Tandem organization and highly disparate expression of the two laccase genes *lcc1* and *lcc2* in the cultivated mushroom *Agaricus bisporus*

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Two non-allelic laccase genes (*lcc1* and *lcc2*) in *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 *lcc1* and the start codon of *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 *lcc1* and *lcc2*, it has been shown that the level of *lcc2* mRNA is approximately 300 times higher than that of *lcc1* mRNA in malt extract liquid cultures; in compost cultures *lcc2* mRNA is almost 7000 times more abundant than *lcc1* mRNA.

Keywords: *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 *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 plants laccase has a role in the lignification of differentiating xylem tissues (Bao *et al.*, 1993; O'Malley *et al.*, 1993). In fungi the role of laccase is often uncertain. In plant-pathogenic fungi, such as *Botrytis cinerea*, laccases are one of a range of

'attack' enzymes produced during the infective process (Marbach *et al.*, 1985; Viterbo *et al.*, 1993; Rigling & Van Alfen, 1991, 1993). Conidiation in *Aspergillus nidulans* is accompanied by a laccase activity that functions in the formation of a dark-green pigment, which protects the conidia against UV radiation (Clutterbuck, 1972). More recently the involvement of laccase in lignin degradation has regained favour (Smith *et al.*, 1997). Laccase from *Trametes (Coriolus) versicolor*, in the presence of mediating substrates such as ABTS, can oxidatively cleave C α –C β linkages of non-phenolic components of kraft pulp lignin *in vitro* and oxidize veratryl alcohol to veratryl aldehyde (Bourbonnais & Paice, 1990; Bourbonnais *et al.*, 1995). The presence of ABTS also prevented the repolymerization of the phenoxy radicals produced by the oxidation of the lignin phenolic groups. Eriksson's group have convincingly shown that laccase is essential for lignin degradation by *Pycnoporus cinnabarinus*, that the fungus produces a natural mediating substrate for laccase and that the laccase/mediator combination will depolymerize DHP *in vitro* (Eggert *et al.*, 1996a, b, 1997). It is likely, therefore, that the very widespread occurrence of laccase in white-rot and leaf-litter-degrading fungi is (at least in part) as a component of the ligninolytic system characteristic of these fungi.

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 *Neurospora crassa*

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylthiazoline-6-sulphonate); DHP, dehydrogenative polymer (synthetic lignin); ME, malt extract.

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

(Germann & Lerch, 1986; Germann *et al.*, 1988). Subsequently several others have been reported, with the two laccase genes (*lcc1* as genomic DNA and *lcc2* as cDNA) from *Agaricus bisporus* being the first to be identified as a fungal laccase gene-family (Perry *et al.*, 1993b). More recently, four distinct laccase genes have been characterized from the plant-pathogenic fungus *Rhizoctonia solani* (Wahleithner *et al.*, 1996) and five from the white-rot fungus *Trametes villosa* (Yaver *et al.*, 1996; Yaver & Golightly, 1996), and although only a single laccase gene has been isolated from the filamentous ascomycete *Podospira anserina*, Southern analysis and restriction mapping suggest the presence of at least three others (Fernández-Larrea & Stahl, 1996). All these sequences encode polypeptides of about 520–600 amino acid residues including an N-terminal secretion peptide. In sequence comparisons the overall amino acid similarity is generally not very high, but importantly they all show strict conservation in and around the single cysteine and ten histidine residues proposed as copper ligands. It is also apparent that there is some basic similarity with the other two enzymes in the large blue copper family, the plant ascorbate oxidase and the vertebrate plasma protein ceruloplasmin (Germann *et al.*, 1988; Messerschmidt & Huber, 1990; Messerschmidt, 1997).

Laccase gene regulation is another feature of these enzymes that differs between organisms. Some fungal laccases are inducible by substrates such as 2,5-xylylene (see Bourbonnais *et al.*, 1995, for an example). Some are 'induced' by protein synthesis inhibitors such as cycloheximide (see for example Germann & Lerch, 1986), but the extent to which this is solely a consequence of laccase mRNA being more stable than average is very difficult to measure; the alternative, that cycloheximide is a natural inducer, has yet to be given a plausible explanation. Many fungal laccases are apparently constitutive, but whether they are mechanistically constitutive (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 × 5 × 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 triisopropyl-naphthalene sulphonate and phenol/cresol, 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 Pustell). Sequence comparison and alignments were performed using GCG programs (Devereux *et al.*, 1984).

Competitive RT-PCR. All RNA preparations were treated with RQ1 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 3877–3880 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 (8.6% 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. Harmsen *et al.* (1992) have shown the presence of a (pseudo)gene (*GPD^{Ag1}*) similar to the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD^{Ag2}*). These two sequences are closely linked and while the deduced amino acid sequence of *GPD^{Ag1}* conserves many important structural features associated with *GPD^{Ag2}*, 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

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^{-6} , 0.8×10^{-6} , 1.6×10^{-7} , 0.8×10^{-7} , 1.6×10^{-8} , 0.8×10^{-8} , 1.6×10^{-9} , 0.8×10^{-9} , 1.6×10^{-10} mol.

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' boxes and a single 'CAAT' box previously identified in *lcc1*, only the second TATA box is found in both genes. 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 *cel1* (Raguz *et al.*, 1992), *cel3* (Chow *et al.*, 1994) and *GPD^{Ag2}* (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 *cel1* gene (Raguz *et al.*, 1992) and laccase 1 gene from *Aspergillus nidulans* (Aramayo & Timberlake, 1990) have the symmetrical sequence TATA and the *GPD^{Ag2}* gene has TACAAAAA. The inverted repeat and regions of similarity with *Neurospora 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, 1995) 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 NGANN 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 *mnp1* and *mnp2* 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 *Phanerochaete 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, conforming to the higher eukaryotic consensus AATAAA (Proudfoot & Brownlee, 1976) is present in the intergenic 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

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

Source of mycelium...	Specific mRNA (mol per µg total RNA)*	
	ME medium	Compost culture
<i>lcc1</i>	$1.0 \pm 0.1 \times 10^{-17}$	4.5×10^{-19}
<i>lcc2</i>	$3.0 \pm 0.4 \times 10^{-15}$	3.0×10^{-15}
Ratio <i>lcc2/lcc1</i>	300	~ 7000

* Values for RNA from ME culture are the mean $\pm$ 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 suppose 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-xylydine 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). The plant-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 *cel1* and *cel3* genes may be controlled in part by mRNA degradation (Yagüe *et al.*, 1994; Chow *et al.*, 1994). For both *cel1* and *cel3* 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 to control 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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REFERENCES

- Aramayo, O. R. & Timberlake, W. E. (1990). Sequence and molecular structure of the *Aspergillus nidulans* *yA* (laccase 1) gene. *Nucleic Acids Res* 18, 3415.
- Bao, W., O'Malley, D. M., Whetten, R. & Sederoff, R. R. (1993). A laccase associated with lignification in loblolly pine xylem. *Science* 260, 672–674.
- Bourbonnais, R. & Paice, M. G. (1990). Oxidation of nonphenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett* 267, 99–102.
- Bourbonnais, R., Paice, M. G., Reid, I. D., Lanthier, P. & Yaguchi, M. (1995). Lignin oxidation by laccase isozymes from *Trametes*

- versicolor* and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in Kraft lignin depolymerisation. *Appl Environ Microbiol* **61**, 1876–1880.
- Breathnach, R. & Chambon, P. (1981). Organisation and expression of eukaryotic split genes coding for proteins. *Annu Rev Biochem* **50**, 349–383.
- Chow, C.-M., Yagüe, E., Raguz, S., Wood, D. A. & Thurston, C. F. (1994). The *cel3* gene of *Agaricus bisporus* codes for a modular cellulase and is transcriptionally regulated by the carbon source. *Appl Environ Microbiol* **60**, 2779–2785.
- Clutterbuck, A. J. (1972). Absence of laccase from yellow spored mutants of *Aspergillus nidulans*. *J Gen Microbiol* **70**, 423–435.
- Devereux, J., Haeblerli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Eggert, C., Temp, U., Dean, J. F. D. & Eriksson, K.-E. L. (1996a). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett* **391**, 144–148.
- Eggert, C., Temp, U. & Eriksson, K.-E. L. (1996b). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterisation of the laccase. *Appl Environ Microbiol* **62**, 1151–1158.
- Eggert, C., Temp, U. & Eriksson, K.-E. L. (1997). Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. *FEBS Lett* **407**, 89–92.
- Fernández-Larrea, J. & Stahl, U. (1996). Isolation and characterisation of a laccase gene from *Podospira anserina*. *Mol Gen Genet* **252**, 539–551.
- Germann, U. A. & Lerch, K. (1986). Isolation and partial nucleotide sequence of the laccase gene from *Neurospora crassa*: amino acid sequence homology of the protein to human ceruloplasmin. *Proc Natl Acad Sci USA* **82**, 8854–8858.
- Germann, U. A., Muller, G., Hunziker, P. E. & Lerch, K. (1988). Characterisation of two allelic forms of *Neurospora crassa* laccase: amino- and carboxyl-terminal processing of a precursor. *J Biol Chem* **263**, 885–896.
- Gold, M. H. & Alic, M. (1993). Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Microbiol Rev* **57**, 605–622.
- Harmsen, M. C., Schuren, F. H. J., Moukha, S. M., Carin, M. Z., Punt, P. J. & Wessels, J. G. H. (1992). Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. *Curr Genet* **22**, 447–454.
- Kojima, Y. M., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaino, M. & Kita, Y. (1990). Cloning, sequence analysis, and expression of lignolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. *J Biol Chem* **265**, 15224–15230.
- Leonard, J. C., Dunham, S. M. & Thurston, C. F. (1981). Isolation of RNA from *Chlorella fusca* var. *vacuolata*. *New Phytol* **87**, 39–51.
- Li, D., Alic, M., Brown, J. A. & Gold, M. H. (1995). Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress and molecular oxygen. *Appl Environ Microbiol* **61**, 341–345.
- Loftus, M. G., Moore, D. & Elliott, T. J. (1988). DNA polymorphisms in commercial and wild strains of the cultivated mushroom, *Agaricus bisporus*. *Theor Appl Genet* **76**, 712–718.
- Marbach, I., Harel, E. & Mayer, A. M. (1985). Pectin, a second inducer for laccase production by *Botrytis cinerea*. *Phytochemistry* **24**, 2559–2561.
- Messerschmidt, A. (1997). *Multi-copper Oxidases*. Singapore: World Scientific Publishing Co.
- Messerschmidt, A. & Huber, R. (1990). The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin, modelling and structural relationships. *Eur J Biochem* **187**, 341–352.
- O'Malley, D. M., Whetten, R., Bao, W., Chen, C.-L. & Sederoff, R. (1993). The role of laccase in lignification. *Plant J* **4**, 751–757.
- Perry, C. R., Matcham, S. E., Wood, D. A. & Thurston, C. F. (1993a). The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. *J Gen Microbiol* **139**, 171–178.
- Perry, C. R., Smith, M., Britnell, C., Wood, D. A. & Thurston, C. F. (1993b). Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. *J Gen Microbiol* **139**, 1209–1218.
- Proudfoot, N. J. & Brownlee, G. G. (1996). 3' non-coding region sequences in eukaryotic messenger RNA. *Nature* **263**, 211–214.
- Raguz, S., Yagüe, E., Wood, D. A. & Thurston, C. F. (1992). Isolation and characterisation of a cellulose-growth-specific gene from *Agaricus bisporus*. *Gene* **119**, 183–190.
- Rigling, D. & van Alfen, N. K. (1991). Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. *J Bacteriol* **173**, 8000–8003.
- Rigling, D. & van Alfen, N. K. (1993). Extra- and intracellular laccases of the chestnut blight fungus *Cryphonectria parasitica*. *Appl Environ Microbiol* **59**, 3634–3639.
- Sachs, A. B. (1993). Messenger RNA degradation in eukaryotes. *Cell* **74**, 413–421.
- Selleck, S. B. & Majors, J. (1987). Photofootprinting *in vivo* detects transcription-dependent changes in yeast TATA boxes. *Nature* **325**, 173–177.
- Smith, M. (1995). *Molecular analysis of the laccase genes from Agaricus bisporus*. PhD thesis, University of London.
- Smith, M., Thurston, C. F. & Wood, D. A. (1997). Fungal laccases: role in delignification and possible industrial applications. In *Multi-copper Oxidases*, pp. 201–224. Edited by A. Messerschmidt. Singapore: World Scientific Publishing Co.
- Sodhi, H. S. (1992). *Enzyme mutants of Coprinus bilanatus and recombinant DNA technology for strain improvement in Agaricus bisporus*. PhD thesis, University of London.
- Sorger, P. K. (1991). Heat shock factor and the heat shock response. *Cell* **65**, 363–366.
- Tsai, Y.-L. & Olson, B. H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl Environ Microbiol* **58**, 2292–2295.
- Viterbo, A., Yagen, B. & Mayer, A. M. (1993). Cucurbitacins, 'attack' enzymes and laccase in *Botrytis cinerea*. *Phytochemistry* **32**, 61–65.
- Wahleithner, J. A., Xu, F., Brown, K. M., Brown, S. H., Golightly, E. J., Halkier, T., Kauppinen, S. & Pederson, A. (1996). The identification and characterisation of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. *Curr Genet* **29**, 395–403.
- Wood, D. A. (1980a). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J Gen Microbiol* **117**, 327–338.
- Wood, D. A. (1980b). Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. *J Gen Microbiol* **117**, 339–345.
- Yagüe, E., Wood, D. A. & Thurston, C. F. (1994). Regulation of transcription of the *cel1* gene in *Agaricus bisporus*. *Mol Microbiol* **12**, 41–47.
- Yaver, D. S., Feng, X., Golightly, E. J., Brown, K. M., Brown, S. H.,

Rey, M. W., Schneider, P., Halkier, T., Mondorf, K. & Dalbøge, H. (1996). The purification, characterisation, molecular cloning and expression of two laccase genes from the white-rot basidiomycete *Trametes pinsitus*. *Appl Environ Microbiol* **62**, 834–841.

Yaver, D. S. & Golightly, E. J. (1996). Cloning and characterisation of three laccase genes from the white-rot basidiomycete *Trametes*

villosa: genomic organisation of the laccase gene family. *Gene* **181**, 95–102.

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