The full-length cDNA of the gene PoLOX1 encoding a lipoxygenase (LOX) and its corresponding genomic DNA were isolated from the basidiomycete mushroom *Pleurotus ostreatus* strain H1. The deduced amino acid sequence of PoLOX1 showed similarity to a valencene dioxygenase of *Pleurotus sapidus*, putative LOX-like proteins from ascomycete, basidiomycete, and deuteromyctye fungi, and known LOXs from plants, animals, and bacteria. PoLOX1 also contained the LOX iron-binding catalytic domain in the C-terminal region, but not the polycystin-1, lipoxygenase, alpha-toxin (PLAT) domain, which is usually found in the N-terminal region of eukaryotic LOXs. Genomic sequence analysis revealed that PoLOX1 was interrupted by one intron, and that the promoter region included TATA and CAAT boxes. Southern blot analysis indicated that it is transcribed more abundantly in the stipes of the fruit bodies than in the caps.

**Key words:** aroma; lipoxygenase; mushroom; 1-octen-3-ol; *Pleurotus ostreatus*

Lipoxygenase (LOX) is a non-heme iron-containing dioxygenase widely distributed in nature. LOXs have been characterized from plants, animals, algae, fungi, and bacteria. Plant and animal LOXs, in particular, have been extensively studied. This enzyme catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) containing a Z,Z-1,4-pentadiene moiety, such as linoleic acid, linolenic acid, and arachidonic acid, yielding the corresponding hydroperoxides. These hydroperoxides are subsequently metabolized via several secondary pathways to form important physiological molecules. Animal LOXs are involved in the biosynthesis of leukotrienes and lipoxins, which are potent mediators of inflammatory responses. Plant LOXs participate in the biosynthesis of signaling compounds such as jasmonates, antimicrobial compounds such as 2-hexenal, and antifungal compounds such as divinyl ethers. They also play roles in the production of volatile molecules that can positively or negatively influence the flavor and aroma of many plant products.

Oxygen insertion into PUFAs by LOX is regio- and stereo-specific, and this specificity is used as the decisive criterion for LOX classification. In plants, linoleic acid and linolenic acid are the primary substrates of LOX, because they are the most abundant fatty acids. Plant LOXs are classified as 9- or 13-LOXs with respect to their positional specificity of linoleic acid oxygenation. Animal LOXs are classified as 5-, 8-, 9-, 11-, 12-, or 15-LOXs with respect to their positional specificity of the oxygenation of arachidonic acid, the predominant substrate of animal LOXs. Furthermore, LOXs are classified as S- or R-LOXs on the basis of the chirality of their hydroperoxide products.

The essential structural features of plant and animal LOXs are highly conserved, although they show a low level of amino acid sequence similarity. Plant and animal LOXs consist of a single polypeptide chain that has two domains: a smaller N-terminal domain and a larger C-terminal domain. The N-terminal domain forms a β-barrel domain composed of two sheets of four strands each, and it is one of the defining members of the polycystin-1, lipoxygenase, alpha-toxin (PLAT) domain family. The N-terminal domain might play a role in the interaction of the enzyme with biological membranes.

On the other hand, the C-terminal domain, composed primarily of α-helices, is the catalytic domain, because it contains amino acid residues that are essential for catalysis, iron binding, and substrate positioning. In this domain, five highly conserved amino acid ligands for non-heme iron and several primary determinants of regio- and stereo-specificity have been identified.

Fungal LOXs have been characterized in molds such as *Fusarium proliferatum* and mushrooms such as *Morchella esculenta*. Among these, a unique LOX (Mn-LOX), which contains Mn as the catalytic metal, has been well studied in a take-all fungus, *Gaeumannomyces graminis*, but information on the molecular structure and biological function of fungal LOXs is limited. To our knowledge, the only reports on fungal LOX genes are those on the *G. graminis Mn-LOX-Gga* gene and the *Aspergillus flavus A/LOX* gene.

Aroma is an important factor in determining the quality of mushrooms as food materials. The volatile compounds in mushrooms have been reported to be composed primarily of a series of aliphatic 8-carbon compounds, including 1-octen-3-ol, 3-octanone, and 3-octenol. Among these, 1-octen-3-ol, which is the most abundant in many mushrooms, is considered the
major contributor to the characteristic mushroom aroma. The biosynthesis of 1-octen-3-ol in mushrooms is believed to proceed via enzyme-catalyzed oxidation and cleavage of linoleic acid. To date, three biosynthetic pathways have been proposed. Tressl et al.\(^2\) suggested that linoleic acid is converted to 13-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPOD) by LOX, and that 13-HPOD is cleaved into 1-octen-3-one and 10-carbon compounds by a hydroperoxide cleavage enzyme. The former is finally reduced to 1-octen-3-ol by alcohol oxidoreductase. On the other hand, Wurzenberger and Grosch\(^2\) found that 10-hydroperoxy-8E,12Z-octadecadienoic acid (10-HPOD) is formed from the hydroperoxidation of linoleic acid by LOX, and is subsequently cleaved by hydroperoxide lyase (HPL) into 1-octen-3-ol and 10-oxo-trans-8-decenoic acid. Combet et al.\(^3\) proposed a novel pathway, in which 1-octen-3-ol is generated from linoleic acid by a novel oxidizing enzyme, heme dioxygenase, and HPL via 10-HPOD as intermediate, but little is known about the biosynthetic pathways leading to the formation of 1-octen-3-ol in mushrooms, because the enzymes involved in the pathways have not been identified and characterized. On the other hand, in the moss Physcomitrella patens, 1-octen-3-ol is formed from arachidonic acid by a bifunctional LOX (PpLOX1) via a 12-hydroperoxide of this fatty acid as intermediate.\(^4\)

**Pleurotus ostreatus** is a wood-rotting basidiomycete, and a popular edible mushroom in many countries. We previously reported for the first time the homogeneous purification and characterization of a mushroom LOX from *P. ostreatus*.\(^5\) It exhibited preferential activity towards linoleic acid and converted it into 13-HPOD, the main oxidative product. These data suggest the presence of a 1-octen-3-ol biosynthetic pathway with 13-HPOD as intermediate in *P. ostreatus*. However, it has not yet been confirmed that LOX is involved in the formation of 1-octen-3-ol. To achieve a better understanding of *P. ostreatus* LOX and the formation of 1-octen-3-ol, a LOX gene, designated *PoLOX1*, was isolated from *P. ostreatus* strain H1 in this study. Here we report the deduced amino acid sequence and the genomic structure of the *PoLOX1* gene and its expression in fruit bodies.

### Materials and Methods

**Fungal strain and culture conditions.** Fresh *P. ostreatus* mushrooms (probably strain H1) purchased from the Shitome Garden (Niigata, Japan) were used to determine the partial amino acid sequence of LOX. A commercial dikaryotic strain of *P. ostreatus* strain H1 (Onuki Kinjin, Utsumonima, Japan) was used in the remaining experiments. Mycelia of strain H1 were cultured for 7 d at 25 °C in YM medium (3% w/v yeast extract, 3% w/v malt extract, 5% w/v peptone, and 10% w/v glucose) and were used as the source for genomic DNA isolation. For the production of *P. ostreatus* fruit bodies, the mycelia were cultivated in a sawdust medium containing beech sawdust and rice bran at a ratio of 3:1 (v/v). The sawdust medium was adjusted a moisture content of 65% with tap water and packed into a culture bottle (850 mL). The sawdust medium was autoclaved and incubated with approximately 5 g of mycelial cultures grown in the sawdust medium. The cultures were incubated at 20 °C at 70% humidity in the dark for 30–35 d. Subsequently, to induce fruit-body development, the culture was shifted to 15 °C at 90% humidity under a 12-h light-dark cycle. After 9–11 d, fruit bodies with approximately 15-mm-diameter caps were formed and were harvested, and soon thereafter some of them were separated into two parts, the cap containing the gills, and the stipe. Some of the mushroom samples were immediately used to study 1-octen-3-ol formation and enzyme activity, and the others were immediately frozen in liquid nitrogen and stored at −80 °C until use.

**Amino acid sequencing of purified lipoygenase.** A LOX was purified from fresh *P. ostreatus* fruit bodies (probably strain H1), as described previously.\(^6\) The purified LOX was subjected to proteolysis with V8 protease (Wako, Osaka, Japan) by a method described by Cleveland et al.\(^7\) The resulting peptide fragments were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by Laemmli’s method,\(^8\) and then transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-P, Atto, Tokyo) by electroblotting. The N-terminal amino acid sequence was determined using a protein sequencer (PPSQ-21; Shimadzu, Kyoto, Japan).

**DNA and RNA isolation.** Genomic DNA was isolated from the mycelia using Isolamp II (Nippon Gene, Tokyo). Total RNA was isolated from the fruit bodies by the phenol–SDS method,\(^9\) except that a room-temperature extraction buffer was used at the extraction step instead of the hot-extraction buffer. Poly(A)\(^+\) RNA was purified from total RNA with a Poly(A)\(^+\) Isolation Kit (Nippon Gene).

**3′ rapid amplification of cDNA ends and cloning.** 3′ rapid amplification of cDNA ends (RACE) was performed using an RNA PCR Kit (AMV) Ver.3.0 (Takara Bio, Shiga, Japan). The two degenerate polymerase chain reaction (PCR) primers used were *PoLOX1*-F1 and *PoLOX1*-F2, designed based on the internal partial amino acid sequence (AVIVAVQR) of purified LOX. The primers used in this study are listed in Table 1. Poly(A)\(^+\) RNA was used to synthesize first-strand cDNA. Semi-nested PCR was performed using the synthesized cDNA as template and adaptor primer M13 primer M4 and adaptor primer M13 primer F1 primers for the first PCR, and adaptor primer M13 primer M4 and *PoLOX1*-F2 primers for the second PCR. The second PCR product was cloned into vector pGEM-T (Promega, Madison, WI), followed by sequencing. DNA sequencing was performed with a BigDye Terminator Cycle Sequencing Ready Reaction Kit with a Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

**cDNA library construction and screening.** A cDNA library of *P. ostreatus* strain H1 fruit bodies was constructed in *E. coli* vector pTriEx2 plasmid by a SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA) following the manufacturer’s protocol. An aliquot of the library containing approximately 5 × 10\(^8\) recombinant clones was screened by plaque hybridization on nylon membranes (Hybond-N+; GE Healthcare, Piscataway, NJ). The *PoLOX1* cDNA was labeled with digoxigenin (DIG) with a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) and used as probe. The membrane was hybridized overnight with the probe in DIG Easy Hyb (Roche Diagnostics) at 40 °C and washed twice in 2 × SSC containing 0.1% w/v SDS at room temperature for 5 min, and twice in 0.2 × SSC containing 0.1% w/v SDS at 68 °C for 15 min. Signals were detected using a DIG DNA Labeling and Detection Kit. Positive *E. coli* plasmids were converted to pTriEx2 plasmids following the manufacturer’s protocol.

### Table 1. Primers Used in This Study

<table>
<thead>
<tr>
<th>Primers Used in This Study</th>
<th>Usage</th>
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<tbody>
<tr>
<td><em>PoLOX1</em>-F1</td>
<td>GCGTNATHTNGTNGCTGT</td>
</tr>
<tr>
<td><em>PoLOX1</em>-F2</td>
<td>ATHTNGCNCTNGNCAACG</td>
</tr>
<tr>
<td><em>PoLOX1</em>-R1</td>
<td>AAGAAATGGCTGCGGGAAGACG</td>
</tr>
<tr>
<td><em>PoLOX1</em>-R2</td>
<td>CAACCCAAATGTTCGCGTTCG</td>
</tr>
<tr>
<td><em>PoLOX1</em>-R3</td>
<td>TCCTCTTTCTTCTCCTGCGGTT</td>
</tr>
<tr>
<td><em>PoLOX1</em>-F3</td>
<td>CTTTCTGCGAATGGCTTTGTA</td>
</tr>
<tr>
<td><em>PoLOX1</em>-F4</td>
<td>AGCAGACACGCGACATCTCATA</td>
</tr>
<tr>
<td><em>PoLOX1</em>-F4</td>
<td>TGTCATCTCTCCTGCGGTTT</td>
</tr>
<tr>
<td><em>PoLOX1</em>-R5</td>
<td>ACTGGAAGATACGAGAACGCTCG</td>
</tr>
<tr>
<td><em>PoLOX1</em>-F5</td>
<td>GAACACGGCGTACATGTTGC</td>
</tr>
<tr>
<td><em>PoLOX1</em>-R6</td>
<td>ATGACCTCGCCAATGAGGAC</td>
</tr>
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</table>

Abbreviations: A, T/G/C; H, A/T/C; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction
5' RACE. A primer for reverse transcription and two primers for amplification of the 5' end of the cDNA were designed using the sequence of the PoLOX1 cDNA. First-strand cDNA was synthesized from poly(A)⁺ RNA using a SMART cDNA Library Construction Kit (Clontech) with a 5'-adapter SMART IV oligonucleotide and PoLOX1-R1 primer. Semi-nested PCR was performed using the synthesized cDNA as template and the 5'-PCR primer and PoLOX1-R2 primer for the first PCR, and the 5'-PCR primer and PoLOX1-R3 primer for the second PCR.

Southern blot analysis. Genomic DNA (10µg) was digested with BamHI, HindIII, KpnI, or PstI (Toyobo, Osaka, Japan), electrophoretically separated on a 0.8% agarose gel, and transferred to a Hybond-N+ membrane (GE Healthcare). Hybridization with the full-length PoLOX1 cDNA probe was performed as described for plaque hybridization. Hybridization signals were detected using a DIG Luminescent Detection Kit (Roche Diagnostics).

PCR. Genomic DNA was digested with HindIII, and was self-ligated overnight at 16°C with a DNA Ligation Kit (Takara Bio). PCR and inverse PCR were performed using Ex Taq DNA polymerase (Takara Bio) and genomic DNA and the self-ligated DNA respectively as template. PCR primers were designed using the sequence of the PoLOX1 cDNA. The primers for PCR were PoLOX1-F3 and PoLOX1-R4. The primers for inverse PCR were PoLOX1-F4 and PoLOX1-R5 for the first PCR and PoLOX1-F5 and PoLOX1-R6 for the second PCR.

Northern blot analysis. Total RNA (20µg/lane) was loaded on a 1.2% agarose gel containing formaldehyde, separated by electrophoresis, and blotted onto a Hybond-N+ membrane (GE Healthcare). To compare the amounts of RNA loaded in each lane before transfer, the gel was stained with SYBR Green II (Lonza, Rockland, ME), and the presence of intact 18S and 28S rRNA was verified. The membrane was hybridized overnight with a PoLOX1 cDNA probe and processed as for Southern blot analysis, except that the hybridization temperature was 50°C. The intensities of the hybridization signals and the stained 28S rRNA bands were quantified by densitometric scanning using the LuminVision IMAGER program (Aisin Seiki, Kariya, Japan). PoLOX1 mRNA levels were normalized by dividing the intensity of the signal for the PoLOX1 probe by that of the 28S rRNA band.

Preparation of crude extracts. Fresh mushroom samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) at a ratio of 1:2 (w/v) at 13,500 rpm for 2 min at 4°C using a homogenizer (Ultra-Turrax T25; Janke and Kunkel IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 15,000 g at 4°C for 30 min, and the supernatant was used as crude extract for enzyme assays and 1-ocet-3-ol determination.

Enzyme and protein assays. A substrate solution was prepared as described by Ben-Aziz et al. 250 LOX activity was determined by a spectrophotometric procedure based on the formation of conjugated diene, as described previously. 251 The protein concentration was determined by the Bradford method with bovine serum albumin as standard. 250

Extraction and determination of 1-ocet-3-ol. One milliliter of the above-mentioned crude extract was homogenized with 1 mL of n-pentane containing 0.15 mg of n-onyl alcohol as internal standard. The homogenate was centrifuged at 2,000 × g for 5 min, and the upper organic phase was collected. The organic extract was analyzed by gas chromatography (GC) using a Shimadzu gas chromatograph (GC-2014AFSP); equipped with a flame ionization detector and a DB-WAX capillary column (60 m length × 0.25 mm i.d. × 0.25 µm film thickness; J&W Scientific, Folsom, CA). The column temperature was programmed to increase from 50°C to 230°C at a rate of 6°C/min. The injection and detector temperatures were 200°C and 250°C respectively. The flow rate of the carrier gas (He) was maintained at 1 mL/min.

Statistical analysis. Northern blot analysis data, LOX activity, and 1-ocet-3-ol content were expressed as mean ± standard error values. Statistical significance was assessed by Student’s t test. A p value of < 0.05 was considered statistically significant.

Results

Determination of the partial amino acid sequence of the purified LOX
To obtain information for cloning of the PoLOX1 gene encoding P. ostreatus strain H1 LOX, we analyzed the partial amino acid sequence of a LOX purified from P. ostreatus fruit bodies (probably strain H1). The N-terminal amino acid sequence of the purified LOX was not detected, suggesting that its N-terminus was blocked. Hence, the purified LOX was digested with V8 protease, and three peptide fragments, V8-I (29 kDa), V8-II (21 kDa), and V8-III (20 kDa), were obtained. The N-terminal region of the peptide fragment V8-I was sequenced and determined to be AVIVAYQRTLDP.

Isolation and sequence analysis of PoLOX1 cDNA
To isolate the PoLOX1 cDNA, 3' RACE was performed using degenerate primers based on the sequence of V8-I. This approach generated a partial cDNA fragment of 1,017 bp, which was cloned and sequenced. The amino acid sequence deduced from the fragment contained the V8-1 sequence. Next, to isolate the full-length cDNA, we isolated nine positive cDNA clones by screening a cDNA library of the P. ostreatus strain H1 fruit body, and sequenced their cDNA inserts. All the DNA sequences determined contained sequences identical to the partial cDNA sequence of PoLOX1. The longest cDNA insert among the isolated clones consisted of 1,837 bp, but the cloned cDNA lacked the 5'-end region. Hence, 5' RACE was performed, and the resulting product was cloned. Sequence analyses of the five resulting clones revealed that four cDNAs were 302 bp long and one cDNA was 5'-truncated and two nucleotides shorter than the other four cDNAs. The full-length cDNA of PoLOX1 (DDBJ accession no. AB472334) is 2,078 bp long and contains an open reading frame (ORF) of 1,920 nucleotides encoding 640 amino acid residues with a molecular mass of approximately 72 kDa and a predicted pI of 5.0 (Fig. 1).

Among these cDNAs, the sequences surrounding the first ATG and the second ATG (10 bp downstream of the first ATG) codons were TCACAATTGC and CCACCATGTC, which agree fairly well with the Kozak consensus sequence CCA(A/G)CCATGG (251) and the sequence TC(A/C)A(A/C)ATG(T/G)TC highly conserved in filamentous fungi. 252 Hence, we designated the first ATG (positions +1 to +3) the tentative translation initiation codon of PoLOX1 cDNA. In addition, 5' RACE results suggested that the major transcription start site is located 49 bp upstream of the tentative ATG initiation codon (Fig. 1). The amino acid sequence from 332 Ala to 344 Asp of PoLOX1 was identical to the sequenced N-terminus of the peptide fragment V8-I (Fig. 2).

Comparative analysis of the PoLOX1 amino acid sequence
A BLAST search of sequence databases done using the deduced amino acid sequence of PoLOX1 revealed that PoLOX1 shared highest identity with a valencene dioxygenase of Pleurotus sapidus (PsaoX1, 77% identity), 253 ~50% identity with putative LOX-like proteins.
from ascomycete, basidiomycete, and deuteromycete fungi, and ~29% identity with known LOXs from animals, plants, and bacteria. The regions in the middle and C-terminal portions of the proteins showed substantial sequence similarity, whereas their N-terminal portions were divergent. Using the CLUSTAL W program at DDBJ (http://clustalw.ddbj.nig.ac.jp/), the C-terminal region of the PoLOX1 protein sequence was aligned with the PsoX1 sequence and LOX sequences of Homo sapiens 12R-LOX (Hs12R-LOX), 34) Glycine max LOX1 (GmLOX1), 35) and Nostoc punctiforme LOX1 (NpLOX1) 36) (Fig. 2). Six His residues (central 6-His box) highly conserved in LOXs were detected: five His residues in the 317–354 amino acid region and one at position 507 in PoLOX1. Conserved amino acid residues involved in iron-ligand binding, three His, one Asp, and the C-terminal Ile, were found at 322His, 327His, 507His, 511Asn, and 640Ile in PoLOX1. Four amino acid determinants of regio- and stereo-specificity of LOXs have been identified at conserved amino acid positions. At the position first described by Sloane et al. 30) a Phe residue, a primary determinant of positional specificity in 13-LOXs, was conserved at 380Phe in PoLOX1. At the position first described by Borngräber et al. 31) a Trp residue (314Trp) was found in PoLOX1 instead of a Phe residue, a primary determinant of positional specificity in mammalian 15-LOXs. At the position first described by Hornung et al. 32) an Arg residue, reported to determine inverse substrate orientation in plant LOXs, was not conserved, and was replaced by a Lys residue (324Lys) in PoLOX1. At the position first described by Coffa and Brash, 13) the amino acid residue that determines the S or R stereochemistry of LOXs was an Ala residue 365Ala) in PoLOX1, which is conserved in S-LOXs, whereas a Gly residue is conserved in R-LOXs, as seen in Hs12R-LOX. These results suggest that the PoLOX1 enzyme is a 13S-LOX.

Sequence analyses performed using motif search programs PROSITE (http://prosite.expasy.org/) and HMMPfam (http://pfam.sanger.ac.uk/) indicated that PoLOX1 contains a LOX iron-binding catalytic domain (PS51393) and a LOX domain (PF00305) at positions 162–640 and 181–605 in the C-terminal region respectively, but the N-terminal region of PoLOX1 showed no similarity to the PLAT domain 31) usually found in eukaryotic LOXs. The three-dimensional protein structure of PoLOX1 was predicted using automated protein structure homology-modeling server SWISS-MODEL (http://swissmodel.expasy.org/), with rabbit 15-LOX (PDB ID, 2P0M) as template. The theoretical results indicated that the PoLOX1 protein is folded into a two-domain structure. The small N-terminal domain consists mainly of two β-sheets and four α-helices, but this domain is structurally different from the N-terminal β-barrel domain of rabbit 15-LOX. The larger C-terminal domain is mainly helical in structure, and most of the α-helices correspond to a structurally similar helix in rabbit 15-LOX. In addition, sequence analysis of PoLOX1 using the program SosuI (http://bp.nuap.nagoya-u.ac.jp/sosui/) predicted that it is a soluble protein. The subcellular localization predicting program WoLF PSORT (http://wolfsort.org/) suggested a mitochondrial localization for PoLOX1 with the following scores: mitochondria, 11.0; cytoplasm, 7.0; nucleus, 7.0; cytoplasm, 6.0; and peroxisome, 3.0. In contrast, the ProtComp (http://linux1.softberry.com/berry.phtml) and SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) prediction programs suggested a cytoplasmic localization with an integral prediction score of 6.1 and a reliability index of 1 and expected accuracy of 56% respectively. These predictions suggest a high probability of PoLOX1 localization to the mitochondria or cytoplasm and/or the nucleus.

Using a genome database (JGI database; http://genome.jgi-psf.org/) of 144 fungal genomes, we tried to identify LOX by a combination of an HMMPfam search using a typical LOX domain (PF00305) and a BLAST search using the deduced amino acid sequence of PoLOX1. The HMMPfam search showed 55 gene models coding for putative LOX-like proteins with the LOX domain present in 37 genomes. A BLAST search on the models using a 10−3 E-value cut-off revealed 39 gene models with similarity to PoLOX1 at the protein level in 27 genomes. In addition, no gene models other than the above-mentioned ones were found in a BLASTP search with a 10−3 E-value cut-off using the 5 His-containing region (positions 317–354) of the central 6-His box conserved in PoLOX1 without a HMMPfam search. PoLOX1 showed 37%–44% and 45%–95% identity at the protein level with 24 and 15 gene models in the genomes of 20 ascomycete and seven basidiomycete species respectively. Of the basidiomycetes, 1–4 putative LOX-like gene models were present in the genomes of Gymnopus luxurians, Hebeloma

**Fig. 1.** Structure of the PoLOX1 Gene and Its Deduced Protein.

A. The horizontal line represents the genomic sequence of the PoLOX1 gene. White and black boxes on the horizontal line indicate exons and an intron respectively. ATG, tentative initiation codon; TAG, termination codon. The restriction enzyme sites used in Southern blot analysis (Fig. 4) are shown. B. The open box indicates the deduced amino acid sequence of PoLOX1. The lipoygenase iron-binding catalytic domain (LOX domain; PROSITE, PS51393) is shaded.
cylindrosporum, Hypholoma sublateritium, Laccaria bicolor, P. ostreatus monokaryotic protoclines PC15 and PC9, and Sphaerobolus stellatus. These findings raise the possibility that many fungi, including basidiomycete mushrooms, have no genes encoding LOX-like proteins. Furthermore, 16 gene models excluded by the BLAST search were in 14 ascomycete genomes, and they displayed 35%–56% identity at the protein level with the G. graminis Mn-LOX-Gga gene. In the genome of P. ostreatus PC15 (v2.0), which is similar to P. ostreatus strain H1, two putative LOX-like gene models (IDs, 1056091 and 1068582) were located on scaffold 05:1025166-1027727 and scaffold 11:352312-354690 respectively. The models, 1056091 and 1068582, contained ORFs of 1,983 and 1,920 nucleotides encoding 661 and 640 amino acid residues with molecular masses of approximately 74 and 72 kDa and predicted pIs of 5.2 and 4.9 respectively. The deduced amino acid sequences of 1056091 and 1068582 were 73% identical and shared 74% and 93% identity with that of PoLOX1 respectively. In addition, the amino acid sequences from 353 Ala to 364 Pro and from 332 Ala to 343 Asp of 1056091 and 1068582 were 73% and 100% identical to the sequenced N-terminus of peptide fragment V8-I respectively. These data indicate that the predicted proteins of the B. cinerea Csa Sl H. oestrilactis Ho Fig. 2. Partial Amino Acid Sequence Alignment of Pleurotus ostreatus PoLOX1 with a Valencene Dioxygenase and Other LOXs. PsaOx1, Hs12R-LOX, GmLOX1, and NpLOX1 indicate Pleurotus sapidus valencene dioxygenase (accession no. CA05788), Homo sapiens 12R-LOX (accession no. O75342), Glycine max LOX1 (accession no. P08170), and Nostoc punctiforme LOX1 (accession no. YP001869751) respectively. The N-terminal amino acid sequence determined from peptide fragment V8-I is underlined. Positions with identical amino acid residues are indicated by asterisks below the sequence. Colons and dots indicate the positions of amino acid residues with strong and weak similarity respectively. The His residues conserved in the central 6-His box characteristic of LOXs are indicated by diamonds. The conserved residues involved in non-heme iron ligation (three His, one Asp, and one Ile residues) are in bold. The three determinants of the substrate- and regio-specificity of LOXs are framed and are indicated by letters (Bo following Borngräber et al.,11 Sl following Sloane et al.,10 and Ho following Hornung et al.,12)). The determinant of stereo-specificity is framed and is indicated by the letters Co following Coffa and Brash.13)
Fig. 3. Unrooted Tree Based on the Deduced Amino Acid Sequences of *Pleurotus ostreatus* PoLOX1, LOXs from Fungi, Plants, Animals, Algae, Coral, and Bacteria, and a *P. sapidus* Valencene Dioxygenase. Amino acid sequences were aligned using the CLUSTAL W program, and the phylogenetic tree was constructed by the neighbor-joining method using the MEGA5 program. Values represent percentages of 1,000 bootstrap replications. Scale bar corresponds to 0.1 amino acid substitutions per position. PoLOX1 of *P. ostreatus* strain H1 is in bold. Putative LOX-like proteins are indicated by asterisks. The sequences used and their accession numbers are as follows: *P. ostreatus* PoLOX1, BAB9788; *P. sapidus* PsaoX1, CAQ87588; *Laccaria bicolor* LbLOX, XP_001881489 (partial sequence); *Aspergillus fumigatus* AfLOX, AF479686; *Neosartorya fischeri* NfLOX, XP_001262720; *Neurospora crassa* NcLOX, Q8NIU6; *Gaemumannomyces graminis* var. *avenae* Mn-LOX-Gga, AAK81882; *Homo sapiens* Hs-5-LOX, P009917; *H. sapiens* Hs12S-LOX, P18054; *H. sapiens* Hs12R-LOX, O75342; *H. sapiens* Hs15-LOX, P16050; *Mus musculus* MmLOX, P39654; *Oryctolagus cuniculus* OcLOX, XP_001923685; *Plexaura homomalla* PhLOX, AAC47283; *Glyceria max* GmLOX1, P08170; *Cucumis sativus* CsLOX, CAA63483; *Hordeum vulgare* HvLOX1, P29114; *Solanum tuberosum* StLOX1, CAA64766; *Momordica charantia* AmLOX, AAK81882; *Porphyra purpurea* PaLOX, AF479686; *Nostoc punctiforme* NpLOX1, XP_001869751; and *Aeropyrum pernix* K11403, XP_001881489.

To deduce the evolutionary relationships between LOXs, putative LOX-like proteins selected from a wide range of different organisms, and a *P. sapidus* valencene dioxygenase, phylogenetic analysis was performed using their full-length amino acid sequences, except in the case of a putative LOX-like protein of *L. bicolor*. The phylogenetic tree indicated that PoLOX1 is closely related to the valencene dioxygenase and the putative LOX-like protein of the basidiomycetes *P. sapidus* and *L. bicolor* respectively, and that these proteins form a fungal group with putative LOX-like proteins of ascomycetes (Fig. 3). On the other hand, Mn-LOX-Gga of the ascomycete *G. graminis* was clearly separated from the fungal group.

Fig. 4. Southern Blot Analysis of the PoLOX1 Gene. Genomic DNA (10 μg) was digested with *Bam*HI (lane B), *Hind*III (lane H), *Kpn*I (lane K), or *Pst*I (lane P), and separated by agarose gel electrophoresis. Following transfer to a nylon membrane, the blot was hybridized with a labeled PoLOX1 cDNA probe. The sizes of DNA markers are indicated to the left.

Expression of PoLOX1 in fruit bodies

To investigate the expression of *PoLOX1* in fruit bodies, total RNA was isolated from their caps and stipes and subjected to Northern blot analysis with the *PoLOX1* cDNA probe. Moreover, to analyze quantitatively the expression of the *PoLOX1* gene, the levels of *PoLOX1* mRNA were normalized to those of the 28S rRNAs. A 2.0-kb transcript of *PoLOX1* was detected in both the cap and the stipe, but the band intensity was higher in the stipe than in the cap (Fig. 5A). The relative mRNA level of *PoLOX1* in the stipe was 1.9-fold that in the cap (Fig. 5B). These results suggest that in fruit bodies with approximately 15-mm-diameter caps, *PoLOX1* is expressed more abundantly in the stipe than in the cap.

Lipoxygenase activity and 1-octen-3-ol formation

To determine the relationship between LOX activity and 1-octen-3-ol formation, enzyme activity (with
1.9-fold higher than that in the cap.

The specific activity in the stipe were 1.7- and 2.7-fold higher respectively than those in the cap (Table 2). The activity on a fresh-weight basis and the fruit bodies. The activity on a fresh-weight basis and the fruit bodies was separated by formaldehyde agarose gel electrophoresis. Following transfer to a nylon membrane, the blot was hybridized with a labeled PoLOX1 cDNA probe (upper panel). The estimated sizes of the mRNAs hybridized with the PoLOX1 probe are indicated to the right of the panel. SYBR Green II-stained 28S rRNA bands (lower panel) were quantified, and were used to normalize PoLOX1 mRNA levels. B. Relative mRNA levels of PoLOX1. The intensities of the PoLOX1 transcripts were determined by scanning densitometry and were normalized to the stained 28S rRNA bands. Error bars indicate standard error for four independent experiments. The asterisk indicates a significant difference between the cap and the stipe (p < 0.05, Student’s t test).

Table 2. Lipoygenase Activity and 1-Octen-3-ol Contents of the Cap and the Stipe of the Pleurotus ostreatus Strain H1 Fruit Body

<table>
<thead>
<tr>
<th></th>
<th>Cap</th>
<th>Stipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>542.4 ± 52.3</td>
<td>944.4 ± 51.4*</td>
</tr>
<tr>
<td>Specific activity</td>
<td>46.1 ± 3.1</td>
<td>123.4 ± 10.6*</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>2.2 ± 0.2</td>
<td>4.1 ± 0.5*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error for four independent experiments. Asterisks indicate significant differences between the cap and the stipe (p < 0.05, Student’s t test).

Linoleic acid as substrate) and 1-octen-3-ol content were measured in the caps and stipes of P. ostreatus strain H1 fruit bodies. The activity on a fresh-weight basis and the specific activity in the stipe were 1.7- and 2.7-fold higher respectively than those in the cap (Table 2). The 1-octen-3-ol content per mg of protein in the stipe was 1.9-fold higher than that in the cap.

Discussion

In this study, we investigated the deduced amino acid sequence, genomic structure, and expression of the PoLOX1 gene. The deduced amino acid sequence of PoLOX1 shared similarity with LOXs from plants, animals, and bacteria (Fig. 2). PoLOX1 also contained the C-terminal lipoygenase iron-binding catalytic do-
with increases in 1-octen-3-ol content per mg of biomass during mycelial growth. These findings suggest the possibility that the LOX enzyme is associated with 1-octen-3-ol formation, but do not provide evidence that it is involved in 1-octen-3-ol formation. Hence, at present, a significant relationship between LOX expression and 1-octen-3-ol formation remains to be confirmed. Further expression and functional analyses of PoLOX1 are underway to clarify this point.

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References