Tandem organization and highly disparate expression of the two laccase genes \textit{lcc1} and \textit{lcc2} in the cultivated mushroom \textit{Agaricus bisporus}

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Two non-allelic laccase genes (\textit{lcc1} and \textit{lcc2}) in \textit{Agaricus bisporus} have been mapped to the same cosmid clone and are close together, in tandem. The intergenic region consists of 1562 bp between the stop codon of \textit{lcc1} and the start codon of \textit{lcc2}. Differences between the 5' non-coding regions of the two genes suggest the potential for their differential regulation. By employing competitive RT-PCR and specific primer pairs that discriminate between \textit{lcc1} and \textit{lcc2}, it has been shown that the level of \textit{lcc2} mRNA is approximately 300 times higher than that of \textit{lcc1} mRNA in malt extract liquid cultures; in compost cultures \textit{lcc2} mRNA is almost 7000 times more abundant than \textit{lcc1} mRNA.

**Keywords**: \textit{Agaricus bisporus}, laccase genes, differential regulation, polyphenol oxidase

INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a member of the blue multi-copper family of enzymes and is produced abundantly by \textit{Agaricus bisporus}, constituting 2% of mycelial protein during vegetative growth (Wood, 1980a). The enzyme is secreted into the medium and is a glycoprotein of 65 kDa apparent molecular mass, that exists as a dimer (Wood, 1980a; Perry et al., 1993a). Laccases catalyse the one-electron oxidation of a wide range of organic substrates, typically including mono-, di-, and polyphenols, aromatic amines, methoxyphenols and ascorbate, coupled to the four-electron reduction of dioxygen to water. The occurrence of laccase is widespread amongst fungi and higher plants and the enzyme appears to have different functions in different organisms. In plant laccase has a role in the lignification of differentiating xylem tissues (Wood, 1980a). During recent years fungal laccase gene and/or cDNA sequences have been reported from a number of sources, the first of which was the ascomycete \textit{Neurospora crassa}.

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\[ \text{Laccase} \quad \text{(benzenediol:oxygen oxidoreductase, EC 1.10.3.2)} \]

The intergenic sequence between \textit{lcc1} and \textit{lcc2} has been submitted to GenBank as an amendment to the genomic sequence of the laccase gene \textit{lcc1} (accession number L10664).

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intervals. Cultures were grown in (2%, w/v) malt extract proportion to biomass in laboratory liquid cultures malt extract agar at 25 °C and subcultured at monthly different members within them. Thus, whilst some basis for understanding the functions constitutive (no sequences for modulation of transcription much to be found out about the regulation of laccase and its structure are developing, there is still difficult to measure although laccase activity is present in a constant situation is likely to occur in the large blue copper family, the plant ascorbate oxidase enzymes that differs between organisms. Some fungal metabolism of the fungus remains unknown. The latter is a natural inducer, has yet to be given a plausible explanation. Many fungal laccases are apparently con- structive, but whether they are mechanistically consti- tutive (no sequences for modulation of transcription present) or being induced by an unidentified product of metabolism of the fungus remains unknown. The latter situation is likely to occur in A. bisporus because although laccase activity is present in a constant proportion to biomass in laboratory liquid cultures (Wood, 1980a), during the fruiting cycle in compost culture, laccase activity shows dramatic changes (Wood, 1980b).

Thus, whilst some basis for understanding the functions of laccase and its structure are developing, there is still much to be found out about the regulation of laccase synthesis, the organization of the members of gene families (where they are present) and the functions of the different members within them.

METHODS

Organism. Agaricus bisporus strain D649 was maintained on malt extract agar at 25 °C and subcultured at monthly intervals. Cultures were grown in (2%, w/v) malt extract (ME) liquid medium (Boots Co.) as described previously (Perry et al., 1993a).

Harvesting and storage of A. bisporus mycelium for total RNA isolation. For RNA analysis, a series of 250 ml conical flasks containing 50 ml ME medium were each inoculated with 1 ml homogenized mycelial suspension from a 10-d-old ME starter-culture. Mycelium was harvested from up to ten individual flasks by filtering off onto a fine nylon mesh. The mycelium was washed with 500 ml deionized water, 'squeezed' dry and cut into 5 x 5 x 5 mm pellets. The pellets were immediately frozen in liquid nitrogen and stored at −70 °C. A. bisporus mycelium was also grown axenically in commercial mushroom compost to the colonization stage and stored at −70 °C until required for RNA extraction.

Total RNA isolation. The frozen mycelial pellets, or approximately 4 g colonized compost, were blended to a fine powder by 2–3 min treatment in an electric coffee bean grinder (Braun) in the presence of approximately 2 vols solid CO₂ pellets. The mycelial powder and residual solid CO₂ were transferred to 50 ml polythene tubes (Falcon). After allowing the residual CO₂ to sublime, but before the mycelial fragments thawed, RNA was extracted using triisopropylsilphonylphosphate sulphonate and phenol/creosol, followed by ethanol precipitation as described previously (Leonard et al., 1981).

Removal of humic acids from compost RNA. Samples (100 μl) of total RNA solution were centrifuged through 5 ml sterile syringe barrels packed with 1.0 cm plastic wool and 1.5 cm Sephadex G-200 (Pharmacia) as described by Tsai & Olson (1992).

DNA isolation. DNA from the cosmid clone LA16E2 (Sodhi, 1992) was isolated using Wizard Maxi-prep kits (Promega) for sequencing and as competitor in RT-PCR.

Sequence analysis. Sequencing by Sanger’s dideoxy chain-termination method was carried out on double-stranded templates using Sequenase version 2 (USB) with specifically synthesized primers where necessary. All sequence reported was determined on both strands. Sequence data were assembled using MacVector software (IBI Pestell). Sequence comparison and alignments were performed using GCG programs (Devereux et al., 1984).

Competitive RT-PCR. All RNA preparations were treated with RQI DNase (Promega) to remove any contaminating DNA; according to the manufacturer’s instructions, using 50 μl total RNA in a final reaction volume of 500 μl. The concentration of each RNA sample was determined spectrophotometrically and adjusted to 2 μg μl⁻¹ in sterile, double-deionized water. Reverse transcription and competitive RT-PCR were carried out as described by Smith (1995). Primers used were: lcc1-specific primers, RP3 (5’-3’ residues 1751–1771 in lcc1), RP4 (3’-5’ residues 2633–2613 in lcc1), RP5 (equivalent to 5’-3’ residues 1751–1771 in lcc1, but specific sequence of lcc2) and RP6 (equivalent to 3’-5’ residues 2343–2323 in lcc1, but specific sequence of lcc2; Perry et al., 1993b). Competitor DNA for both assays was serial dilutions of the genomic clone LA16E2 (which contains one copy of each gene). Analyses were carried out directly from 1.5% (w/v) ethidium-bromide- stained agarose gels using the Herolab EASY Plus enhanced analysis system running EASY Plus version 4.16 software. This system allows quantification of irradiance from individual fluorescent bands so that RNA-derived and competitor-DNA-derived band intensities can be plotted to interpolate the equivalent concentration.
RESULTS AND DISCUSSION

DNA sequence

Evidence for the existence of two non-allelic laccase genes including the complete genomic sequence of lcc1 and the close to full-length cDNA sequence of lcc2 has been reported previously (Perry et al., 1993b). Further restriction mapping and Southern analysis of cosmid clones from a library (Sodhi, 1992) of Agaricus bisporus

Fig. 1. (a) Arrangement of the lcc1 and lcc2 genes. The distance between the two ORFs is 1562 bp. Only the beginning of the lcc2 ORF is shown, as this sequence has largely been analysed as cDNA. (b) Comparison (BESTFIT) of the lcc2-proximal part of the intergenic sequence (upper sequence) with the 5' non-coding region of lcc1 (lower sequence). The coding sequence of lcc1 starts at residue 460 (not shown) and the coding sequence for lcc2 starts at residue 4327. Putative regulatory sequences are identified as follows. 1, CAAT boxes. Eight further CAAT motifs (not shown) are present in the intergenic sequence at residues 3871-3880 and positions further upstream from the start of lcc2, but not at equivalent positions upstream of lcc1, in the region that has so far been sequenced. 2, Heat-shock response element binding site. 3, Residues identical to the 5' region of the laccase gene of Coriolus hirsutus and the yeast GAL4 binding site. 4, TATA boxes.

C-54-carb-8 (a fungicide-resistant mutant; Loftus et al., 1988) had shown that both laccase sequences were present in the cosmid clone LA16E2, approximately 2 kb apart. PCR with all possible combinations of primers for extension out of the ends of both genes gave a consistent product only with the combination 5'-3' primer close to the 3' end of lcc1 combined with 3'-5' primer close to the 5' end of lcc2, indicating a tandem arrangement with lcc1 upstream of (5' to) lcc2 (results not shown). Although the nucleotide sequences of lcc1 and lcc2 are largely the same (86.6% difference in the coding regions) there are small regions of divergence, particularly within the 5' coding region of the genes, which permitted the design of sequence-specific primers. This enabled the nucleotide sequence of the intergenic region to be sequenced directly from the clone LA16E2. The 3'-5' sequencing primer complementary to the 5' region of lcc2 had a 4 base mismatch at its 3' end compared to lcc1 sequence. Because very little 3' non-coding sequence from lcc2 was available for comparison, however, the initial 5'-3' sequencing primer from the 3' non-coding region of lcc1 (complementary to residues 2874-2893 in lcc1) was designed beyond the stop codon (residues 2762-2764), but with 115 bp of known lcc1 sequence downstream for comparison to ensure correct lcc1-specific priming. Thereafter, specific, sequential primers were designed to obtain the full sequence in both strands. The organization of the two laccase genes, with 1562 bp separating the stop codon of lcc1 and the start codon of lcc2 is shown in Fig. 1(a).

Multiplicity of laccase genes differing only slightly in sequence is apparently not uncommon in fungi. This is sometimes simply due to allelic differences as found in Neurospora crassa (Germann et al., 1988) and the laccase genes (PO1 and PO2) of Coriolus hirsutus (Kojima et al., 1990). In contrast, of the four laccase genes isolated from Rhizoctonia solani, three are closely linked in tandem (and the fourth is an allele of one of the others; Wahleithner et al., 1996). Five non-allelic laccase genes have been characterized from the white-rot basidiomycete Trametes villosa (Yaver et al., 1996; Yaver & Golightly, 1996). Two genes (lcc1 and lcc2) correspond to the purified laccase protein-forms 1 and 3 respectively. Under the conditions tested, no RNA species representing lcc3, 4 and 5 could be detected. The organization of these genes in relation to one another has not been established. Considering Agaricus bisporus genes other than laccase, there is presently only one report of two non-allelic homologous genes. Harmes et al. (1992) have shown the presence of a (pseudo)gene (GPDAR1) similar to the glyceraldehyde-3-phosphate dehydrogenase gene (GPDAR3). These two sequences are closely linked and while the deduced amino acid sequence of GPDAR1 conserves many important structural features associated with GPDAR3, transcripts of this gene have not been found in either fruit bodies or mycelium.

The alignment of the 3' region of the intergenic sequence (the 5' non-coding region of lcc2) with the 5' non-coding region of lcc1 is shown in Fig. 1(b). The sequences show...
significant similarity. Allowing gaps as shown in Fig.
1(b), 61% of residues are identical. A number of unique
and common, putative, transcription-factor-binding
sites have been identified. Of the two putative 'TATA'
sites and common, putative, transcription-factor-binding
boxes and a single 'CAAT' box unique to
lccl, possible 'CAAT' boxes unique to lcc2 are numerous,
with examples at residues 3877–3880, 4087–4090 and
4126–4129, 392, 182 and 147 bp from the common
TATA box (the closer of which are shown in Fig. 1b).
The cell1 (Raguz et al., 1992), cel3 (Chow et al., 1994)
and GPD<sup>AE2</sup> (Harmsen et al., 1992) genes from A.
bisporus contain similar 'TATA' sequence elements
that generally conform to the asymmetric consensus
sequence TAT(A/T)AA (Breathnach & Chambon,
1981), as do all the fungal laccase genes sequenced to
date. However, the cell1 gene (Raguz et al., 1992)
and laccase 1 gene from Aspergillus nidulans (Aramayo &
Timberlake, 1990) have the symmetrical sequence
TATA and the GPD<sup>AE2</sup> gene has TACAAAAA. The
inverted repeat and regions of similarity with Neuro-
spora crassa and the La France disease-associated
double-stranded M1 RNA virus previously reported for
the 5' region of lcc1 (Perry et al., 1993b; Smith, 1993)
are not present in the intergenic sequence.

The 'core' heat-shock elements (HSE) present in both
non-coding regions (feature 2 in Fig. 1b) typically form
part of contiguous arrays of variable numbers of the
6 bp sequence NGANNN arranged in alternating
orientations (Sorger, 1991). It is thought that a minimum
of two units are required for high-affinity heat-shock
binding and such arrays are present in both sequences.
HSE involvement in control of ligninolytic enzymes has
been extensively studied with respect to manganese
peroxidases of Phanerochaete chrysosporium (Gold &
Alic, 1993; Li et al., 1995). There may be other
similarities between manganese peroxidase and laccase
gene expression, as both may be controlled by metal
response elements (MREs). MRE consensus sequences
are found in the promoter regions of mnpl and mnmp2
of P. chrysosporium (Gold & Alic, 1993) and at residues
4298–4304 close to the start of lcc2.

A short sequence also found in the 5' region of the
laccase gene from Coriolus hirsutus is present (Fig. 1b),
that also matches the binding sequence for the yeast
Gal4 transcription initiator (Selleck & Majors, 1987).
Differences in the sequences for putative regulatory sites
suggest a potential for differential regulation between
lcc1 and lcc2. This is comparable to the many lignin
peroxidase and manganese peroxidase genes of Phaner-
ochaete chrysosporium (Gold & Alic, 1993). For all the
sequence motifs discussed above, however, it will remain
uncertain as to whether any of them are functional
transcription factor recognition sites until a reliable
transformation system is developed for A. bisporus.

A potential polyadenylation signal sequence, conform-
ing to the higher eukaryotic consensus AATAAA
(Proudfoot & Brownlee, 1976) is present in the inter-
genic sequence (residues 3394–3399). This, together with
the 1563 bp of coding sequence, is in agreement with the
previous estimate for the size of laccase mRNA at
2.3 ± 0.15 kb (Perry et al., 1993b). The function of this

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**Fig. 2.** Competitive RT-PCR analysis of mRNA from the laccase genes lcc1 (a) and lcc2 (b) of A. bisporus. Total RNA was extracted from mycelium grown in ME liquid medium. Reverse transcriptase reactions were carried out with 2 μg total RNA. Individual PCRs were 'spiked' with serial dilutions of the competitor genomic clone LA16E2. In each case 30% of the PCR mixture was resolved on a 1% (ethidium-bromide-containing) agarose gel. In both (a) and (b) lanes 1 and 11 contained 1 kb ladder (BRL). The competitive genomic fragments (881 bp in a, 592 bp in b) and cDNA-derived fragments (500 bp in a, 386 bp in b) are indicated with arrows as gDNA and cDNA, respectively. In both (a) and (b), dilutions for the competitor genomic clone contained (lanes 2–10, respectively): 1·6 × 10<sup>-4</sup>, 9·8 × 10<sup>-5</sup>, 1·6 × 10<sup>-5</sup>, 9·8 × 10<sup>-6</sup>, 1·6 × 10<sup>-6</sup>, 9·8 × 10<sup>-7</sup>, 1·6 × 10<sup>-7</sup> mol.

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expression of Agaricus bisporus laccase genes

Table 1. Quantification of lcc1 and lcc2 mRNA by competitive RT-PCR

<table>
<thead>
<tr>
<th>Source of mycelium...</th>
<th>Specific mRNA (mol per μg total RNA)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ME medium</td>
</tr>
<tr>
<td>lcc1</td>
<td>1.0 ± 0.1 × 10^{-17}</td>
</tr>
<tr>
<td>lcc2</td>
<td>3.0 ± 0.4 × 10^{-15}</td>
</tr>
<tr>
<td>Ratio lcc2/lcc1</td>
<td>300</td>
</tr>
</tbody>
</table>

* Values for RNA from ME culture are the mean ± sd of four independent samples; those from compost culture are the mean of duplicate assays of a single sample.

sequence remains speculative, as no cDNA clones for laccase that include (part of) the poly(A)-tail have so far been isolated.

Laccase mRNA levels

The results of the discriminatory lcc1-lcc2 competitive RT-PCR from 10-d-old ME liquid culture and colonization-stage compost mycelium are shown in Fig. 2 and Table 1. The highest value obtained for laccase mRNA (lcc2) was 3 × 10^{-15} mol per μg total RNA and was the same for both ME-grown and compost-grown mycelium. It is clear that lcc2 mRNA was very much more abundant than lcc1 mRNA in both culture conditions. In ME medium, levels of lcc2 mRNA were nearly 300 times higher than those of lcc1 mRNA; in compost, lcc2 mRNA was almost 7000 times more abundant than lcc1. This result is paradoxical in the sense that the species which varies in concentration, and which is therefore apparently under some form of regulation, is very much the minor component and consequently cannot have any significant direct effect on overall laccase expression. This suggests that the two laccase gene products have different functions and/or that the two genes are controlled by different regulatory circuits, that respond to different conditions. The latter proposition would suggest that there are conditions (which we have yet to find) where lcc1 expression is relatively high.

Differential expression in laccase gene-families has recently been reported from two other fungi. In the basidiomycete Trametes villosa, lcc1 mRNA could be induced approximately 17-fold by the addition of 2,5-xylyline to the culture. This corresponded to a 20-fold increase in enzyme activity; however, lcc2 mRNA could not be induced and was present at a constitutive level which was approximately half that of the uninduced lcc1 mRNA (Yaver et al., 1996a, b). Theplant-pathogenic fungus Rhizoctonia solani has a family of four laccase genes, three of them located within a 12 kb fragment of the genome. These are present at low constitutive levels and are further suppressed by addition of p-anisidine to the cultures. The mRNA for the fourth laccase is present at higher basal levels and its transcription is increased in response to p-anisidine (Wahleithner et al., 1996).

There are now several examples of systems in which mRNA degradation may contribute significantly to the control of expression of fungal genes (see for example Sachs, 1993). In Agaricus bisporus, the cell1 and cell3 genes may be controlled in part by mRNA degradation (Yagüe et al., 1994; Chow et al., 1994). For both cell1 and cell3 expression, although no mRNA accumulated under repressing conditions (as measured by Northern analysis), de novo transcripts were produced in nuclear run-on experiments, at comparable levels tocontrol messages that did accumulate on Northern blots. This suggests that, at least under repressing conditions, the cel mRNAs are subject to rapid turnover.

Further mechanistic detail of the control of expression of genes for secreted enzymes of A. bisporus will not be easily acquired, as simple correlations between rate of enzyme accumulation, mRNA level and rate of transcription do not obtain in laboratory liquid culture (Yagüe et al., 1994; Chow et al., 1994). At the same time, the methods described here have enabled us to examine individual mRNA levels in solid (compost) culture. Expression of genes in compost mycelium during the whole fruiting cycle will be described elsewhere.

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