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Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*

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Abstract

A gene encoding laccase has been isolated from a genomic library of the white-rot basidiomycete *Ceriporiopsis subvermispora* constructed in Lambda GEM-11. This gene (*Cs-lcs1*) contains an open reading frame of 2215 bp, encoding a mature protein of 499 amino acids with a 21-residue signal peptide. The protein sequence exhibits between 63 and 68% identity with laccases from other basidiomycetes and shares with all of them 10 conserved histidines and one cysteine involved in the coordination of copper atoms at the active site of the enzyme. The gene possesses 11 introns, with splicing junctions and internal lariat formation sites adhering to the GT-AG and CTRAY rules, respectively. The upstream region of *Cs-lcs1* contains a TATA box, two CAAT sites, five putative metal response elements and a ACE1 element. In agreement with the presence of the latter element, transcription of *Cs-lcs1* is activated by copper and silver, as shown by Northern blot and reverse transcription followed by DNA amplification analyses. Based on Southern blot analysis, *Cs-lcs1* appears to be the only gene encoding laccase in *C. subvermispora*.

Keywords: Lignin biodegradation; White-rot fungus; Phenol oxidase; Genomic clone; ACE1; Metal response element

1. Introduction

Ceriporiopsis subvermispora is a white-rot basidiomycete that displays high selectivity towards lignin when growing on wood [1]. Enzymes secreted by this fungus that are thought to participate in lignin decay include manganese-dependent peroxidase (MnP) and laccase [2]. The former oxidizes Mn(II) to Mn(III), which then acts as an oxidant of lignin residues. Laccase is a copper-containing oxidase that attacks phenolic compounds by abstraction of one electron [3,4]. Laccase can also oxidize non-phenolic compounds and manganese in the presence of suitable mediators [5,6], a property that strongly supports its involvement in ligninolysis. A distinct role for laccase in lignin breakdown is also suggested by its presence in degraded wood cell walls, as shown by immunocytochemical methods [7]. Moreover, using a laccase-less mutant of the fungus *Pycnoporus cinnabarinus*, it was recently demonstrated that this enzyme is essential for lignin biodegradation [8].

When growing in liquid cultures, *C. subvermispora* produces laccase as a family of isoenzymes with iso-

Abbreviations: Lcs1, laccase enzyme encoded by gene *Cs-lcs1*; MnP, manganese-dependent peroxidase; MRE, metal response element; RT-PCR, reverse transcription followed by DNA amplification by the polymerase chain reaction

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electric points that vary according to the composition of the medium [9]. These isoenzymes exhibit different substrate specificities, whereas N-terminal sequencing suggests that they may be encoded by more than one gene [9]. This phenomenon of isoenzyme multiplicity is commonly observed among ligninolytic fungi, although its physiological significance is not known.

A suitable approach to investigate the role of the different laccase isoenzymes is to study both the structure and transcription regulation of the gene(s) encoding this phenol oxidase [10–12]. Indeed, molecular genetics studies addressed to the elucidation of the genomic organization and expression pattern of the ligninolytic enzymes produced by white-rot fungi are allowing significant progress in this field [13]. Hence, as a first step to reach our objective, in this work we report the cloning, structure and expression in liquid cultures of *Cs-lcs1*, the first laccase gene identified in *C. subvermispora*.

2. Materials and methods

2.1. Genomic DNA library

A genomic DNA library from *C. subvermispora* (strain FP105757, Forest Products Laboratory, Madison, WI) was constructed in Lambda Gem-11, as previously described [14].

2.2. RNA purification

The method for RNA isolation was adapted from Logemann et al. [15]. The fungus was grown for 10 days in YMPG medium [16], as well as in the same medium supplemented with either 25 μM $CuSO_4$, 25 μM $AgNO_3$ or 25 μM $ZnSO_4$. The mycelium was separated from the culture fluid by filtration through Miracloth and immediately frozen in liquid nitrogen. The frozen mycelium was ground to a powder in a mortar containing liquid nitrogen. The powder was then further homogenized in the same mortar by addition of 2 volumes of ice-cold guanidine buffer (8 M guanidine hydrochloride, 50 mM Tris-HCl pH 7.0, 20 mM EDTA and 50 mM mercaptoethanol). This homogenate was extracted with 1 volume of phenol:chloroform and left on ice for 20 min. After centrifugation at 13 000 rpm for 30 min at 4°C, the RNA was precipitated from the supernatant by addition of 1/20 volume of 1 M acetic acid and 0.7 volume of cold absolute ethanol. RNA was pelleted by centrifugation and washed with 3 M sodium acetate pH 4.8. The salt was removed by a final wash with cold 70% ethanol. The RNA pellet was air dried and subsequently dissolved in sterile water. All the solutions and glassware were treated with diethyl pyrocarbonate and sterilized by autoclave.

2.3. Preparation of laccase homologous probes

A probe for screening the genomic library was prepared by PCR amplification using total DNA. Two degenerated oligonucleotide primers were designed considering the codon usage frequency of *C. subvermispora*. The sequence of the forward primer (5′-GAGATYACYGACGCDTTCGTC-3′) was based on the N-terminal sequence EITDAFV previously determined for three laccase isoenzymes from this fungus [9]. The reverse primer (5′-GAAVCGRATRGTGACGTTGTC-3′) corresponds to the amino acid sequence DNVTIRF located approximately 60 amino acids before the C-terminus, which is conserved among all known laccases from different basidiomycetes. PCR conditions were as previously described [17], using 100 pmol of each degenerate primer and an annealing temperature of 52°C. A 1.8-kb PCR product was obtained. Thereafter, it was cloned and partially sequenced, showing similarity with sequences of other laccase genes when aligned and analyzed by pairwise comparison. This fragment was used to screen the genomic library under high stringency hybridization conditions.

A second probe was prepared for Northern blot analysis. Messenger RNA was obtained from total RNA of the fungus by fractionation on a oligo(dT)-cellulose column (Gibco BRL). Then, cDNA was synthesized using the conditions provided in the SuperScript Lambda System (Gibco BRL). For PCR amplification, conditions were the same as those utilized for the genomic probe. Partial sequencing of this probe showed full identity with the expected cDNA sequence corresponding to the *Cs-lcs1* gene.

2.4. Hybridization conditions

The hybridization solution used to screen the genomic library and perform Northern blots under high stringency conditions consisted in 50% formamide, 0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 0.06% Ficoll, 0.06% polyvinylpyrrolidone, 0.06% BSA, 0.1% SDS and 50 μg/ml of denatured salmon sperm DNA. For these experiments, the hybridization temperature was 42°C. For the low stringency conditions employed in Southern blot analysis, the concentration of formamide in the hybridization solution was lowered to 35% and the temperature was 37°C.

2.5. RT-PCR

Reverse transcription reactions were performed in 20 μl incubation mixtures containing 2 μg of total RNA, 10 mM DTT, 1×first strand buffer (Gibco BRL), 200 U of M-MLV reverse transcriptase (Gibco BRL), 0.5 mM (each) deoxynucleotide triphosphates and 50 pmol of a laccase-specific reverse primer (see below). The RNA was first mixed with the primer. Then, the solution was heated at 70°C for 10 min and quickly cooled on ice. After the addition of the other components, reactions were conducted at 42°C for 60 min. 5 μl of each reaction was used for PCR amplification in a 50 μl mixture containing 1 mM $MgCl_2$, 0.2 mM (each) deoxynucleotide triphosphates, 1×PCR buffer (Gibco BRL), 2.5 U of Taq DNA polymerase (Gibco BRL) and 50 pmol each of laccase-specific forward (5′-GGTGACAACTTCCAGATC-3′) and reverse (5′-GAAGCGGTAGCGTTTGCCCT-3′) primers. To avoid possible amplification of laccase fragments from contaminating genomic DNA, the latter was designed from a region interrupted by an intron. To have an internal control of amplification, 20 ng of a MnP full-length cDNA (MnP-2, GenBank accession number AF036254) cloned in pZL1 (Gibco BRL), as well as 25 pmol each of MnP-specific primers were also added to the reaction mixtures. The PCR program consisted of an initial cycle of denaturation (3 min, 94°C), annealing (1 min, 52°C) and prolonged extension (20 min, 72°C), followed by 29 cycles of denaturation (30 s, 94°C), annealing (1 min, 52°C) and extension (2.5 min, 72°C). A final 15 min extension step at 72°C was also included.

3. Results and discussion

3.1. Cloning and characterization of the Cs-lcs1 gene

A genomic library of *C. subvermispora* prepared in Lambda Gem-11 [14] was screened with a laccase homologous probe obtained as indicated above. Six out of 19 phage clones isolated were selected for further screening by PCR with the same primers employed for the preparation of the probe. One of the three positive clones obtained was selected for subcloning and sequencing.

Fig. 1 shows the complete nucleotide and predicted amino acid sequence of *Cs-lcs1* gene (GenBank accession number AF053472). It consists of 2215 bp in the coding region, which includes 11 introns plus information for a mature protein of 499 amino acids with a leader sequence of 21 amino acids. The deduced molecular mass of the mature protein is 53.7 kDa, which is 68% of the experimentally determined molecular mass [9]. This difference can be attributed, at least partially, to glycosylation of the protein, as the deduced sequence presents eight potential residues for *N*-glycosylation. Indeed, upon treatment of laccase with endoglycosidase F, the molecular mass of the protein decreased from 79 to 63 kDa [9]. It is likely that laccase may also undergo *O*-glycosylation, since 14% of the total amino acids correspond to

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Fig. 1. The nucleotide and deduced amino acid sequence of the *Cs-lcs1* gene (GenBank accession number AF053472) from *C. subvermispora*. Standard M13 and internal sequence-derived oligonucleotide primers were used for DNA sequencing with T7 Sequenase version 2.0 (Amersham Life Science) and *fmol* DNA cycle sequencing system (Promega). The underlined 21 amino acid sequence correspond to the signal peptide. TATA and CAAT boxes are shown in bold. A putative ACE1 transcription factor binding site is double underlined. Direct (⊓) and inverted (⊔) metal response elements (MREs) are also shown. Introns are in lower case. The probable polyadenylation site is underlined with a dashed line.

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CTTGTTACCCTGGATAAGAGGCCTCGAGTCACTTCTATGTCATCCTTGTCATCCTTTCCGATCAACCTGGCGCGCCGGTCGGGCCACAACGCTCGTTGCC  100

TCATGTTCAGAGCGTCCTTGAACCAGAACTGAATGCAGCGCGTCAGTCAAGCTTGAATGCCGCTGATACTTCCGCGCGTCTCGAAGACATAACCGCGCCG  200

TGACTGAGCACCTTCGACGGGCCAGGCCTTTCAGAAGAAGCCGAGACTCTCGGCTTGCCGAGCAAGTCAATTGCGCTACCAGGATAGCCTTCGGACCAAT  300

GCGCTGGCGCTTCTCGAAAGAGCATACGAGAAGAGACATATGTGCGGAAAGCGCTCCAGGGTTGGCTGAGAGCGCTCAGACCTCCGGCCCCAAGGCACCG  400

TACGTGCGCGCATTTCGCGCCGGCCGACACACCCCATCATGGTGGGCTCCATTCAATTGCCGCTTTCAGCGCCCACGCTCGCCGGACGTGATGTGCAGGT  500

GCGATGCGCATGCGCCCACACCGTTCCCACCGTGTTTCTGTACGTACTCCGTTGCACAGGCGACAAAACATGAAATCCGACGGTATAAAGGCTCGGGGTC  600

GAGCTGGAGTGATCTGCATCTCGGGTTCACTCCTTTGAGAGCAGTTCACTCACAGCTCTCGCATTCCTCTCGAGACACCTATCTTTGAATATGGCCCGTT  700
                                                                                M A R                  3

TCCAGACGCTCTCCGCCGCCGTCGCCCTCGGCTTGTCCCTGGGCGCGTTCGCCGCCATTGGGCCTGTTACCGACCTCGAAATCACCGACGCGTTTGTCAG  800
F Q T L S A A V A L G L S L G A F A A I G P V T D L E I T D A F V S  37

TCCCGATGGTCCTGGTCTCGGGCGAGAGGCTGTTCTCGCTGGTGGTACTTTCCCCGGACCTCTTATCCAGGGTAACAAGgtaacgctcctcaacatatac  900
  P D G P G L G R E A V L A G G T F P G P L I Q G N K                                                  63

gcaattacgatgacgatagcttactgttatgcatgaagGGTGACAACTTCCAGATCAATGTCGTCAACAACTTGACGAACCACACTATGCTGAAGACTAC  1000
                                        G D N F Q I N V V N N L T N H T M L K T T                      84

GTCTATTgtgcgtctacaaaccaaaccactgaatacggggttagggcttacctacgtcatgctcttcggctgacgtctatctaatctccggttgcggatc  1100
  S I                                                                                                  86

tagCACTGGCATGGTTTGTTCCAACACGGGACAACGTGGGCGGACGGTCCGGCCTTCGTAAGCCAGTGCCCGATTGCGTCTGGGAACTCTTTCCTGTACA  1200
    H W H G L F Q H G T T W A D G P A F V S Q C P I A S G N S F L Y                                      118

ATTTCAATGTTCCAGACCAGGCCGgtgagtccgccgaataagattggtgccttccgagtattttcggagtaactgatatggtggccacgtacagGCACTT  1300
N F N V P D Q A                                                                          G T           128

TCTGGTACCACAGTCACCTCGCAACTCAGTACTGCGATGGTCTCCGTGGACCTCTGGTTGTTTACGATCCCAATGACCCTCATGCGGATTTGTACGATGT  1400
F W Y H S H L A T Q Y C D G L R G P L V V Y D P N D P H A D L Y D V                                      162

AGATGATGgtcagttcaacttcggtccctggcgcgaacgtggtcaatgacgattttacctagAGAGCACCGTCATCACTCTGTCTGACTGGTACCACGCG  1500
  D D                                                                E S T V I T L S D W Y H A          177

GCGGCGTCGACTCTGACATTCCCGCGAgttctggtcctcttagacaaatgcgtgatcacgtgtcactgattctggttccggacattagACGTTCGATACC  1600
 A A S T L T F P R                                                                       T F D T           190

ACTCTCATCAATGGTCTCGGTCGCTTCGCTGGTACTGGTGGTTCTGACTCGAACCTTACTGTGATCACCGTCGAGCAGGGCAAACGgtatgtttacagat  1700
 T L I N G L G R F A G T G G S D S N L T V I T V E Q G K R                                              219

gtaatcagtgttgatgatactgaagttgaacacagCTACCGCTTCCGTCTTGTCTCAATTTCTTGCGATCCGAACTGGGTCTTCTCCATCGATCAGCACG  1800
                                    Y R F R L V S I S C D P N W V F S I D Q H                          240

AGCTGACTGTTATCGAGGTTGATGGGGTTAATGCTGTGCCCCTCACCGTCGATGCGATCCAGATTTTCGCAGCTCAGCGCTACTCGTTTGTGgtacgttc  1900
E L T V I E V D G V N A V P L T V D A I Q I F A A Q R Y S F V                                          271

taccggtgctcctctgcatgaggtttgttctcatgttggtttgcagCTTAACGCTAACCAAACTGTCGACAACTACTGGATTCGCGCTAATCCCAATAAT  2000
                                                L N A N Q T V D N Y W I R A N P N N                    289

GGTAACATGGGCTTCGCAAATGGAATCAACTCAGCCATCTTGCGCTACGTTGGTGCCGATGATGTAGAGCCGACCTCCACCGGCACGACTGCGAACTTAC  2100
 G N M G F A N G I N S A I L R Y V G A D D V E P T S T G T T A N L                                     322

TGAACGAGGCTGACTTGAGCCCCCTGTCCTCTGCGGCTGTAgtactgattggcgcatacgaacgatgctgaaactaatcacaaattttcagCCGGGTGCT  2200
L N E A D L S P L S S A A V                                                                P G A      339

CCCAACCAGGACTTTGACGCTGTCGATGTTCCTATGAACCTGAACTTTACCTTCgtatgtccatccatatattcacagtccagcgggtcttaacgtctat  2300
 P N Q D F D A V D V P M N L N F T F                                                                    357

cgcagAACGGCACGAACCTCTTCATCAACGGTGCCACCTTCGTCCCGCCCTCCGTCCCCGTGCTGACGCAGATCCTGAGTGGTGCGATGACCGCTCAGGA  2400
      N G T N L F I N G A T F V P P S V P V L T Q I L S G A M T A Q E                                  389

GCTACTTCCCGCGGGTAGCGTGTACACCCTCCCGCGCAACGCGACGGTGCAGCTCTCGTTGCCGGGTAACATCATCGCTGGCCCTCACCCCTTCCACTTG  2500
  L L P A G S V Y T L P R N A T V Q L S L P G N I I A G P H P F H L                                     422

CACGGCCACACGTTCAGTGTCATCCGTTCCGCCGGCCAATCCGACTACAACTACGTCGACCCCATCCAGCGTGACGTTGTTAGCATTGGTGGTGCTACCG  2600
 H G H T F S V I R S A G Q S D Y N Y V D P I Q R D V V S I G G A T                                      455

ATAACGTCACCATCCGCTTCACAgtgagttgctccctacgtcgtcaaaagtgtgcgatgctgaatgctggctggcgtgcagACCGATAACCCCGGCCCGT  2700
D N V T I R F T                                                                          T D N P G P    469

GGTTCTTCCACTGCCACATCGACTGGCACTTGCAGGCCGGTTTCGCCATCGTCTTCGCTGAGGAGACCGCAGATGTCGCTTCCGCCAACCCTGTCCCTGg  2800
W F F H C H I D W H L Q A G F A I V F A E E T A D V A S A N P V P                                      502

taagtttaccaatgtgccaatttcagcgcgaagctgacaatccatgcagCGGATTGGAGTGCCTTGTGCCCGACCTACGATGCCCTTTCCGACGCGGATC  2900
                                                A D W S A L C P T Y D A L S D A D                      519

ACTAAGTTGGTTTATCGACACAAGTAACTGGGTCTTTACTGGGTTTGGTATTAGACATTTGTCACGGACTTCGACATTGTAGTATGAATGTGAATTGTGG  3000
H -                                                                                                    520

TGCCTCAATGGCTGCGAATATAATGGATG
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C.s. LCS1     MARFQTLSAAVALGLSL--GAFAAIGPVTDLEITDAFVSPDGPGLGREAVLAGG---------TFPGPL  58
C.h. PO       MSRFQSLLAFVVASLAA--VAHAAIGPTADLTISNAEVSPDG--FARQAVVVNN---------VTPGPL  56
T.vill.LCC1   MSRFHSLLAFVVASLTA--VAHAGIGPVADLTITNAAVSPDG--FSRQAVVVNG---------GTPGPL  56
PM1 LAC1      MAKFQSLLTFITLSLVA--SVYASIGPVADLTISNGAVSPDG--FSRQAILVND---------VFPSPL  56
I-62 LCC1     MAKLQFSNFFVTLAVVT--GALAAVG-EADLTITNAVVAPDG--FSRDAVVVNG---------VFPGPL  55
P.r. LAC      MHTFLRSTALVVAGLSA--RALASIGPVTDFHIVNAAVSPDG--FSRQAVLAEG---------VFPGPL  56
T.vers.LCC1   MGRFSSLCALTAVIHSFG-RVSAAIGPVTDLTISNADVSPDG--FTRAAVLANG---------VFPGPL  57
P.o. POX1     MFPGARILATLTLALHLLHGTHAAIGPTGDMYIVNEDVSPDG--FTRSAVVARSDPTTNGTSETLTGVL  67
              *           .      . *.:*   *: * :  *:***  : * *::. .            .. *

C.s. LCS1     IQGNKGDNFQINVVNNLTNHTMLKTTSI H W H GLFQHGTTWADGPAFVSQCPIASGNSFLYNFNVP 123
C.h. PO       VAGNKGDRFQLNVIDNLTNHTMLKSTSI H W H GFFQKGTNWADGPAFVNQCPISSGHSFLYDFQVP 121
T.vill.LCC1   ITGNMGDRFQLNVIDNLTNHTMVKSTSI H W H GFFQKGTNWADGPAFINQCPISSGHSFLYDFQVP 121
PM1 LAC1      ITGNKGDRFQLNVIDNMTNHTMLKSTSI H W H GFFQHGTNWADGPAFVNQCPISTGHAFLYDFQVP 121
I-62 LCC1     ITGKKGDRFQLNVIDNLTNHTMLKSTSI H W H GFFQAGTNWADGPAFVNQCPISTGHAFLYDFHVP 120
P.r. LAC      IAGNKGDNFQINVIDELTNATMLKTTTI H W H GFFQHGTNWADGPAFINQCPIASGDSFLYNFQVP 121
T.vers.LCC1   ITGNKGDNFQINVIDNLSNETMLKSTSI H W H GFFQKGTNWADGAAFVNQCPIATGNSFLYDFTAT 122
P.o. POX1     VQGNKGDNFQLNVLNQLSDTTMLKTTSI H W H GFFQSGSTWADGPAFVNQCPIASGNSFLYDFNVP 132
              : *: **.**:**:::::: **:*:*:* * * * *:** *:.****.**:.****::*.:***:* ..

C.s. LCS1     DQAGTFWY H S H LATQYCDGLRGPLVVYDPNDPHADLYDVDDESTVITLSDWYHAAAST-LTFPRT 187
C.h. PO       DQAGTFWY H S H LSTQYCDGLRGPFVVYDPNDPHASLYDVDNDDTVITLADWYHTAAKLGPAFPLG 186
T.vill.LCC1   DQAGTFWY H S H LSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLVDWYHVAAKLGPAFPLG 186
PM1 LAC1      DQAGTFWY H S H LSTQYCDGLRGPIVVYDPQDPHKSLYDVDDDSTVITLADWYHLAAKVGPAVPT- 185
I-62 LCC1     DQAGTFWY H S H LSTQYCDGLRGPIVVYDPLDPHAFRYDVDDESTVITLSDWYHTAATLGLGSRLG 185
P.r. LAC      DQAGTFWY H S H LSTQYCDGLRGPFVVYDPADPYLDQYDVDDDSTVITLADWYHTAARLGSPFP-A 185
T.vers.LCC1   DQAGTFWY H S H LSTQYCDGLRGPMVVYDPSDPHADLYDVDDETTIITLSDWYHTAASLGAAFPIG 187
P.o. POX1     DQAGTFWY H S H LSTQYCDGLRGPFIVYDPSDPHLSLYDVDNADTIITLEDWYHVVAPQNAVLP-T 196
              ******** * * * *:**********::**** **    ****:  *:*** **** .*

C.s. LCS1     FDTTLINGLGRFAGTGGSDSNLTVITVEQGKRYRFRLVSISCDPNWVFSIDQHELTVIEVDGVNAVPLT 256
C.h. PO       ADATLINGLGRSPST--TAADLAVINVTKGKRYRFRLVSLSCDPNHTFSIDGHDLTIIEVDSINSQPLV 253
T.vill.LCC1   ADATLINGKGRSPST--TTADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHNMTIIETDSINTAPLV 253
PM1 LAC1      ADATLINGLGRSINT--LNADLAVITVTKGKRYRFRLVSLSCDPNHTFSIDGHSLTVIEADSVNLKPQT 252
I-62 LCC1     ADATLINGLGRSSST--PTANVTVINVQHGKRYRFRLVSLSCDPNHTFSIDGHNLTVIEVDGVNSKPLT 252
P.r. LAC      ADTTLINGLGRCGEAGCPVSDLAVISVTKGKRYRFRLVSISCDSFFTFSIDGHSLNVIEVDATNHQPLT 254
T.vers.LCC1   SDSTLINGLGRFAGG--DSTDLAVITVEQGKRYRMRLLSLSCDPNYVFSIDGHNMTIIEADAVNHEPLT 254
P.o. POX1     ADSTLINGKGRFAGG--PTSALAVINVESNKRYRFRLISMSCDPNFTFSIDGHSLQVIEADAVNIVPIV 263
               *:***** **         : ::**.*  .****:**:*:***.  .**** *.: :**.*. *  * .

C.s. LCS1     VDAIQIFAAQRYSFVLNANQTVDNYWIRANPNNG-NMGFANGINSAILRYVGADDVEPTSTGTTAN-LL 323
C.h. PO       VDSIQIFAAQRYSFVLNADQDVGNYWIRANPNFG-NVGFAGGINSAILRYDGADPVEPTTTQTTPTKPL 321
T.vill.LCC1   VDSIQIFAAQRYSFVLEANQAVDNYWIRANPNFG-NVGFTGGINSAILRYDGAAAVEPTTTQTTSTAPL 321
PM1 LAC1      VDSIQIFAAQRYSFVLNADQDVDNYWIRALPNSG-TRNFDGGVNSAILRYDGAAPVEPTTTQTPSTQPL 320
I-62 LCC1     VDSIQIFAAQRYSFVLNANQTVGNYWIRANPNFG-TTGFAGGINSAILRYQGAPIIEPTTVQTTSVIPL 320
P.r. LAC      VDELTIYAGQRYSFILTADQDVDNYWIRANPGIGITTGFAGGINSAILRYDGADVVEPTTTQATSPVVL 323
T.vers.LCC1   VDSIQIYAGQRYSFVLTADQDIDNYFIRALPSAG-TTSFDGGINSAILRYSGASEVDPTTTETTSVLPL 322
P.o. POX1     VDSIQIFAGQRYSFVLNANQTVDNYWIRADPNLG-STGFDGGINSAILRYAGATEDDPTTTSSTST-PL 330
              ** : *:*.*****:* *:* :.**:*** *. * . .* .*:******* **    :**:. :..   *

C.s. LCS1     NEADLSPLSSAAVPGAPNQDFDAVDVPMNLNFTFNGTNL-F-INGATFVPPSVPVLTQILSGAMTAQEL 388
C.h. PO       NEVDLHPLATMAVPGSP--VAGGVDTAINMAFNFNGTNF-F-INGASFVPPTVPVLLQIISGAQNAQDL 386
T.vill.LCC1   NEVNLHPLVTTAVPGSP--VAGGVDLAINMAFNFNGTNF-F-INGTSFTPPTVPVLLQIISGAQNAQDL 386
PM1 LAC1      VESALTTLEGTAAPGNP--TPGGVDLALNMAFGFAGGRF-T-INGASFTPPTVPVLLQILSGAQSAQDL 385
I-62 LCC1     VETNLHPLVPTIVPGLP--VSGGVDKAINLGFNFNGTNF-F-INNATFTPPTVPVLLQILSGASTAQDL 385
P.r. LAC      SESNLAPLTNAAAPGLP--EVGGVDLALNFNLTFDGPSLKFQINGVTFVPPTVPVLLQILSGAQSAADL 390
T.vers.LCC1   DEANLVPLDSPAAPGDP--NIGGVDYALNLDFNFDGTNF-F-INDVSFVSPTVPVLLQILSGTTSAADL 387
P.o. POX1     EETNLVPLENPGAPGPA--VPGGADININLAMAFDVTNFELTINGSPFKAPTAPVLLQILSGATTAASL 397
               * * .*    .** .   ...* :*: : *    :   **. .* .*:.*** **:**: .* .*
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Fig. 2.

either T or S. Surprisingly, there is a L residue at position 8 of the mature Lcs1 protein instead of the expected I residue found by N-terminal sequencing in three laccase isoenzymes from *C. subvermispora* [9], suggesting that Lcs1 corresponds to a fourth isoenzyme.

The protein sequence exhibits 63–68% identity with those of laccases from other basidiomycetes

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C.s. LCS1     LPAGSVYTLPRNATVQLSLPGNIIAG---P H PF H L H GHTFSVIRSAGQSDYNYVDPIQRDVV 449
C.h. PO       LPSGSVYSLPSNADIEISFPATAAAPG-AP H PF H L H GHAFAVVRSAGSTVYNYDNPIFRDVV 447
T.vill.LCC1   LPSGSVYSLPSNADIEISFPATAAAPG-AP H PF H L H GHAFAVVRSAGSTVYNYDNPIFRDVV 447
PM1 LAC1      LPSGSVYSLPANADIEISLPATSAAPG-FP H PF H L H GHTFAVVRSAGSSTYNYANPVYRDVV 446
I-62 LCC1     LPPGSVYPLPAHSSIEITLPATTLAPG-AP H PF H L H GHVFAVVRSAGSTAYNYVDPIFRDVV 446
P.r. LAC      LPSGSVYALPSNATIELSLPAGALGG---P H PF H L H GHTFSVVRPAGSTTYNYVNPVQRDVV 449
T.vers.LCC1   LPSGSLFALPSNSTIEISFPITATNAPGAP H PF H L H GHTFSIVRTAGSTDTNFVNPVRRDVV 449
P.o. POX1     LPSGSIYSLEANKVVEISIPALAVGG---P H PF H L H GHTFDVIRSAGSTTYNFDTPARRDVV 456
              **.**::.*  :  :::::*          * * ** * * * **.* ::*.**.:  *:  *  **** 

C.s. LCS1     SIGGA--TDNVTIRFTTDNPGPWFF HCH IDW H LQAGFAIVFAEETADVASANPVPAD------- 504
C.h. PO       STGTPAAGDNVTIRFRTDNPGPWFL HCH IDF H LEAGFAVVFAEDIPDVASANPVPQA------- 504
T.vill.LCC1   STGTPAAGDNVTIRFRTDNPGPWFL HCH IDF H LEAGFAVVFAEDIPDVASANPVPQA------- 504
PM1 LAC1      STGSP--GDNVTIRFRTDNPGPWFL HCH IDF H LEAGFAVVMAEDIPDVAATNPVPQA------- 501
I-62 LCC1     STGTPAAGDNVTIRFHTDNPGPWFL HCH IDF H LEAGFAIVFAEDVADVKAANPVPKA------- 503
P.r. LAC      SIGNT--GDNVTIRFDTNNPGPWFL HCH IDW H LEAALPLSSLRTSLTLRPLTLSPRTGPTCALS 511
T.vers.LCC1   NTGTA--GDNVTIRFTTDNPGPWFL HCH IDF H LEAGFAIVFSEDTADVSNTTTPSTA------- 504
P.o. POX1     NTGTD-ANDNVTIRFVTDNPGPWFL HCH IDW H LEIGLAVVFAED---VTSITAPPAA------- 509
              . *     ******* *:******: *** **: * *: .:.:   .   :   .  .

C.s. LCS1     ---WSALCPT-YDALSD---------ADH-------- 520
C.h. PO       ---WSDLCPI-YDALDV----------------NDQ 520
T.vill.LCC1   ---WSDLCPT-YDALDP----------------SDQ 520
PM1 LAC1      ---WSDLCPT-YDALSP----------------DDQ 517
I-62 LCC1     ---WSDLCPT-YDALAEGDL---------------- 519
P.r. LAC      TTLWTHLITSGFASIIQWMMGGNGLFAPHALSFLGSQ 548
T.vers.LCC1   ---WEDLCPT-YNALDSSDL---------------- 520
P.o. POX1     ---WDDLCPI-YDALSDSDKGGIA------------ 529
                 *  * .  : ::
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Fig. 2. Alignment of Lcs1 amino acid sequence with other known basidiomycete laccase amino acid sequences. Sequences were aligned using the CLUSTAL X algorithm. Conserved histidinyl and cysteinyl residues are boxed. The asterisks and dots denote identical and conserved residues, respectively, whereas colons indicate partial identity. Numbers refer to the amino acid sequence. C.s., *C. subvermispora*; C.h., *Coriolus hirsutus* [18]; T.vill., *Trametes villosa* [12]; PM1, basidiomycete PM1 [20]; I-62, basidiomycete CECT 20197 [23]; P.r., *Phlebia radiata* [19]; T.vers., *Trametes versicolor* [22]; P.o., *Pleurotus ostreatus* [21].

[12,18–23]. Totally conserved among all laccases are the 10 histidines and the cysteine residue required to coordinate the four copper atoms at the active site of the enzyme [20] (Fig. 2). Laccases also have an additional residue involved in the coordination of type 1 copper atoms, located 10 residues downstream of the aforementioned cysteine. This residue, which appears to have a role in determining the redox potential of the enzyme [24], can be methionine, leucine or phenylalanine, giving rise to the categorization of laccases into classes 1, 2 and 3, respectively [25]. Based on this criterion, Lcs1 from *C. subvermispora* is a class 3 enzyme, the one possessing the highest redox potential of the three [24].

In the upstream region of the *Cs-lcs1* gene, there is a putative ACE1 transcription factor binding site centered at position −532 (see Fig. 1). ACE1 activates the transcription of the *Saccharomyces cerevisiae* copper, zinc superoxide dismutase [26] and metallothionein [27] genes in response to copper. The alignment between the ACE1 binding sites of *Cs-lcs1* promoter, *S. cerevisiae* copper, zinc superoxide dismutase and metallothionein genes and the basidiomycete PM1 laccase gene [20] shown in Fig. 3 reveals significant homology among them. Also in the upstream region of *Cs-lcs1* there are five putative metal response elements (MREs) adhering to the consensus sequence TGCRCNC [28]. Three of them are present in a direct orientation with respect to the direction of transcription and are centered at positions −176, −282 and −285, whereas the other two elements are in inverted sense and centered at positions −281 and −514. Interestingly, three of these elements are clustered and overlap in a short segment

```
Cs-lcs1   CAGTCAAGC-----TTGAATGCCGCTGATAC--------TTCC
SOD1      CAGT-AAGCGGCATTTGC-----GCTGTCATCG--------CCTT
CUP1      GAGCGATGCGTCTTTT-----CCGCTGAACCG--------TTCC
lac1      CAGTCA--------TTGT-----GCTGTGTAACGATGGACGCCTT
```

Fig. 3. Alignment of the putative ACE1 transcription factor binding site present in *Cs-lcs1* gene with ACE1 sites from other fungal genes. Conserved bases are in bold. *Cs-lcs1*, *C. subvermispora* laccase; *SOD1*, *S. cerevisiae* copper, zinc superoxide dismutase [26]; *CUP1*, *S. cerevisiae* metallothionein [27]; *lac1*, basidiomycete PM1 laccase [20].

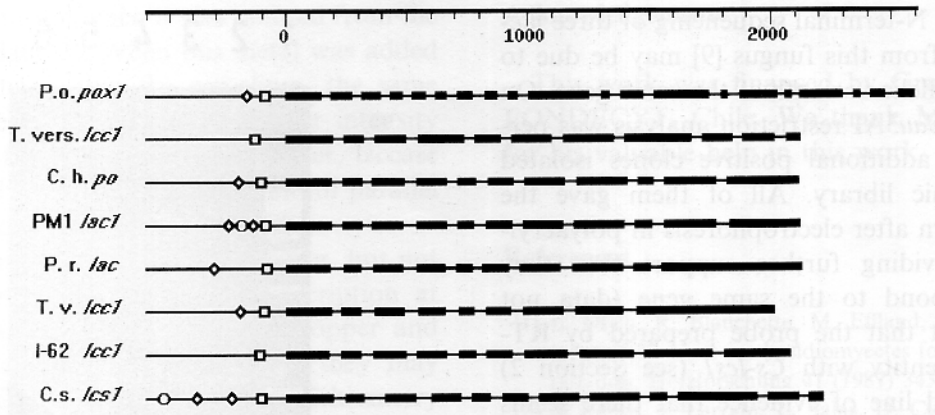

Fig. 4. Comparison of the intron/exon structure and promoter elements between *Cs-lcs1* from *C. subvermispora* and laccase genes from other basidiomycetes. Exons are indicated by solid lines, whereas the thick lines correspond to introns. The relative location of the promoter elements TATA box (□), CAAT box (◇) and ACE1 binding site (○) is indicated. C.s., *C. subvermispora*; C.h., *Coriolus hirsutus* [18]; T.v., *Trametes villosa* [12]; PM1, basidiomycete PM1 [20]; I-62, basidiomycete CECT 20197 [23]; P.r., *Phlebia radiata* [19]; T.vers., *Trametes versicolor* [22]; P.o., *Pleurotus ostreatus* [21].

of 11 bp (−278 to −288), whereas another one is adjacent to the ACE1 element (−514) (Fig. 1). The promoter region of *Cs-lcs1* also presents a TATA box and two CAAT boxes (Figs. 1 and 4).

At the 3′ end, an apparent polyadenylation site is found 112 bp after the termination codon. This sequence AATATA differs in one base from the consensus AATAAA [29], which is in agreement with other authors that have reported polyadenylation signals in filamentous fungi and yeast that do not match the consensus exactly [30–32].

As mentioned above, the gene possesses 11 short introns, with splicing junctions and internal lariat formation sites adhering to the GT-AG rule and the consensus sequence CTRAY, respectively [33]. As observed in Fig. 4, the intron-exon structure of several laccase genes from basidiomycetes is highly conserved, some of them being almost identical. The exception is *Pleurotus ostreatus pox1* gene, which has 19 introns instead of the average 10 present in other fungi.

3.2. Southern analysis of genomic DNA

Southern blot analysis was used as an approach to estimate the number of laccase genes in the *C. subvermispora* genome. A single band was detected with each restriction enzyme used to digest the genomic DNA (Fig. 5), none of which cleaves the *Cs-lcs1* gene. The use of low-stringency hybridization conditions practically ensures that the probe will recognize any laccase gene present, regardless of its homology. The fact that a single band was obtained in each case suggests that *C. subvermispora* possesses only one laccase gene. If this was the case, the minor differ-

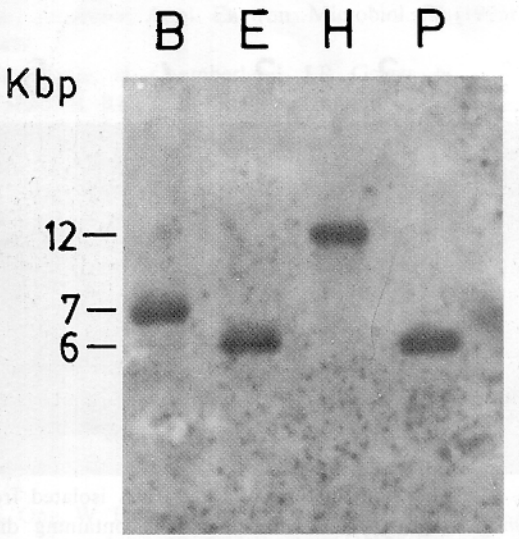

Fig. 5. Southern blot of *C. subvermispora* genomic DNA. 10 μg of total DNA from *C. subvermispora* were digested with *Bam*HI (lane B), *Eco*RI (lane E), *Hin*dIII (lane H) and *Pst*I (lane P). The resultant DNA fragments were resolved by electrophoresis in a 0.8% agarose gel and blotted onto a nylon membrane. The blot was probed under low-stringency hybridization conditions with a 1800-bp laccase genomic fragment amplified by PCR (section 2.3) and labeled with biotin using the BioNick Labeling System (Gibco BRL). The approximate sizes of the bands are indicated on the left.

ences detected by N-terminal sequencing of three laccase isoenzymes from this fungus [9] may be due to the presence of allelic variants of the same gene. On the other hand, *Sau*3AI restriction analysis was performed with 12 additional positive clones isolated from the genomic library. All of them gave the same band pattern after electrophoresis in polyacrylamide gels, providing further support that they probably correspond to the same gene (data not shown). The fact that the probe prepared by RT-PCR has full identity with *Cs-lcs1* (see Section 2) represents a third line of evidence that there seems to be only one gene encoding laccase in *C. subvermispora*.

3.3. Expression of laccase in cultures of *C. subvermispora*

We have reported that the presence of copper in the growth medium of *C. subvermispora* is essential for the detection of extracellular laccase activity [9]. Since copper is present at the active site of the enzyme, it is conceivable that the apoenzyme may be produced in an inactive form in the absence of copper, as shown by Huber and Lerch [34] in the case of laccase from *Neurospora crassa*. Another possibility is that copper may exert its action activating gene transcription, as described for the *S. cerevisiae* copper, zinc superoxide dismutase [26] and metallothionein [27] genes, as well as for a *Trametes versicolor* laccase gene [35]. The finding of a putative ACE1 binding site in the upstream region of the *Cs-lcs1* gene from *C. subvermispora* suggested initially that copper acts at the level of gene transcription.

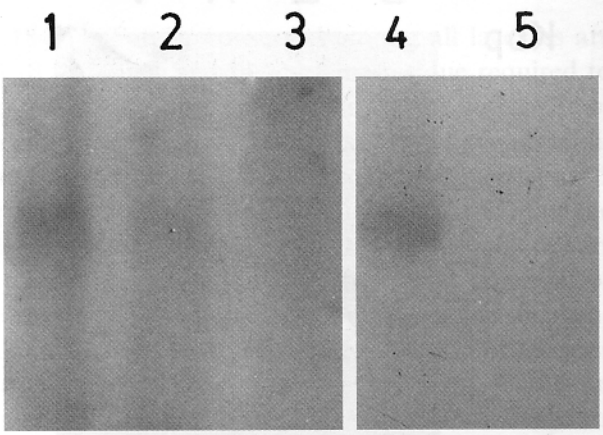

Fig. 6. Northern blot analysis of total RNA isolated from *C. subvermispora* grown in YMPG medium containing different metals as possible inducers of *Cs-lcs1* transcription. YMPG medium was supplemented with: 25 μM $CuSO_4$ (lane 1); 25 μM $CuSO_4$ added 1 day before harvesting the mycelium (lane 2); not supplemented (lane 3); 25 μM $AgNO_3$ (lane 4); 25 μM $ZnSO_4$ (lane 5). For this experiment, 30 μg of total RNA was electrophoresed in 1% agarose-2.2 M formaldehide gels at 100 V. After transfer to a Hybond N nylon membrane (Amersham Life Science), the blots were hybridized under high stringency conditions using a laccase cDNA fragment amplified by PCR as a probe (see Section 2.3).

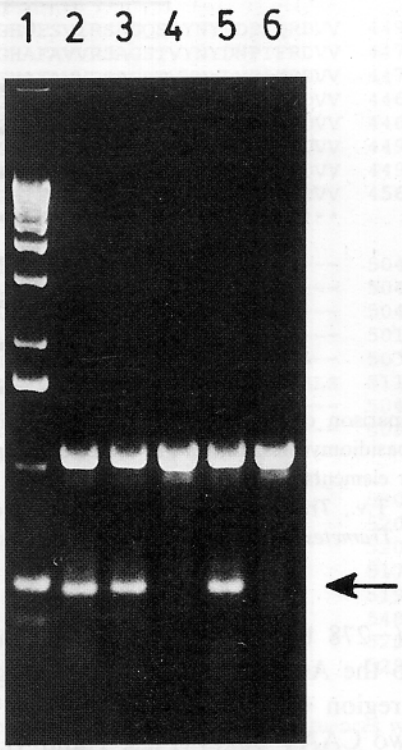

Fig. 7. RT-PCR analysis of total RNA isolated from *C. subvermispora* grown in YMPG medium supplemented as follows: 25 μM $CuSO_4$ (lane 2); 25 μM $CuSO_4$ added 1 day before harvesting the mycelium (lane 3); not supplemented (lane 4); 25 μM $AgNO_3$ (lane 5) and 25 μM $ZnSO_4$ (lane 6). Lane 1 contains a 1-kb DNA ladder (Gibco BRL) as size marker. RT-PCR products were electrophoresed in a 1% agarose gel and stained with ethidium bromide. The shorter fragment (shown by the arrow) corresponds to the amplified laccase cDNA, whereas the larger one corresponds to manganese peroxidase cDNA MnP-2 used as control (see Section 2.5).

Northern blot analysis was carried out with total RNA isolated from mycelium grown during 10 days in YMPG medium [16], either in the presence or in the absence of 25 μM $CuSO_4$. A single transcript of about 1.9 kb was detected when copper was present

(Fig. 6, lane 1), but not when it was omitted from the medium (Fig. 6, lane 3). When this metal was added one day before harvesting the mycelium, the same band was detected, although with a lower intensity (Fig. 6, lane 2). In the latter case, however, laccase activity measured in the extracellular fluid of parallel cultures became detectable only after 48 h.

On the other hand, in *S. cerevisiae*, silver, but not zinc, induces metallothionein gene transcription at similar levels than copper [27]. Having copper and silver a similar electronic configuration, they may bind to yeast ACE1 with comparable stoichiometry and coordination properties [36]. To investigate if the laccase gene from *C. subvermispora* responds to silver and zinc, Northern blot analyses were carried out with RNA isolated from the fungus grown in medium containing either 25 μM $AgNO_3$ or 25 μM $ZnSO_4$ instead of $CuSO_4$. A band with similar size and intensity as that observed with the copper-containing medium was detected with silver (Fig. 6, lane 4) but not with zinc (Fig. 6, lane 5) present in the cultures. However, laccase activity was not detected at any time in the extracellular fluid of cultures containing silver (data not shown).

To confirm the results obtained by Northern blot analysis, laccase mRNA was detected by RT-PCR. As shown in Fig. 7, laccase transcripts are produced in the fungus grown in medium containing copper or silver, whereas these were undetectable in cultures lacking copper or containing zinc. In this experiment, a MnP full-length cDNA with its corresponding primers was used as an internal control of the DNA amplification reaction (see Section 2).

These results are in agreement with the finding of a putative ACE1 binding site in the upstream region of *Cs-lcs1*, suggesting that an 'ACE1-like' transcription factor could be participating in the induction of this gene in *C. subvermispora*. Surprisingly, transcription of the laccase gene from *T. versicolor* is also activated by copper, although it lacks an apparent ACE1 transcription factor binding site [35]. On the other hand, although basidiomycete PM1 laccase gene possesses a putative ACE1 binding site [20], laccase levels in basal medium are very low even in the presence of copper [37], suggesting that other factors may contribute to activate expression of laccase in lignino-lytic fungi.

Acknowledgements

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