FULL PAPER

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Cloning and characterization of a laccase gene from the white-rot basidiomycete *Pleurotus ostreatus*

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Abstract The gene *lccK* encoding a laccase of the white-rot basidiomycete *Pleurotus ostreatus* wild-type strain collected in Japan has been cloned, sequenced, and characterized. The isolated gene consists of 2929 bp with the coding region interrupted by 19 introns and flanked by an upstream region in which putative CAAT and TATA elements were identified. Two putative N-glycosylation sites and four putative copper-binding sites found in other fungal laccase are conserved in *lccK*. The cDNA contains an open reading frame of 1599 bp and the gene encodes 533 amino acids preceded by a signal peptide of 23 amino acids. The nucleotide sequence of the *lccK* cDNA showed high homology with those of laccases of other basidiomycetes.

Key words Copper-binding sites · Intron · Laccase gene · N-Glycosylation sites · *Pleurotus ostreatus* · Promoter

Introduction

Laccase (EC 1.10.3.2) is one of the important enzymes that contribute to lignin degradation in conjunction with lignin peroxidase and manganese peroxidase in white-rot basidiomycetes (Youn et al. 1995). The enzyme contains sugar and copper atoms, which are extracellularly excreted, as its major characteristic. Several studies have been carried out on the applications of laccase in the pulp and paper industry, such as biopulping and biobleaching processes (Reid

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and Paice 1994; Decarvalho et al. 1999). Moreover, much attention has been paid to the application of laccase to the detoxification of environmental pollutants (Martirani et al. 1996; Kadhim et al. 1999; Ullah et al. 2000; Abadulla et al. 2000; Pointing 2001; Leontievsky et al. 2001). Laccase genes from various basidiomycetes, such as Coriorus hirsutus (Wulf.: Fr.) Pilát (Kojima et al. 1990), Phlebia (Ph.) radiata Fr. (Saloheimo et al. 1991), basidiomycete PM1 (Coll et al. 1993), Agaricus (Ag.) bisporus (J. Lange) Imbach (Perry et al. 1993), Pleurotus ostreatus (Jacq.: Fr.) Kummer (type, Florida) (Giardina et al. 1995, 1996, 1999), Trametes villosa (Fr.) Kreisel (Yaver et al. 1996), Trametes versicolor (L.: Fr.) Lloyd (Ong et al. 1997), Pycnoporus (Py.) cinnabarinus (Jacq.: Fr.) P. Karst. (Eggert et al. 1998), Schizophyllum commune Fr.: Fr. (Hatamoto et al. 1999), and Lentinula edodes (Berk.) Pegler. (Zhao and Kwan 1999) have been cloned and characterized.

Pleurotus ostreatus shows strong laccase activity among edible mushrooms, and it is relatively easy to culture in a medium. We have been studying the heterologous expression of laccase from *P. ostreatus* (Okamoto et al. 1995), and we have previously reported the purification and characterization of laccase from *P. ostreatus* as part of our study (Okamoto et al. 2000). Although there have been various reports on laccase cDNA already described, the comparison of the promoter region and intron of the laccase gene from various fungi has not been described in detail. Here we report the sequences of the laccase gene including those of the promoter region and introns from *P. ostreatus* wild-type strain collected in Japan, comparing it with sequences of other fungal laccases.

Materials and methods

Organism and culture conditions

The basidiomycete *Pleurotus ostreatus* K16-2 is a monokaryon screened from the protoplasts of a dikaryon collected in Kanagawa prefecture (Okamoto et al. 2000). The strain was maintained on 1.5% agar slants containing 1% (w/v) malt extract (Difco, Detroit, MI, USA), 0.4% (w/v) yeast extract (Difco), and 0.4% (w/v) glucose (MYG medium, pH 6.3). For the preparation of genomic DNA and total RNA encoding laccase, the mycelia grown on an agar slants were suspended in 8ml MYG medium, transferred to a 500-ml Erlenmeyer flask containing 80ml MYG medium, and incubated at 30°C for 7 days in a static culture.

mRNA isolation

Total RNA was isolated from 7-day-old mycelia of *P.* ostreatus K16-2 that were snap-frozen and ground in liquid N_2 , with a RNeasy plant minikit (Qiagen, Hilden, Germany). Poly (A)⁺ mRNA was purified using an OligotexTM-dT30 (Super) kit (Roche Diagnostics, Basel, Switzerland).

cDNA synthesis

Purified poly $(A)^+$ mRNA was used to synthesize the cDNA using a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Primers for cDNA amplification were designed based on the sequence of the N-terminus and the internal peptides of purified P. ostreatus laccase. The forward primer NF (5'-CTGGCAACATGTACATCG TCAACG-3') was designed on the basis of the N-terminal sequence, and the reverse primer IR (5'-TGCTCGTCGA CGAGGTTGTGGTG-3') was based on the sequence of the internal peptides. With these primers, an approximately 900-bp cDNA fragment was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequenced. Then, primers for rapid amplification of cDNA ends (RACE) were designed from the cDNA sequence: RT5R (5'-GAAACTATCCCCAGAGGCGAC-3') for 5' and RT3F (5'-TTGCTCGTCATTGAAGCAGATGC-3') for 3' RACE. RACE PCR was carried out using the two primer pairs, RT5R or RT3F and an adapter primer (AP1, 5'-CCATCCTAATACGACCACTATAGGGC-3'), and the PCR products were sequenced.

Genomic DNA preparation

Genomic DNA as a template for PCR amplification was isolated from frozen mycelia of *P. ostreatus* by cetyltrime-thylammonium bromide treatment, chloroform–octanol extraction, and isopropanol precipitation (Ausubel et al. 1994).

PCR primers

The primers designed from the nucleotide sequences of the cDNA from *P. ostreatus* laccase and used for PCR amplifications were as follows: N1, 5'-ATGTTTCCAGGC GCACGGATTCTT-3'; M361, 5'-GTCGCCTCTGGGG ATAGTTTC-3'; M490R, 5'-CGTATAAACTCAAGT GCGGATCCGA-3'; M532, 5'-ACGGTCATTACGC TTGAGGATTGG-3'; M661, 5'-ATCAACGTCGAAA GCAACAAGCG-3'; M753R, 5'-CATCTGCTTCAA TGACGAGC-3'; M966, 5'-CACCACAACCTCGTCGA CGAGC-3'; M1004R, 5'-AGGATTCTCAAGCGGAA CAAGGTTGG-3'; M1395R, 5'-ACGAAGCGAATAG TGACGTTGTCG-3'; and M1578R, 5'-TTAGGACGG AACGATGCCTCCTTTG-3'. The full-length laccase genomic DNA from P. ostreatus K16-2 was amplified by PCR using the primer pairs N1 and M1578R. A 2.6-kb fragment was isolated and analyzed partially by PCR using five primer pairs: N1 and M490R, M361 and M753R, M532 and M1004R, M661 and M1395R, and M966 and M1578R. The upstream region including the promoter of the gene was isolated by inverse PCR using AatII-cleaved genomic DNA.

PCR amplification

The reaction mixture for the DNA amplification contained 2.5 U ProofStart DNA polymerase (Qiagen), 1X ProofStart PCR buffer (Qiagen), 2.5 mM MgSO₄, 300 µM each dNTP, 100 ng template DNA, and 1.0 µM each of the primers in a 50-µl reaction volume. A mineral oil overlay that completely covers the solution was added to prevent condensation. PCR amplification was conducted with a DNA thermal cycler model 480 (Perkin-Elmer, Norwalk, CT, USA), using an initial denaturation step of 94°C for 2 min followed by 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 57°C for 2 min, and primer extension at 72°C for 2min, with a final extension at 72°C for 5min. Aliquots (10µl) of the amplification products were separated by gel electrophoresis through a 0.8% horizontal agarose gel at 100V in Tris-acetateethylenediaminetetraacetic acid buffer for 2h. Gels were stained with ethidium bromide and visualized under UV light. Molecular sizes were determined by comparison with a λ DNA-EcoT14I digest marker. A target amplification product was recovered from the agarose gel using the QIAquick gel extraction kit (Qiagen).

DNA sequencing

Sequencing reaction was performed using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequence data were analyzed using DNASIS-Mac v. 3.7 software (Hitachi Software, Yokohama, Japan). DNA sequences and deduced protein sequences obtained in this study were compared using the BLAST program.

Nucleotide sequence accession number

The nucleotide sequence data reported in this article has been submitted to DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB089612.

P.o K16-2 LccK P.o Florida Pox2 P.o Florida Pox1 C.h Po1 T.vil Lcc1 T.ver Lac1 PY.c Lcc3-1 PM1 Lac1 Ph.r Lac S.c Lac L.e Lac1 Ag.b Lcc1	חחחחחחחחח	MEPGARILATLTLALHLLHGTHAAIGPTGNMYIVNEDVSPDGFARSAVVARSVPATDPTPATVSIPGVLVQGNKGDNFQLNVVNQLSDTMLKTT MEPGARILATLTLALHLHGAHAAIGPTGDMYIVNEDVSPDGFARSAVVARSVPATDPTPATASIPGVLVQGNKGDNFQLNVVNQLSDTMLKTT MEPGARILATLTLALHLHGHAAIGPTGDMYIVNEDVSPDGFARSAVVARSVPATDPTPTATASIPGVLVQGNKGDNFQLNVVNQLSDTMLKTT MSRFQSLLAFLVASLAAVAHAAIGPTADLTISNAEVSPDGFTRSAVVARSVPATDPTPTATASIPGVLVQGNKGDNFQLNVINQLSDTMLKTT MSRFQSLLAFLVASLAAVAHAAIGPTADLTISNAEVSPDGFARQAVVVNNQTPGPLVQGNKGDNFQLNVIDNLTNHTMLKST MSRFQSLLAFLVASLAAVAHAAIGPVADLTISNAEVSPDGFSRQAVVNNQVPGPLITGNKGDRFQLNVIDNLTNHTMLKST MSRFQSLLSFVUSLAAVANAAIGPVADLTISNAEVSPDGFSRQAVVNNQVPSPLITGKKGDRFQLNVIDNLTNHTMLKST MSRFQSLLSFVUSLAAVANAAIGPVADLTISNGAVSPDGFSRQAVLVNQVPSPLITGKKGDRFQLNVIDNLTNHTMLKTT MAKFQSLLTFIILSLVASYASIGPVADLTISNGAVSPDGFSRQALLVNDVFPGPLIAGOKGDRFQLNVIDNLTNHTMLKTT MAKFQSLLTFIILSLVASYASIGPVADFIVNAAVSPDGFSRQALLVNDVFPGPLIAGOKGDRFQLNVIDNTNHTMLKST MHTFLRSTALVVAGLSARALASIGPVDFIVNAAVSPDGFSRQALLAEGVFPGPLIAGNKGDNFQLNVIDNTNHTMLKST MLPFVYLLAAELVCLVGGAVERDTTTYGQTVSENLFIVNAAVSPDGFSRQAVLAEGV
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Fig. 1. Amino acid sequence alignment of LccK from *Pleurotus* ostreatus K16-2 and laccases from different basidiomycetes. Possible copper-containing domains are shown in *white with a black back-ground*. Possible N-glycosylation sites (N-X-S/T) are highlighted in gray boxes. Identical residues are shown with asterisks. P.o K16-2 LccK, *Pleurotus ostreatus* K16-2 LccK (GenBank accession number, AB089612); P.o Florida Pox2, P. ostreatus (strain Florida) Pox2 (Q12739); P.o Florida Pox1, P. ostreatus (strain Florida) Pox1

(Q12729); C.h Lac, Coriorus hirsutus Lac (Q02497); T.vil Lcc1, Trametes villosa Lcc1 (Q99044); T.ver LacI, T. versicolor LacI (U44430); Py.c Lcc3-1, Pycnoporus cinnabarinus Lcc3-1 (AF025481); PM1 Lac1, basidiomycete PM1 Lac1 (Z12156); Ph.r Lac, Phlebia radiata Lac (Q01679); S.c Lac, Schizophyllum commune Lac (AB015758); L.e Lac1, Lentinula edodes Lac1 (AF153610); Ag.b Lcc1, Agaricus bisporus Lcc1 (Q12541) Fig. 2. Phylogenetic tree constructed based on the deduced amino acid sequences of fungal laccases. Calculated matching percentages are indicated at each branch point of the dendrogram and were generated using the DNASIS-Mac program



Results and discussion

Nucleotide sequence of *lccK* cDNA

The full-length *lccK* cDNA from *P. ostreatus* was amplified by RT-PCR and RACE. Sequence analysis revealed one open reading frame of 1599 bp and 533 amino acids including a signal sequence of the first 23 amino acids, and its mature laccase with a molecular mass of 56798 Da, which is almost identical to that of the purified enzyme (Okamoto et al. 2000). The open reading frame showed a G+C content of 53%.

The deduced amino acid sequence of laccase from P. ostreatus K16-2 was compared with those of other laccases (Fig. 1). The laccase amino acid sequence shows similarity to those of Pox2 (97% identity) and Pox1 (89% identity) from strain Florida, which is a variety of P. ostreatus, C. hirsutus Po1 (64% identity), T. villosa Lcc1 (62% identity), T. versicolor LacI (62% identity), Py. cinnabarinus Lcc3-1 (61% identity), basidiomycete PM1 Lac1 (61% identity), S. commune Lac (60% identity), Ph. radiata Lac (58% identity), L. edodes Lac1 (54% identity), and Ag. bisporus Lcc1 (47% identity). On the other hand, the primary sequence of LccK exhibits a lower similarity to ascomycetes, such as Neurospora crassa Lac (34% identity) (German et al. 1988) and Aspergillus nidulans YA (30% identity) (Aramayo and Timberlake 1990). These identity values are the same as those obtained when compared with N-terminal amino acid sequences among them (Okamoto et al. 2000). The percentage amino acid identity was calculated for pairwise combinations of fungal laccases and presented as a phylogenetic tree (Fig. 2), which shows that the *P. ostreatus* laccase is slightly different from laccases of other white-rot fungi. The signal amino acid sequence, MFPGARILATLTLALHLL HGTHA, is perfectly conserved among P. ostreatus laccases, but shows low identity (below 26%) to those of other laccases from different fungi. Purified laccase from *P. ostreatus* K16-2 was a copper-containing glycoprotein (Okamoto et al. 2000). It is confirmed that the deduced amino acid sequence of the laccase from *P. ostreatus* K16-2 contains two potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) and four potential copper-binding histidine-rich sites as the other fungal laccases mentioned earlier.

Nucleotide sequence of *lccK* genomic DNA

To determine the upstream N-terminal sequences and introns, the *lccK* genomic DNA from *P. ostreatus* K16-2 was amplified by PCR using some primers designed based on the nucleotide sequence of the *lccK* cDNA as described in Materials and methods. A fragment of 2929bp was amplified by PCR and sequenced. The structure of the laccase gene was characterized by comparison of the sequences of the genomic DNA and cDNA (Fig. 3). Nineteen introns that ranged in size from 48 to 61 bp were found to be localized in the gene, and the sequences at the splicing junctions in their introns adhered to the GT-AG rule (Gurr et al. 1987). The number and localization of introns in the laccase gene were compared with those of other fungal laccases in Fig. 4. There are various numbers of introns among laccases from fungi, such as P. ostreatus (strain Florida) pox1 (19 introns) and pox2 (19 introns), C. hirsutus pol (10 introns), T. villosa lcc1 (8 introns), basidiomycete PM1 lac1 (10 introns), Py. cinnabarinus lcc3-1 (10 introns), Ag. bisporus lccl (14 introns), and A. nidulans yA (5 introns). The variation of intron number and localization in the genes indicate a mutual vestige of divergence. P. ostreatus laccase have a large number of introns as compared with other fungal laccases, which confirms that P. ostreatus laccase is diverged a little differently from laccases of other basidiomycetes, as can be seen in the phylogenetic tree (see Fig. 2).

Analysis of the upstream N-terminal region revealed typical promoter sequences that were the same as those of

15

	AAGAAGCACTTTGAGTCTATTTCGCTCT	-301
-300		-201
-200	TTTCTAACAACACCGCCCGATTCGTTTCAAGATACTCGAGAGATGAGGTACGCCTAACCGAAGCTTCCTAATCCATTCGCCTTCGTTCTCATGCGTATCG	-101
-100	ACTTATAGG <u>TATATTT</u> AAGACCTGTACTGACGATTGCAAACAGCATCGACTTCCAGTTGCTTAACACCTCATCCAGCGCGTACTGCTACACCCTACAAACG	-1
1	ATGTTTCCAGGCGCACGGATTCTTGCTACGCTTACCTTGGCTCTTCATCTTTACATGGGACTCATGCTGCCATCGGGCCCACTGGCAACATGTACATCG	100
101	TCAACGAGGAGGAGGTCTCTCCTGATGGATTCGCTCGTTCgtaagtgtcttcactctcagtgttttgaaaacctcagtgctaattcattatacagGGCGGTTG V N E D V S P D G F A R S Intron I	200
201	TCGCTCGCTCGCTGCGCCGCTACAGATCCGACGCCTGCGACAGTATCCATTCCTGGCGTTCTCGTTCAAGGAAACAAGgtaattcttgttcttctcgatgg V A R S V P A T D P T P A T V S I P G V L V Q G N K Intron II	300
301	$agccgatgttttgctcaccccacgtagGGCGATAACTTCCAGCTGAACGTCGTCAATCAATTGTCGGACACGACCATGTTGAAGACGACCAGTATCgtac\\ G D N F Q L N V V N Q L S D T T M L K T T S I$	400
401	gtacatctgtggttatttcggataaacatttatggtctttgcagCATTGGCACGGTTTCTTCCAAGCCGGATCTTCGTGGGCTGATGgtagggttcatca Intron III H W H G F F Q A G S S W A D	500
501	ctgcgcatttaccatacctcgctaacggcgttttgtagGTCCCGCTTTCGTGACCCATGCCCCGTCGCCTCTGGGGATAGTTTCCTgtaggtgccactg Intron IV G P A F V T Q C P V A S G D S F L	600
601	gctttattgccgcccttgactaatccaaaatatccgcagGTACAATTTCAATGTCCCAGACCAAGCTGGAACGTTTTgtaagttgatcatattgttatcc Intron V Y N F N V P D Q A G T F	700
701	$\label{eq:linear} tttctacatcacctgactacttctacagGGTATCACTCGCATCTTTCCACCCAATATTGTGACGGCCTCAGAGGACCATTCGTGGTgtaagcgatgccat} \\ Intron VI W Y H S H L S T Q Y C D G L R G P F V V \\ \end{array}$	800
801	tccatatgacagaggtagtctggacactgacctgtctgcagATACGACCCCTCGGATCCGCACTTGAGTTTATACGATATTGACAACGgtgagctccgaa Intron VII Y D P S D P H L S L Y D I D N	900
901	cttctctcgacataaactgcagtttactcacgctcttctcagCCGACACGGTCATTACGCTTGGGGACgtcgtcttctgcgaggcctgccaaca Intron VIII A D T V I T L E D W Intron IX	1000
1001	$\label{eq:construct} tctactcatgttctccttagTATCATATCGTGGCTCCCCAAAACGCGGCAATCCCAACTCCGGATAGTACCCTCATCAATGGTAAAGGTCGTTATGCCGG \\ Y H I V A P Q N A A I P T P D S T L I N G K G R Y A G$	1100
1101	GGGCCCTACCTCCCTTTGTCCATCATCAACGTCGAAAGCAACAAGCGCTATCGTTTCAGACTTGTCTCAATGTCTTGTGACCCCAATTTCACGTTCTCG G P T S P L S I I N V E S N K R Y R F R L V S M S C D P N F T F S	1200
1201	ATCGACGGTCACTCTTTGCTCGTCATTGAAGCAGATGCTGTCAACATTGTTCCCATCACCGgtttgtcctttctatggcctcactctgctga I D G H S L L V I E A D A V N I V P I T Intron X	1300
1301	tcacggaccgtcgatatcatagTGGATAGTATTCAGATCTTCGCTGgtgagtcgcacctgccttgtcattgcaactgttgctaagcgtagtaaaagGCCAA V D S I Q I F A Intron XI G Q	1400
1401	eq:cgctactccttcgtcttcgtcttgactgctactcgtgcaactggcgcgcgc	1500
1501	actttcgtctttcgctgactccttatcgtgccagGCTTCGATGGTGGTATCATTCCGCTATTCTTCGGTATGCTGGTGCCACTGAGGATGACCCCACCA Intron XII G F D G G I N S A I L R Y A G A T E D D P T	1600
1601	CAACCTCGTCGACGAGCACCCCATTGCTGGAGAACCAACC	1700
1701	CATCAATCTCGCTATGGCCTTCGACTTCACTAACTTCGACTATCAACGgtacgcagcatctgacctgctttcgttttggatgtttgtt	1800
1801	acgtctatagGCGTTCCTTTCATCCCACCCACTGgtgagtgcagctcgaatgatgccagcaaaggcaattactgatgatactcctgcagCCCCTGTCCTT G V P F I P P T Intron XIV A P V L	1900
1901	CTTCAAATTCTCTCAGGAGCCTCCTCTGCTGCTGCTGCTGCTGCTGCTGCTGGTAGTATTACGCGCTGGAGCCTAATAAGGTTGTTGAAATCTCGATGCCTG L Q I L S G A S S A A S L L P S G S I Y A L E P N K V V E I S M P	2000
2001	$\begin{array}{cccc} CGCTGGCTGTCGGGGGACCCgtaagttctaccctgctctgcggccgtccatttgctaatgctacgcgcctagCATCCATTCCATCTCCACGGCgtgagta \\ A \ L \ A \ V \ G \ G \ P & Intron \ XV & H \ P \ F \ H \ L \ H \ G \end{array}$	2100
2101	atgtggtcaccgggagttccatttggctaatttatttcgtatagCACACCTTCGACGTTATTAGGAGTGCGGGTTCCACTACAACTTTGACACTCC Intron XVI H T F D V I R S A G S T T Y N F D T P	2200
2201	$\label{eq:constraint} \begin{array}{cccc} TGCGCGCCGCGACGACACACCTGGCGCGAACGACAACGTCACTATTCGCTTCGTGACCGACAACCCAGGGCCGTGGTTCCTCCACTGgtga \\ A & R & D & V & N & T & G & T & G & A & N & D & N & V & T & I & R & F & V & T & D & N & P & G & P & W & F & L & H & C \end{array}$	2300
2301	gtatttcgcgcaatgtatacggcgcatgctgatagtttccccagTCACATTGATTGGCATCTCGAAATgtaagtggcagtctttaccacctcgtac Intron XVII H I D W H L E I Intron XVIII	2400
2401	ttgatactaaacgccgcttagTGGTCTCGCTGTCGTTTTCGCCGAAGACGTGACATCCATTTCGGCCCCACCTGgtatgcatccttcctgccgcggcccg G L A V V F A E D V T S I S A P P Intron XIX	2500
2501	$\label{eq:constraint} tgtcccttctaacttgcatttctccagCCGCGTGGGACGACGACGACGACGACGACGACGACGACGACGACGA$	2600
2601	Α	

Fig. 3. Nucleotide and deduced amino acid sequences of the *lccK* genomic DNA from *P. ostreatus* K16-2. The putative CAAT element, TATA element, Sp1, heat shock element (HSE), and metal response element (MRE) are *underlined*. The predicted signal peptide is *boxed*.

The 19 putative introns are indicated in *lowercase type* and *numbered* (I-XIX). Recognition site for restriction endonuclease *Aat*II is highlighted in the gray box

Fig. 4. Comparison of intron/ exon structure of *lccK* from *P.* ostreatus strain K16-2, *pox1* and *pox2* from *P.* ostreatus (strain Florida), *po1* from *C.* hirsutus, *lcc1* from *T.* villosa, *lcc3-1* from *Py.* cinnabarinus, *lac1* from basidiomycete PM1, *lcc1* from *Ag.* bisporus, and *yA* from *A.* nidulans. The exons are in black boxes; white boxes correspond to the introns



P.o K16-2 lccK P.o Florida pox2 P.o Florida pox1 C.hirsutus po1 T.villosa lcc1 Py.cinnabarinus lcc3-1 PM1 lac1 Ag.bisporus lcc1 A.nidulans yA	-328 -334 -327 -336 -307 -300 -336	AAGAAGCACTTTGAGTCTATTTCGCTCTAGTAGCCTTCCATTGTTCACGGACTCTTCTTCGTATCCCCGAATACAT GCCATGAAAAGAAGCACTTTGAGTCTATTTCGCTCTAGTAGCCTTCCATTGTTCACGGAGTCTTCTTCGTATCCCCGAATACAT GCTATGAATAGAAGCACTTTGAGTCTATTTCGCTCTAATAGCCTTCCATCGCGACCTCTCCTCGCGACTCTCCCGAATATAAC CCGACACTGTTCGGGACGCGCGTCTTACCGCCGTGAGAACGCCAGGGCGTGTCGCGCACCTCTGCAAGCTCACACGCTTACCAGGGG AAGCCGGGTCTCTCCCGCCCTCTGTTGGCTCTCACGCATTCCGCATGGTTCGCGCGAC CGTGAGAGGGCTTCCTACTCCCTCCTCTCTCCCCGCCTCTCTCCCCAGCCCC AAACTGAACCACAGTACAACGCCTGCAAGACACGCGCGTGTCAATACCAAACGTTGAACATCTATGCGCCTGTAACGGGGA	
P.o K16-2 lccK P.o Florida pox2 P.o Florida pox1 C.hirsutus po1 T.villosa lcc1 Py.cinnabarinus lcc3-1 PM1 lac1 Ag.bisporus lcc1 A.nidulans yA	-252 -250 -243 -252 -229 -252 -252 -252	MRE Sp1 CCAGGCACTGCACTCCCTGGAGACGATCATCGCTCCCTCATGACAAACAGATTTCTAACAACACCGCCCGATTCGTTTCAAGA CCAGGCACTGCACT	
P.o K16-2 lccK P.o Florida pox2 P.o Florida pox1 C.hirsutus po1 T.villosa lcc1 Py.cinnabarinus lcc3-1 PM1 lac1 Ag.bisporus lcc1 A.nidulans yA	-168 -166 -159 -168 -168 -168 -168 -168 -143	HSE TACTCGAGAGATGAGGTACGCCTAAC <u>GGAAGCTTCC</u> TAATCCATTCGCCTTCGTTCTCATGCGTATCGACTTATAGGTATATTT TACTCGAGATGAGGTACGCCTAAC <u>GGAGCTTCC</u> TAATCCATTCGGCTTCGTTCTCATGCGTATCGACTTATAGATATATTT TCCTTGGACTGAGGTACGCCTAAC <u>GGAGCTTCC</u> TAATCCGTTCATTCGTTCTCATGCATATCGTTCTGAGGTATAATTT TCGTCCTTGGTTTGCTGGAGGCGCCCACCGTTGAGCCTTGGCCATACAGAGCGCGCTGTCCTTCGACGGGGTATAAAGGATGCCGC TCGGGGACTGGCGCCGCGCGCCCACCGTTGGCCATTCACTCTACCAGAGCGCTGGCCTTGGCCGAGGTATAAAGGATGTTGCGCG TTTCCTGCCCCGCCGCCGCTCGTCCACCGTTGGCTGTCCTCGTCCACGGCGCTCTGACTTGCGCGG TTTCCTGCCCCGCCGCCTACCCTCGCCCACGGCGTGTTGCTGTACAGGACTTCAAGGGCGCTCTGTCCTGGCCAGGCATAGAGGATGACGCTGCCACGGCGTCCACCGTTACACAGCACAATGACGCCACGACGCACGC	
P.o K16-2 lccK P.o Florida pox2 P.o Florida pox1 C.hirsutus po1 T.villosa lcc1 Py.cinnabarinus lcc3-1 PM1 lac1 Ag. bisporus lcc1 A.nidulans yA	- 84 - 84 - 77 - 84 - 84 - 84 - 84 - 84	AAGACCTGTACTGACGATTGCAAACAGCATCGACTTCCAGTTGCTTAACACCTCATCCAGCGCGTACTGCTACACCCTACAAACG AAGACCTGTACTGACGATTGCAAACAGCATCGACTTCCAGTTGCTTAACACCTCATCCAGCGCGTACTGCTACACCTACCAACG AAGACGTGCATGGACGACCGAAAATATCATCATCAACTTAACACCTCATCCAGCGCGTACTGCTACACCTACCAACG AGGGAACTCCCAACAGCACAACTCGAGCCCCGCTTGAGTTTCTACGAGGTCCTGCAAACCACTGCCTCCTCCCGGTCACACCACG ACGCCTCAACACCCCAACTCAGGCCCCGCTTGAGTTTCTACGAGGTCCTCCACAACCACTCACT	ATG ATG ATG ATG ATG ATG ATG

Fig. 5. Comparison of nucleotide sequence of the region upstream of the *lccK* gene of *P. ostreatus* with those of other basidiomycetes. The CAAT and TATA elements are highlighted in *gray boxes*. The puta-

tive Sp1, heat shock element (HSE), and metal response element (MRE) are boxed

previously characterized laccase genes (Fig. 5). The promoter region of this gene contains a TATA element that is an important sequence for initiating transcription (Ballance 1986) and an inverted CAAT element. The putative TATA and CAAT elements were localized at nucleotide positions -91 and -289, respectively, of the 5'-flanking region. A putative inverted Sp1 transcription factor recognition site (GGGCGG) (Dynan and Tjian 1985) was found at -188. A putative heat shock element (NGAANNTTCN) (Bonner et al. 1994) was found at -142, whereas a putative metal re-

sponse element (TGC(A/G)CNC) (Thiele 1992) was found at -244. The positions of CAAT and TATA elements differ among fungal laccase genes. No characteristic common to all except for the TATA and CAAT elements was found in a promoter region. The important point to note, however, is that there are some consensus sequences beyond the genus among the same group, revealing phylogenetic proximity (see Fig. 2). Two consensus sequences, 5'-TATAAAGGATG...CAACTC(G/A)AG(C/T)CCC (G/A)CTTGAG(T/C)TT...CGAG-3', 5'-(G/C)A(C/G)AC C(G/A)GTG(T)CAAT...TGACA(C/G)TG(T/C)(G/A)(C/ A)CAAC(C/T)G(G/A)G(T/C)AAG(G/T)CTCGTC-3', could be seen at nucleotide positions -102 to -32, and -235 to -164, respectively, among C. hirsutus pol, T. villosa lcc1, and Py. cinnabarinus lcc3-1. These motifs may have some relation to promoter activity for strict conservation within these genes. So far as we know, various reports published previously have made no mention of the matter. This is an interesting finding supplementing dendogram and intron analyses to determine divergence among fungal laccases.

From what has been discussed here, we can conclude that *P. ostreatus* possesses a slightly different type of laccase from others. The sequence reported here should be helpful in the mass production of fungal laccase, which is expected to be an application to various methods. As the next step, we are now investigating the heterologous expression of laccase from *P. ostreatus* K16-2 in yeast cells.

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