLac-2_gDNA  ACCCTTACAAGAAGATTATTGAGCTGGTTCAAGGAACGTTATCGAGGCTGATGGCGTGAGCATTGTGCCTAGGGAAACTGATCAAATCCAAATTTTCGCCGGACAAAGAT 1210
fvLac-2_cDNA  ------------- Intron 8 ---------GAACGTTATCGAGGCTGATGGCGTGAGCATTGTGCCTAGGGAAACTGATCAAATCCAAATTTTCGCCGGACAAAGAT 787

fvLac-2_gDNA  ACTCGTTCGTGGTGAGTTGATTGTCCAGGTTATACACGTCTTCCCATCTAACCGTTCCACAGTTAACCGCCGACCAAGAAATTGCTAATTATTGTACGAATGCTTCTCCG 1320
fvLac-2_cDNA  ACTCGTTCGTG------------------- Intron 9 ---------------------TTAACCGCCGACCAAGAAATTGCTAATTATT---------------- 829

fvLac-2_gDNA  CTCGACGCTGGTTTTCAGATTGACTAGTCTTCTAGGGATTCGTGCTATTCCCAATCTTCCCGCGCCTCCAGCCAATCCAGTCGAAAACGGCATCAACTCTGCTATCCTTC 1430
fvLac-2_cDNA  ---------- Intron 10 -------------GGATTCGTGCTATTCCCAATCTTCCCGCGCCTCCAGCCAATCCAGTCGAAAACGGCATCAACTCTGCTATCCTTC 904

fvLac-2_gDNA  GTTATGTCACCGCAGACGTCGCGGAGCCTCTAGACGATTCAATCGACATCCGTAACCCTCTGGATGAGAACGCCCTGGTGGTATGTGCTAATTCCCGATCTCAAAGCAAT 1540
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fvLac-2_gDNA  GACATTCATATCCCCCACAGCCTCTCGAGAATCCTGGAGCTGTACGTGACAACCAGTTTCTCCCAAAATTCGCAGTCTAAATATCATTTCAGCCTACCGGAACCATCTTC 1650
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fvLac-2_cDNA  CCCGTCAATCTCCAGTTCGGTTTTAAC------------------------ Intron 13 --------------------CCGGCCAACTTGCAGTTCACCGTTAAC 1077

fvLac-2_gDNA  GGCGCTCCCTTCATTCCCCCAGACGTACCAGTGCTGCTCCAACTTCTGAGTGGCGCTCAAACGCCCGACACACTCATGCCAGGGGGCTCCATAATTCGTCTACCGGCGAA 1870
fvLac-2_cDNA  GGCGCTCCCTTCATTCCCCCAGACGTACCAGTGCTGCTCCAACTTCTGAGTGGCGCTCAAACGCCCGACACACTCATGCCAGGGGGCTCCATAATTCGTCTACCGGCGAA 1187

fvLac-2_gDNA  CGCTGTTGTCGAACTTTCGATGCCATCTGGTGGGCTCGCTGGAAGTCCTCATCCATTCCATCTGCACGGTGTACGTTCTTCTGCTCTCTCATTGTCAGTGATATCCGAGC 1980
fvLac-2_cDNA  CGCTGTTGTCGAACTTTCGATGCCATCTGGTGGGCTCGCTGGAAGTCCTCATCCATTCCATCTGCACGGT------------------------- Intron 14 ---- 1257

fvLac-2_gDNA  TCAACTTCTATAGCACGTATTCGACGTGATTCGCGGCCCAGGACAGCCAGCCAACACGGAGCCCAATCTTACGAACCCTCCTCGTCGAGATACAGTGAGCATTGGAAATG 2090
fvLac-2_cDNA  -------------CACGTATTCGACGTGATTCGCGGCCCAGGACAGCCAGCCAACACGGAGCCCAATCTTACGAACCCTCCTCGTCGAGATACAGTGAGCATTGGAAATG 1354

fvLac-2_gDNA  CTGGCGATAACGTTACAGTTCGGTTCGTCACTGACAATCCTGGACCATGGTTCCTCCATTGGTACGCCTACCTTACTATACAGCGGAGCATGTAACCTTGACTGACTCTC 2200
fvLac-2_cDNA  CTGGCGATAACGTTACAGTTCGGTTCGTCACTGACAATCCTGGACCATGGTTCCTCCATTG---------------------- Intron 15 --------------- 1415

fvLac-2_gDNA  ACCTTCGTATCTAGCCACATTGACTGGCATCTTGAACTGTAAGAACATCTCCCACATTCTTATACTATTCGTCGTTCTCATGCTTCTTCTAGGGGGCTGGCTGTTGTGTT 2310
fvLac-2_cDNA  -------------CCACATTGACTGGCATCTTGAACT---------------------- Intron 16 ---------------------GGGGCTGGCTGTTGTGTT 1457

fvLac-2_gDNA  TGCGGAGGACACTGATCGATGGGTGTCCAACATCGACCCATCGCGTAAGCTTCTCTTCTGCCTTATAGCGAGCGATCTGCTAATATCTTCCGACCAGCCGCTTGGGACGA 2420
fvLac-2_cDNA  TGCGGAGGACACTGATCGATGGGTGTCCAACATCGACCCATCGC-------------------- Intron 17 ---------------------CCGCTTGGGACGA 1514

fvLac-2_gDNA  GCTCTGTCCTATCTACTCCGCTTTGCCTCCGGGTGATCTCTAA 2463
fvLac-2_cDNA  GCTCTGTCCTATCTACTCCGCTTTGCCTCCGGGTGATCTCTAA 1557
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Supplementary Fig. 2. Comparison of the nucleotide sequences of *fvLac-2* genomic DNA and cDNA. Introns are numbered based on the comparison of genomic DNA and cDNA. Asterisks indicate the splicing junctions based on the GT-AG rule.

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fvLac-3_cDNA  ATGTATCCTGTCTCTTCTACTGTCTCTGCTGTGGTGTTCTCTTTGGCCGCATGCGCCTACGCGGCCATTGGTCCTTCGTCCAATATGAGGATCGTCAACGCCAATATCTC 110

fvLac-3_gDNA  CCCCGATGGCTTCGAGAGAGCGTCAGTGACCTGCGCTACTTTCGTATTTGAGCTGACTGGAGCTTGCGTAGTGCTGTTCTGGCTGGCGGTACCTTCCCTGGTCCACTCGT 220
fvLac-3_cDNA  CCCCGATGGCTTCGAGAGAGC-------------------- Intron 1 --------------------TGCTGTTCTGGCTGGCGGTACCTTCCCTGGTCCACTCGT 170

fvLac-3_gDNA  TCGGGGAAACAAGGTTAGACACCTACATTTCCCCTCCCTAATCACTCGATTGACGACCTGTTTATTTCAGGGCGATCGTTTCAAAATGAACGTGATAGACCAATTGACCG 330
fvLac-3_cDNA  TCGGGGAAACAAG-------------------------- Intron 2 ---------------------GGCGATCGTTTCAAAATGAACGTGATAGACCAATTGACCG 223

fvLac-3_gDNA  ATAATACGATGTTGAGGAGCACGAGTATCGTAAGTGTCTTGTTCACCTTGGTGAGGGTTGAGGATAACATGGATTTTAGCATTGGCATGGCATGTTCATGGCTGGGAGTA 440
fvLac-3_cDNA  ATAATACGATGTTGAGGAGCACGAGTATC------------------ Intron 3 ----------------------CATTGGCATGGCATGTTCATGGCTGGGAGTA 283

fvLac-3_gDNA  GTTGGGCGGACGGGTATGATCCTTGCTCTCCATATCGTTCAGCGGTCTAATACCATCGTGAACTCCACAGCCCTAGCTTTGTTACCCAGTGTCCAATCGCTGCTGTGAGT 550
fvLac-3_cDNA  GTTGGGCGGACGG--------------------- Intron 4 --------------------------CCCTAGCTTTGTTACCCAGTGTCCAATCGCTGCT------ 330

fvLac-3_gDNA  TCCTTATTTCATTCACATGTTCCTCCCTGACATTGAGTTTCATTCAGAACCACTCGTTCCTGTACGACTTCAAAGTCCCAGATCAGGCGGGGACATTCTGGTATCATTC 660
fvLac-3_cDNA  --------- Intron 5 ------------------------------AACCACTCGTTCCTGTACGACTTCAAAGTCCCAGATCAGGCGGGGACATTCTGGTATCATTC 392

fvLac-3_gDNA  TCATCTCTGTGAGTCGTTGAATTTCCCTGCGCTGGTCGTACATTTGCTGAGTTCTTCTGTAGCGTCGCAATACTGCGATGGGCTGAGAGGGCCAATGGTTGTGTACGATC 770
fvLac-3_cDNA  TCATCTCT------------------- Intron 6 ------------------------CGTCGCAATACTGCGATGGGCTGAGAGGGCCAATGGTTGTGTACGATC 448

fvLac-3_gDNA  CGCGCGACCCGCACAGAAAGCTATATGATGTAGACGACGGTAAGTGAATGGTGTGTGGATGTCAAGATGGAACACATTTACTCACCTGAGATATGTAGAATCAACGATCA 880
fvLac-3_cDNA  CGCGCGACCCGCACAGAAAGCTATATGATGTAGACGACG----------------------- Intron 7 -------------------------AATCAACGATCA 499

fvLac-3_gDNA  TCACCCTCGCAGATTGGTACCACACACCCGCCATGGCCGCTGGCCCCGTCCCTATCTTTGACTCCACGCTCATAAACGGAAAAGGGCGCTACGTCGGCGGAGTAAGTCCA 990
fvLac-3_cDNA  TCACCCTCGCAGATTGGTACCACACACCCGCCATGGCCGCTGGCCCCGTCCCTATCTTTGACTCCACGCTCATAAACGGAAAAGGGCGCTACGTCGGCGGA--------- 600

fvLac-3_gDNA  AACTCTCTCCTTCTCCAACCTAACCATGTTCTGACAAACCACACAGCCAGCCACCCCATTCTTTAAACTTAATGTCGTCCGTGGGCTGCGCTACCGCCTTCGTTTAATCG 1100
fvLac-3_cDNA  ------------- Intron 8 ----------------------CCAGCCACCCCATTCTTTAAACTTAATGTCGTCCGTGGGCTGCGCTACCGCCTTCGTTTAATCG 664

fvLac-3_gDNA  CCATCTCCTGCGACCCAAACTGGGTATTCTCGATCGACGGACACAACATGACCGTCATCGAAGCAGACGGGGAGAACACCAAGCCGCTGCTCGTCGACTCAGTACAGATC 1210
fvLac-3_cDNA  CCATCTCCTGCGACCCAAACTGGGTATTCTCGATCGACGGACACAACATGACCGTCATCGAAGCAGACGGGGAGAACACCAAGCCGCTGCTCGTCGACTCAGTACAGATC 774

fvLac-3_gDNA  TTCGCAGGCCAACGCTACTCCCTCATCCTCCACGCCAACCAGCCCGTGCGCAACTACTGGATGCGCGCCAACCCGAACTTTGGACCCACAGGCTTCGACGGGGGGATCAA 1320
fvLac-3_cDNA  TTCGCAGGCCAACGCTACTCCCTCATCCTCCACGCCAACCAGCCCGTGCGCAACTACTGGATGCGCGCCAACCCGAACTTTGGACCCACAGGCTTCGACGGGGGGATCAA 884

fvLac-3_gDNA  CTCGGGTGTGCTGCATTATGTGGGCGCACCGGGGAATAGGGACCCGGAGTCTGTGCAGGCGCCGAGTGTGAACCCGCTGCTCGAGACAAGCTTGCGGCCGTTGAGGGACC 1430
fvLac-3_cDNA  CTCGGGTGTGCTGCATTATGTGGGCGCACCGGGGAATAGGGACCCGGAGTCTGTGCAGGCGCCGAGTGTGAACCCGCTGCTCGAGACAAGCTTGCGGCCGTTGAGGGACC 994

fvLac-3_gDNA  CGCAGGCCCCGGGAGGGGATGGGGAGGCTGATGTGGTGATTCGGCTCGAGCTGGCGTTTGATTTGACGATGTTCTTGTTTACGGTTAATGGGGTGCCGTTTGTGCCCCCC 1540
fvLac-3_cDNA  CGCAGGCCCCGGGAGGGGATGGGGAGGCTGATGTGGTGATTCGGCTCGAGCTGGCGTTTGATTTGACGATGTTCTTGTTTACGGTTAATGGGGTGCCGTTTGTGCCCCCC 1104

fvLac-3_gDNA  ACTGCACCTGTCTTGCTGCAGATTTTGAGTGGGGCAACAAACGCAGCGGATTTGTTGCCCACGGGGAGTGTGTATGAACTTCCGGCGAACAAGGTCGTCGAGTTGGTCAT 1650
fvLac-3_cDNA  ACTGCACCTGTCTTGCTGCAGATTTTGAGTGGGGCAACAAACGCAGCGGATTTGTTGCCCACGGGGAGTGTGTATGAACTTCCGGCGAACAAGGTCGTCGAGTTGGTCAT 1214

fvLac-3_gDNA  TCCGGGGTTTGCGATTGGAGGACCGGTGAGTTTTTGGTTATTGTTGTGTGGCCGCTTTTTCTGATCCTGTATAGCATCCGTTCCATTTACATGGGTACGCTTCAACTCC 1760
fvLac-3_cDNA  TCCGGGGTTTGCGATTGGAGGACCG------------------ Intron 9 --------------------CATCCGTTCCATTTACATGGG-------------- 1260

fvLac-3_gDNA  TTTTGATCGAGCTCAGGTATTAACATTTGCATAGCATACATTCAGTGTCGTGCGCAGTGCTGGCAGCTCGACATACAACTACGAGAATCCCGTTCGGAGGGATGTCGTC 1870
fvLac-3_cDNA  ---- Intron 10 --------------------CATACATTCAGTGTCGTGCGCAGTGCTGGCAGCTCGACATACAACTACGAGAATCCCGTTCGGAGGGATGTCGTC 1335

fvLac-3_gDNA  TCTATTGGTCAGCCTGGGGACGAGGTTACGATCCGCTTCCTCACTGATAACGCTGGGCCGTGGTTCTTGCATTGGTACGTCAATTGAAGTCTGATGATGTCGCAACTAAC 1980
fvLac-3_cDNA  TCTATTGGTCAGCCTGGGGACGAGGTTACGATCCGCTTCCTCACTGATAACGCTGGGCCGTGGTTCTTGCATTG------------------- Intron 11 ------ 1409

fvLac-3_gDNA  GATCATATAGCCATATCGATTGGCACTTGGAGATGTGAGTAGCTTCGAGTTGGATAAGTGTTCGAACTCATGACATGTACAGTGGTCTTGCGGTGGTATTCGTCGAAGAT 2090
fvLac-3_cDNA  ----------CCATATCGATTGGCACTTGGAGAT------------------ Intron 12 ------------------TGGTCTTGCGGTGGTATTCGTCGAAGAT 1461

fvLac-3_gDNA  ATGGGTGGGATGGCACAGCAAAACCCACCTGGTAAGTCTCAGTTCAGGTGGAAAACATTTTGATTAATGATGACTGGTAGCTGCGTGGGACAAGCTTTGCCCGATCTATG 2200
fvLac-3_cDNA  ATGGGTGGGATGGCACAGCAAAACCCACCTG-------------------- Intron 13 ------------------CTGCGTGGGACAAGCTTTGCCCGATCTATG 1522

fvLac-3_gDNA  ATGCACTTGACCCGTCGCAACTCTAG 2226
fvLac-3_cDNA  ATGCACTTGACCCGTCGCAACTCTAG 1548
```

Supplementary Fig. 3. Comparison of the nucleotide sequences of *fvLac-3* genomic DNA and cDNA. Introns are numbered based on the comparison of genomic DNA and cDNA. Asterisks indicate the splicing junctions based on the GT-AG rule.

```
fvLac-4_gDNA  ATGTTTTCCCTCGCTTTAGGTTTTGCTCTTTCTTTATCTGCGCGGCTTGCTTTTGCTGCTATTGGGCCTGTGACGGACCTTCGCATTCAGAATGCGCATCTCGGACTCGA 110
fvLac-4_cDNA  ATGTTTTCCCTCGCTTTAGGTTTTGCTCTTTCTTTATCTGCGCGGCTTGCTTTTGCTGCTATTGGGCCTGTGACGGACCTTCGCATTCAGAATGCGCATCTCGGACTCGA 110

                                                                                 *
fvLac-4_gDNA  TGGGTATGATCGGAGTGGTGTCTTTGCAGATGGGATGTTCCCAGGTCCTTTAATTATTGGGAATAAGGTATGGTCTTCAAACTTATCCTTCGAGCTTCTGCTGACGTTCA 220
fvLac-4_cDNA  TGGGTATGATCGGAGTGGTGTCTTTGCAGATGGGATGTTCCCAGGTCCTTTAATTATTGGGAATAAG---------------------------- Intron 1 -------- 177

                *                                                                      *
fvLac-4_gDNA  ATAGGGCGATGACTTCAAAATTAATGTTATTAACGAGCTCACCGATGAGGCGATGCTTAAAACTACTTCTATTGTGCGTTCAAACTCCATTTTCTCGAAGCACTCGGTCG 330
fvLac-4_cDNA  ----GGCGATGACTTCAAAATTAATGTTATTAACGAGCTCACCGATGAGGCGATGCTTAAAACTACTTCTATT----------------------- Intron 2 ----- 246

                              *                                           *
fvLac-4_gDNA  AACTTCACTTTGTATAGCACTGGCACGGTCTCCTGCAGAAGGGCACTAATTGGGCCGACGGGTACATTTGCTCGCCTATATGAACACTTGCCTTAGTGCTAATGATTTGC 440
fvLac-4_cDNA  ----------------CACTGGCACGGTCTCCTGCAGAAGGGCACTAATTGGGCCGACGG-------------------------- Intron 3 -------------- 290

              *                                                                                                         *
fvLac-4_gDNA  AGCCCGAGTTTCATTAATCAGTGCCCCATCGCGCCGGGAAATTCTTTCAGCTACGATTTCTCGGCTGCAGACCAGGCGGGTACATTCTGGTACCACTCTCATCTCTGTAC 550
fvLac-4_cDNA  --CCCGAGTTTCATTAATCAGTGCCCCATCGCGCCGGGAAATTCTTTCAGCTACGATTTCTCGGCTGCAGACCAGGCGGGTACATTCTGGTACCACTCTCATCTCT---- 394

                                                            *
fvLac-4_gDNA  ATTCCCACTTGAATTAGGTTTGGTGCAGATACTGACCTGTGGGCAGCAACGCAATACTGTGATGGTCTCCGAGGACCGTTTGTCGTCTACGATCCCGAAGATCCACATGG 660
fvLac-4_cDNA  --------------- Intron 4 ----------------------CAACGCAATACTGTGATGGTCTCCGAGGACCGTTTGTCGTCTACGATCCCGAAGATCCACATGG 458

                                     *                           *
fvLac-4_gDNA  ACATCGTTACGACGTGGATGATGGTGTGTTCTAGCTAATCGTCTTGCGCTACTACCAATCTAATAAGTGTCTCCAGAAAGCACGGTCATAACACTGTCGGATTGGTACCG 770
fvLac-4_cDNA  ACATCGTTACGACGTGGATGATG--------------------- Intron 5 ------------------------AAAGCACGGTCATAACACTGTCGGATTGGTACC- 514

                                                                *                                *
fvLac-4_gDNA  TAAGCAAGTTTCTTGTTGTCATTGGATCATAGATTTACTTTAAACTCAATAGACAAACTTGCTCCTCAGCAAGGAGCTGTCCCGTAAGTGCATCTCATCAGGCTGCCGAC 880
fvLac-4_cDNA  --------------------- Intron 6 ----------------------ACAAACTTGCTCCTCAGCAAGGAGCTGTCCC------------------ Intron 7 545

                                      *
fvLac-4_gDNA  GATAGAGAATGAACTCGATGTAACAGTCTTCCTCAATCTACGTTGATCAACGGACGTGGTCGGTTTCCTCAAGGCCCTCTCAATGACCTAGCAGTGGTTAACGTAGTGCA 990
fvLac-4_cDNA  --------------------------TCTTCCTCAATCTACGTTGATCAACGGACGTGGTCGGTTTCCTCAAGGCCCTCTCAATGACCTAGCAGTGGTTAACGTAGTGCA 629

                       *                                           *
fvLac-4_gDNA  AGGAACTCGGTCAGTGCCATTGCCTTTACCTTTCTCTCTACTAACGACCATTTAGCTACCGCTTCCGTCTCATAGCAATGTCCTGTGCTCCGAACTGGGTATTCTCAATC 1100
fvLac-4_cDNA  AGGAACTCG----------------- Intron 8 --------------------CTACCGCTTCCGTCTCATAGCAATGTCCTGTGCTCCGAACTGGGTATTCTCAATC 693

fvLac-4_gDNA  GACAACCACCTGCTGGAAGTCATCGAGGCCGATGGGATCAACACTCAGCCTCTACTAGTCGACTCTATCCAAATCTTCGCTGGACAACGATACTCCTTCGTCTTGACTGC 1210
fvLac-4_cDNA  GACAACCACCTGCTGGAAGTCATCGAGGCCGATGGGATCAACACTCAGCCTCTACTAGTCGACTCTATCCAAATCTTCGCTGGACAACGATACTCCTTCGTCTTGACTGC 803

                                     *                                                  *
fvLac-4_gDNA  TGATCAAGCAGTGGACAATTACTGTATGTTGAATGATCGACATCCGTAATAGCTTTTGACCTTTTCGTGACAATAGGGATTCGCGCGGACCCAAACGAAGGGAATCAAGG 1320
fvLac-4_cDNA  TGATCAAGCAGTGGACAATTACT---------------------- Intron 9 ---------------------GGATTCGCGCGGACCCAAACGAAGGGAATCAAGG 860

fvLac-4_gDNA  ATTTGAGGGAGGAATCAACTCTGCGGTTCTGCGATATAGCGGTGCACCGGAAACTGAGCCCAGTTACGATGTGACGAAGGAGGTTATACTGTTTAATCCCCTCGTAGAGA 1430
fvLac-4_cDNA  ATTTGAGGGAGGAATCAACTCTGCGGTTCTGCGATATAGCGGTGCACCGGAAACTGAGCCCAGTTACGATGTGACGAAGGAGGTTATACTGTTTAATCCCCTCGTAGAGA 970

                         *                                              *                            *
fvLac-4_gDNA  CGAACCTTCACGTATGTCCCTCTACTTCATGCCCTTCGTCATCACTGACATCGGTAACAGCCATTGGTCACTGAGACTGAAGGAGTTGTGAGTTCACCACTCACTGAATG 1540
fvLac-4_cDNA  CGAACCTTCAC---------------- Intron 10 ---------------------CCATTGGTCACTGAGACTGAAGGAGTT----------------------- 1008

                                             *                                                          *
fvLac-4_gDNA  AGTCTTGCATCTTTGTTTATCTCATTGTCATAGGCTGGTGGTCATTTCAACGACGGTGCTGATGTCAACATCAACCTAGCATTCGCAATTGTAAGCATTATCACGGGTCC 1650
fvLac-4_cDNA  -- Intron 11 --------------------GCTGGTGGTCATTTCAACGACGGTGCTGATGTCAACATCAACCTAGCATTCGCAATT-------------------- 1065

                                            *
fvLac-4_gDNA  TACTCATGTCTTCTCATTGATTTCGATTCAAGGACCCAAAGCTTGGTCGTTTCACTGTGAACGGGGCAACCTACCGGGCACCCACGATGCCGGTGCTCCTCCAAATAATG 1760
fvLac-4_cDNA  ------- Intron 12 --------------GACCCAAAGCTTGGTCGTTTCACTGTGAACGGGGCAACCTACCGGGCACCCACGATGCCGGTGCTCCTCCAAATAATG 1143

                                 *                                                     *
fvLac-4_gDNA  AGTGGTGCTTCATCTGCCCGTGAGCGCTCGAATATCTTGTTCTACTACTTCCGTTTCCTGACGCACAAGGTTCAGAGAACCTGCTTCCCTCCAGCTCGATATACGAGCTC 1870
fvLac-4_cDNA  AGTGGTGCTTCATCTGCCC--------------------- Intron 13 -------------------------AGAACCTGCTTCCCTCCAGCTCGATATACGAGCTC 1197

                                                                    *                                                *
fvLac-4_gDNA  CCATTGAACAAGTCGATTCAGCTGTCCTTCCCTGGTGGAGCGCCTGGCTCCCCGGTGCGTCTCACCTTACCGTCCCACTGATCTTTGGGACTGACGGCACTGAAGCACCC 1980
fvLac-4_cDNA  CCATTGAACAAGTCGATTCAGCTGTCCTTCCCTGGTGGAGCGCCTGGCTCCCCG---------------------- Intron 14 -------------------CACCC 1256

fvLac-4_gDNA  ATTCCATCTGCACGGACACGCCTTTGATGTTGTTCGCAGTGCAGGGAGCAGTCAGTACAACTACATCGACCCCATACGGCGCGATGTAGTGTCCACTGGGGATGTTGGGG 2090
fvLac-4_cDNA  ATTCCATCTGCACGGACACGCCTTTGATGTTGTTCGCAGTGCAGGGAGCAGTCAGTACAACTACATCGACCCCATACGGCGCGATGTAGTGTCCACTGGGGATGTTGGGG 1366

                                                                                             *
fvLac-4_gDNA  ACAACGTAACGATCCGTTTCGTGACTGACAATGCGGGCCCGTGGTTCTTGCATTGCCACGTTGATTGGCATATGGAGGCGTGAGTAGTTTTGTCGCAAGACATGGAGGAC 2200
fvLac-4_cDNA  ACAACGTAACGATCCGTTTCGTGACTGACAATGCGGGCCCGTGGTTCTTGCATTGCCACGTTGATTGGCATATGGAGGC--------------------- Intron 15 1445

                                     *                                                                  *
fvLac-4_gDNA  TATCGACTAACATTGAAGCTTACAGTGGTCTTGCTGTTATCTTCACCGAAGGCACTAACAGGGAAGGGGCCCTGGCAATCCAACTCCTGGTACGCTTGAACAGCATGGC 2310
fvLac-4_cDNA  -------------------------TGGTCTTGCTGTTATCTTCACCGAAGGCACTAACAGGGAAGGGGCCCTGGCAATCCAACTCCTG-------------------- 1510

                                                     *
fvLac-4_gDNA  ATTCTTCTCAGGTTTGATTTGGGAACTAACGGTTCGCGTAGACTCTTGGGACGACCTGTGCCCTGCGTATAACGCTCTGTCTCCAACAGATATTGGCGGTATCATTCCTG 2420
fvLac-4_cDNA  ----------- Intron 16 --------------------ACTCTTGGGACGACCTGTGCCCTGCGTATAACGCTCTGTCTCCAACAGATATTGGCGGTATCATTCCTG 1579

fvLac-4_gDNA  ATATCCTTCCCACCGCTTCATGA 2443
fvLac-4_cDNA  ATATCCTTCCCACCGCTTCATGA 1602
```

Supplementary Fig. 4. Comparison of the nucleotide sequences of *fvLac-4* genomic DNA and cDNA. Introns are numbered based on the comparison of genomic DNA and cDNA. Asterisks indicate the splicing junctions based on the GT-AG rule.

```
fvLac-1_promoter  TGACAGTCTCGATTCCATGGCCGCGAGTTCTAATGAATCTGTTCAATTTGTTACGTGAGGGTACTTATGACTGTTTGTGCCGGAGATATTCCTCATCACTGACTTGTGTG 110
fvLac-1_promoter  TTGAGCCTGTTATGGGCGGCGAATAAACCACTCGGCGGTGGGATTTGGCTGTCTTCTCCGTTCGAGAAATCTGACACTGCGCTTAGACTATAGCCAGGCAACAGGCAACA 220
fvLac-1_promoter  CCCGTTGGTGTCATGCCGCTATGTATGGTTCGTGAACTCGAAACGGTACGATCAGATGAACTACATCATGCCTCCTCACTCAATACAACCAGGGAAGTACCTGACTTATG 330
fvLac-1_promoter  CCTGACGCCTCATTCGAGACGAGGATCGGACGCGAACGATGTTCGGCAAACAAAATACACTATATAAGATGGATCAGTTTCACTCAAGCTCTTCAGTTGCCAGTTCTTCT 440
fvLac-1_promoter  TCGAGCGACAGTCTTTCCTTATTCTTCCCAGTTCATTAAAACTCCCTCTTTCTTAACAAC 500

fvLac-2_promoter  GTCCTGACAAAGACTTTTCTGAAACGCGCTTTCTGGCCAGTGCTCTTAGTGTGCTGGTCGGTCAACCCTTACGGGATAGGGATCATCATGTCGACTGGAATGAAGCCTAG 110
fvLac-2_promoter  GTATATTCAGTTCAGCGATCCTCGCTCGGGTGGCGGTCATGGCTCACCATATTAGTACTCATTCATATTATTTCTCCATGTCCTCTAACCTCGGAACATGGGCCGCTCCT 220
fvLac-2_promoter  CCGTAAAGTCCGTCGGTACCGCGCCGCACTGCGTCTCGAGCACGAAACGCCACTATGAGATGCTCTATCATACGCTCAGCATTCTCAACTCAATTCAATAGCTGGGTGTA 330
fvLac-2_promoter  CCTGACGACTGTCGGACGTTTCGACACCGCTCCACTGGAAGGCGGCTTCATGGAGGAACGATGCTCGCAAAATAGGAAAATCATGACTGGTATAAAGTGTCTCAGAACGT 440
fvLac-2_promoter  TCGTTTACTAGTCGACCACCCTCTTGAGTGATTAGTCTCTCTTTCTACCTTCTTCTCAAT 500

fvLac-3_promoter  GAGAAAAACGGGAACACAGAAGTCGAAGAACTACAAGACCCAGATCGGCTAGTGGCGACCGCGACCGCGAGTGACAGTCCTAATGATGTTTCTCATGAGCACGACTGTCG 110
fvLac-3_promoter  GGTGTAGAGATCATGGCGGGAGGTGGCAAAGGTGATTGTCATCGGAGTTTCAAACACGCCCTTGCTCTCGATCTTCGAACATGGCGCTCTCGAGCTCTCTTGTTCTCCTT 220
fvLac-3_promoter  TTGTCTATACTCCCCTCGTTGGCACCCCACGCGAATACATTGCTTACGTGGTTGGCCGCCCATGGCAATCGTTTCGAAACCATCTGTTCGACGTCCAATCCTTCAGCTTC 330
fvLac-3_promoter  AGGCCCGCGCGACACTTTGGGAGGAAGGCACAAACGTATGCTGTCACCCAAGCGCTCATTTGTCTCATTTGAACCTAGCGTCGGCTGGCTGTATTCATTGTTTTGGTATA 440
fvLac-3_promoter  AAACCCAGACGACGATGAAGATCCTTGGATTCCTCAGGCTTCCTTCCCAGTCCATTCATT 500

fvLac-4_promoter  CTTTGCTAACTATCCACACAGCGTAAGCCACACAATCATGGCAGTGGCGATACTGTTTTGGATCTCCAGGTTCGCGCAGGGTCCACACTTTCAGACTCTTGTCAGTTGTC 110
fvLac-4_promoter  ACTTTACTTGCTACGATTCATCATAGGAGTGGATCGTGGGCATTCAATCCCGACTACTATAGCCTTGGATTGCTCATATCCTTCGCGAATGCTTGCGCGCCAGCGCGTCT 220
fvLac-4_promoter  TCGACAGTTTGTTATGCTCTGCATGGCCTCGACCAAGGACGTCCTTCGAAACTTACACCGTACTTGGCGCGAAAGGAATCTGTTTCGACGCTTTACCAAATAACTTTCCC 330
fvLac-4_promoter  GGGCAGTAAGGGTCACCGTTGGGCGGTGTCTATTAGTATTATATATAAGAACGTCGATGTCCAACGTCGCCTCACCCTTCCACGCCTTCAATCTTCCCTTCATTATCCTT 440
fvLac-4_promoter  CTCTTCCCTTTCCCTAAATCCCTTCTTCCTTTATTTTATTTCTTTTGGGGGATTTAGACG 500
```

Supplementary Fig. 5. Promoter region organization of the *Flammulina velutipes* laccase genes and putative cis-acting elements. TATA boxes are highlighted with gray boxes. CAAT boxes are highlighted in boxes. The antioxidant responsive element; TGACNNNGC) is underlined and the stress responsive element; CCCCT) is underlined with a double line.