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REVIEW

Laccases: a never-ending story

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Abstract Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water. In fungi, laccases carry out a variety of physiological roles during their life cycle. These enzymes are being increasingly evaluated for a variety of biotechnological applications due to their broad substrate range. In this review, the most recent studies on laccase structural features and catalytic mechanisms along with analyses of their expression are reported and examined with the aim of contributing to the discussion on their structure–function relationships. Attention has also been paid to the properties of enzymes endowed with unique characteristics and to fungal laccase multigene families and their organization.

Keywords Multicopper oxidases · Laccase structures · Unusual laccases · Laccase gene family · Laccase regulation

Dedicated to the memory of our missed friend and colleague Sophie Vanhulle who died suddenly and tragically.

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Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2), representing the largest subgroup of blue multicopper oxidases (MCO), use the distinctive redox ability of copper ions to catalyze the oxidation of a wide range of aromatic substrates concomitantly with the reduction of molecular oxygen to water [1, 2]. Amongst more than 200 kinds of oxidases and oxygenases, only six classes of enzymes are capable of catalyzing this type of oxygen reaction (cytochrome-*c* oxidase, laccases, L-ascorbate oxidase, ceruloplasmin, bilirubin oxidase, and phenoxazinone synthase).

A laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* [3]. Since then, laccases have also been found in various basidiomycetous and ascomycetous fungi and, thus far, fungal laccases have accounted for the most important group of MCOs with respect to number and extent of characterization. In fungi, laccases carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen/host interaction, stress defense, and lignin degradation [4, 5]. Laccases have been found in nearly all wood-rotting fungi analyzed so far [6] and are almost ubiquitary enzymes as they have been isolated from plants, from some kinds of bacteria, and from insects too [7–13]. In plants, laccases have been found in the wood and cellular walls of herbaceous species [7], where they participate in lignin biosynthesis [8]. Bacterial laccases appear to have a role in morphogenesis [9], in the biosynthesis of the brown spore pigment and in the protection afforded by the spore coat against UV light and hydrogen peroxide, and in copper homeostasis [10]. The main function of the laccase-type proteins in insects is believed to be sclerotization of the cuticle in the epidermis [12].

Laccases are able to catalyze direct oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines,

and aryl diamines as well as some inorganic ions [1, 5, 14–17]. They couple the four single-electron oxidations of the reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu atoms distributed against three sites, defined according to their spectroscopic properties [1]. Typical metal content of laccases includes one type-1 (T1) copper (Cu1), and one type-2 (T2) and two type-3 (T3) copper ions (Cu2 and Cu3), with Cu2 and Cu3 arranged in a trinuclear cluster (TNC).

Laccase sequences and structures

The majority of the fungal laccases are extracellular monomeric globular proteins of approximately 60–70 kDa with an acidic isoelectric point (p*I*) around pH 4.0; they are generally glycosylated, with an extent of glycosylation ranging between 10 and 25% and only in a few cases higher than 30% [18, 19].

Analysis of the essential sequence features of fungal laccases based on multiple sequence alignments of more than 100 laccases has resulted in the identification of a set of four ungapped sequence regions, L1–L4, useful to identify the laccases and to distinguish them within the broader class of MCOs [20] (Fig. 1a). The 12 amino acid residues acting as the copper ligands are housed within these four identified conserved regions, of which L2 and L4 are in line with the earlier reported copper signature sequences of MCOs, while L1 and L3 are distinctive to the laccases. Furthermore, four loop regions, designated loops I, II, III, and IV and involved in substrate binding, have been identified [21] on the basis of laccase 3D structure superimposition (Fig. 1b).

A number of 3D structures of basidiomycete laccases have been reported so far. Crystal structures have been solved for the laccases Lac-Cc (CcL1A65) from *Coprinus cinereus* [22], LccI (TvL1GYC) [23] and LacIIIb (TvL1KYA) from *Trametes versicolor* [24], *Rl*L (R1G1V10) from *Rigidoporus lignosus* [25], *Lt*L (LtL2QT6) from *Lentinus tigrinus* [26], and *Tt*L (TtL2HRG) from *Trametes trogii* [27]. On the other hand, the only three-dimensional structure so far reported for ascomycete laccases is that of the laccase from *Melanocarpus albomyces*, whose crystal structures have been solved both for the native enzyme MaL (MaL1GWO) [28] and for the recombinant enzyme rMaL (rMaL2Q9O) expressed in *Trichoderma reesei* [29]. Crystal structures of bacterial laccases have also been solved [10, 11]. All these laccases exhibit a similar molecular architecture organized in three sequentially arranged cupredoxin-like domains. Each of them has a greek key β-barrel topology, strictly related to that of small copper proteins such as azurin and plastocyanin [30] and common to all the members of the MCOs family, such as ascorbate oxidase [31] and mammalian ceruloplasmin [32]. The Cu1 is located in domain 3, whilst the TNC cluster is embedded between domains 1 and 3 with both domains providing residues for copper coordination. The structure of basidiomycete laccases is stabilized by two disulfide bridges between domains 1 and 3 and between domains 1 and 2, whereas three disulfide bridges were found in the MaL1GWO structure [28].

It is worth noting that the mapping of regions L1–L4 onto the laccases' three-dimensional structure indicated a specific, more or less C-2 symmetric, protein conformational motif characterizing the active site apparatus of the enzymes [20]. The observed intra-protein homologies

A

Protein	L1	L2	L3	L4
TvL1KYA	^{64}HWHGFFQKGTNWADGPAFINQCPI	^{104}GTFWYHSHLSTQYCDGLRGPF	^{395}HPFHLHGH	^{447}GPWFLHCHIDFHLEAGFAVVF
TvL1GYC	^{64}HWHGFFQAGTNWADGPAFVNQCPI	^{104}GTFWYHSHLSTQYCDGLRGPF	^{395}HPFHLHGH	^{447}GPWFLHCHIDFHLEAGFAIVF
R1G1V10	^{64}HWHGFFQAGTTEMDGPAFVNQCPI	^{104}GTYWYHSHLSTQYCDGLRGAF	^{396}HPFHLHGH	^{446}GPWFLHCHIDWHLEAGLAVVF
CcL1A65	^{64}HWHGLFQRGTNWADGADGVNQCPI	^{104}GTFWYHSHFGTQYCDGLRGPM	^{395}HPFHLHGH	^{446}GPWFFHCHIEFHLMNGLAIVF
LtL2QT6	^{64}HWHGFFQKGTNWADGPAFINQCPI	^{104}GTFWYHSHLSTQYCDGLRGPF	^{394}HPFHLHGH	^{446}GPWFLHCHIDFHLDAGFAVVM
TtL2HRG	^{64}HWHGFFQHGTNWADGPAFVNQCPI	^{104}GTFWYHSHLSTQYCDGLRGPI	^{394}HPFHLHGH	^{444}GPWFLHCHIDFHLEAGFAVVM

B

Protein	Substrate binding loops					
	Loop I		Loop II	Loop III	Loop IV	
TvL1KYA	^{154}VAAK--LG--PA--FPLG	^{203}LSCDPNY	^{261}ANP-----NFGNVG----F	^{330}FNFNGTNF--FI	^{384}FPATAAAPGAP	^{454}HIDFHLEAGF
TvL1GYC	^{154}TAAR--LG--PR--FPLG	^{203}ISCDPNY	^{261}ANP-----NFGTVG----F	^{330}FNFNGTNF--FI	^{384}LPATALAPGAP	^{454}HIDFHLEAGF
R1G1V10	^{154}SLST--VLFPNPNKAPPA	^{208}TSCFPNY	^{266}ANP-----SNGRNG----F	^{334}IGRNATTADFTI	^{390}IP-----GGGN	^{453}HIDWHLEAGL
CcL1A65	^{154}IPAP--SI-----QGAAQ	^{202}LSCDPNW	^{260}AQP-----NKGRNGLAGTF	^{333}LGFSGGRF--TI	^{397}VP--AGVLGGP	^{453}HIEFHLMNGL
LtL2QT6	^{154}VAAK--LG--PR--FPKG	^{203}LSCDPNY	^{261}ANP-----NFGTTG----F	^{329}FNFDGTNF--FI	^{383}FPATTAAPGAP	^{453}HIDFHLDAGF
TtL2HRG	^{154}LAAK--VG--SP-VPT-A	^{202}LSCDPNH	^{260}ALP-----NSGTRN----F	^{329}FGFAGGKF--TI	^{383}LPATAAAPGFP	^{451}HIDFHLEAGF

Fig. 1 Comparison of the laccase signature sequences (**a**) and of the substrate binding loops (**b**) of the laccases with known three-dimensional structure: TvL1KYA from *T. versicolor*, TvL1GYC from *T. versicolor*, R1G1V10 from *R. lignosus*, CcL1A65 from *C. cinereus*, LtL2QT6 from *L. tigrinus*, TtL2HRG from *T. trogii*

between L1 and L3 and between L2 and L4 at both structure and sequence levels suggest that the quasi C-2 symmetric active site conformational motif may have arisen from a structural duplication event.

The T1 copper and the reaction with the reducing substrate

Typically MCOs show a Cu1 tetrahedrically coordinated with an axial fourth ligand, usually a methionine, to complete the first coordination sphere [2]. Vice versa, in laccases Cu1 exhibits a planar triangular coordination with the sulfur atom of a cysteine and with the Nδ1 nitrogen of two histidines. The charge transfer transition SCys → Cu(II) is responsible for an extremely intense absorption band with a molecular absorption coefficient, ε, of 5,300 M^{-1} cm^{-1} at 614 nm giving rise to the deep blue color of the enzyme [33]. The Cu1 is the primary electron acceptor site in a laccase catalyzed reaction, where four single-electron oxidations of a reducing substrate occur. The electrons are transferred through the highly conserved His–Cys–His tripeptide to the TNC, where O_2 is reduced to water (Fig. 2) [34].

The reduction of the Cu1 is the rate-limiting step in the catalytic process and involves the Marcus "outer-sphere" mechanism [35]. In this mechanism, ΔE^0 between E^0 of the Cu1 and that of the reducing substrate determines the electron transfer rate [36]. E^0 values of the Cu1 have been determined using potentiometric titrations for a great number of different laccases, and a substantial variation between 420 and 790 mV versus NHE has been found [19, 36–38]. The lack of the fourth axial ligand in laccases is considered as an important factor determining the higher values of E^0 displayed by laccases in comparison with the other MCOs [39], as shown by mutagenesis experiments in

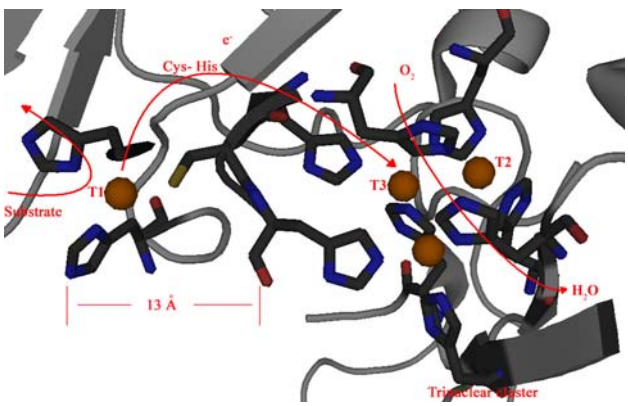

Fig. 2 The structure of the laccase active site with *arrows* marking the flow of substrates, electrons (e^-) and O_2, adapted from [34] with the author's permission

the *Trametes villosa* laccase [37] and recently in a bacterial laccase [40]. A major issue still to be fully clarified is how the different laccases modulate their redox potential despite showing a very similar Cu1 coordination geometry.

The Cu1 occupies a depression of the enzyme surface that makes possible its role as primary electron acceptor site. The Cu1 cavity is fairly wide, and it can accommodate a large variety of substrates that are not tightly buried in it. The structure of TvL1KYA represents the first high resolution structure of a laccase with an organic reducing substrate, 2,5-xylidine, in the substrate binding cavity [24]. Many hydrophobic protein–ligand interactions were shown to take place involving residues belonging to the four loops delineating the cavity (Fig. 3). Moreover, the residues His458 and Asp206 have also been shown to be important for the interaction between the amino group of the reducing substrate and the enzyme. The ring of the His458, coordinated to the Cu1 and highly conserved in all MCOs, is close to the nitrogen of 2,5-xylidine at a hydrogen bond distance (3.2 Å), suggesting that His458 is the entrance door of the electron during its transfer to Cu1.

The amino group of 2,5-xylidine is also hydrogen bound to a terminal oxygen of the Asp206 side chain, which is located at the rear wall of the binding site [23, 24, 41]. Asp206 is well conserved among fungal laccases from basidiomycetes, whereas glutamate can be found among ascomycetes. The interaction of Asp206 with the reducing substrate has been shown to have an important role in determining the pH dependence of laccase activity [42, 43]: as the pH increases from 3 to 5, the K_M values measured for phenolic substrates decrease, and this can be due to the deprotonation of the Asp206 side chain. At pH 5, the carboxylic function of Asp206 (pKa 3.9) [24] is dissociated, conferring a negative charge to the active site, favoring the interaction with substrates bearing the –OH or $–NH_2$ functionality. The role of Asp206 in reaction with phenolic substrates is neatly supported by recent data acquired with mutants of TvL1KYA [44].

Xu et al. [42] postulated that the ascending part of the activity-pH profile observed for all laccases towards 2, 6 dimethoxy phenol (DMP) is generated by the redox potential difference between the reducing substrate and the Cu1 of laccase and is favored by a higher pH. As the pKa of DMP is ~9.9, deprotonation of DMP does not occur spontaneously at acidic pHs. The vicinity of a carboxylic residue interacting with the reducing substrate in the active site favors its deprotonation.

Piontek and coworkers [23] have hypothesized a correlation between the coordination distances around the Cu1 ion and its E^0: the longer the distance the higher is E^0. An elongated Cu–N bond in TvL1GYC could have an effect on the redox potential since the contribution of the free electron pair from the nitrogen to the copper would be decreased, rendering the copper more electron-deficient. This would

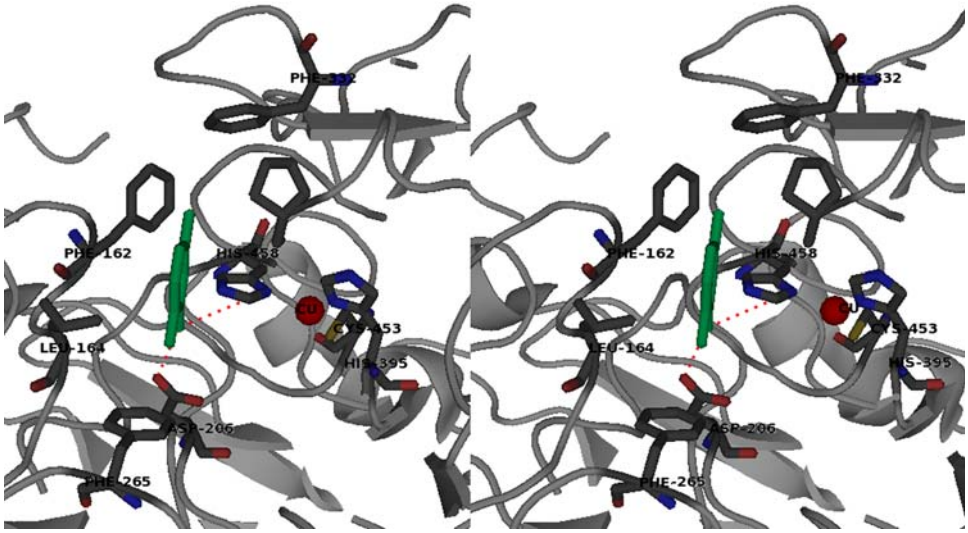

Fig. 3 Stereo view of the active site of TvL1KYA, which binds substrate 2,5-dimethylaniline (in *green*), elaborated with *PyMol* from the crystallographic structure

give rise to a destabilization of the higher oxidation states, thus increasing the copper redox potential. The increase of the Cu–N distance could be a consequence of a hydrogen bond between Ser113 and Glu460, the latter situated in the same helix with His458, thus moving this residue away from Cu1 (Fig. 4). The hypothesis proposed by Piontek and collaborators has been also confirmed by structural observations of Garavaglia and coworkers [25] on R1G1V10, a high E^0 enzyme [45]. In this laccase, the residues Glu459 and Ser113 are engaged in a strong hydrogen bond and appear to play the same role suggested in TvL1GYC. R1G1V10 shows the longest value for the Cu1–His distance (2.20 Å). Sequence data reveal that a glutamic acid in the position corresponding to Glu460 and a serine corresponding to Ser113 in TvL1GYC are highly conserved features among high E^0 enzymes, as well as in some laccases of ligninolytic fungi with unknown E^0 (Fig. 1a).

In the structure of the laccase TtL2HRG, Matera and coworkers [27] have suggested that the occurrence of two hydrophobic residues Phe460 and Ile452 in the near surroundings of the Cu1 contributes to the high redox potential observed. Furthermore, residue Phe460 is additionally surrounded by a large number of hydrophobic residues that would also contribute to increasing the redox potential of the Cu1.

All together these structural observations indicate that the variations in redox potential of the Cu1 observed among laccases cannot be ascribed to a single structural feature but to a sum of factors such as the Cu1 coordination geometry and the nature of the second sphere residues influencing solvent accessibility, hydrogen bonding, and dielectric anisotropy around the site.

Moreover, along with its redox features, steric hindrance of the substrate largely affects the oxidation proficiency of laccases, as recently demonstrated by Tadesse and coworkers [46], in *T. villosa* and *Myceliophthora thermophila* laccases, two enzymes markedly differing in redox potential (0.79 and 0.46 V). The distance between two phenylalanine residues (Phe332 and 265 in TvL1KYA) that mark the entrance to the active site can represent the structural threshold for oxidation of substrates with compatible redox potential (Fig. 3).

The T2/T3 copper cluster and the reaction with the oxygen

The three Cu2/Cu3 ions are arranged in a triangular fashion, as consistently observed in MCOs, and coordinated to a strongly conserved pattern of four His–X–His motifs [2].

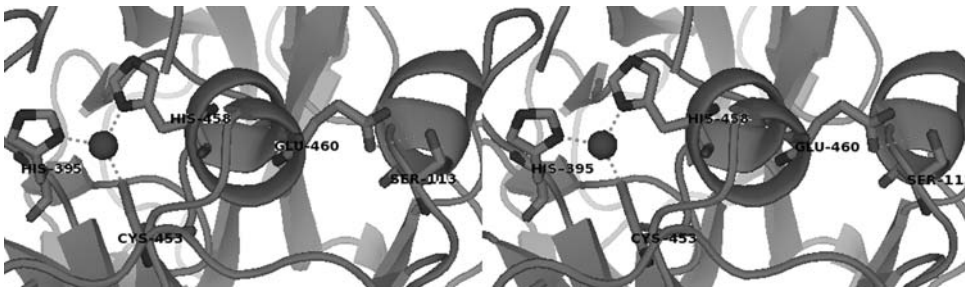

Fig. 4 Close up (stereo view) of the Cu1 site of TvL1GYC showing the α-helical segment moved away from Cu1 as a consequence of the H bond between Ser113 and Glu460 (elaborated with *PyMol* from the crystallographic structure)

Six such histidine residues coordinate the Cu3 copper pair, whereas the Cu2 is coordinated by the remaining two histidine residues. The Cu2 shows a characteristic electron paramagnetic resonance (EPR) spectrum, clearly distinct from that of Cu1, whereas the two Cu3 are antiferromagnetically coupled ions, EPR-silent. Optically, Cu3 absorbs at 330 nm ($\varepsilon_{330} \sim 3{,}600\ M^{-1}\ cm^{-1}$), whereas Cu2 is practically undetectable [47]. TNC acts in dioxygen binding and reduces the molecular oxygen upon receipt of four electrons forwarded from the mononuclear center Cu1 [1, 2, 22] to the two His coordinating the Cu3a and Cu3b copper ions. A detailed description of the catalytic events taking place at the three copper atoms in the dioxygen reduction site is still the subject of intense debate.

The kinetic and spectral features of copper/dioxygen intermediates in MCOs have been investigated in detail mainly by Solomon's group [33, 48, 49]. A considerable number of spectroscopic and kinetic studies have been focused on laccases from *R. vernicifera* [33, 50, 51], and more recently on the laccase-like Fet3p protein from *Saccharomyces cerevisiae* [52, 53]. The molecular mechanism of O_2 reduction to H_2O proposed on the basis of spectroscopic studies of O_2 intermediates in the MCOs is shown in Fig. 5 [34]. The reaction of the fully reduced enzyme with O_2 proceeds via two sequential two-electron steps, generating the peroxy intermediate (PI) and the native intermediate (NI). The first step is rate determining, while the second, involving the $2e^-$ reductive cleavage of the O–O bond, is faster.

In the fully reduced form, TNC is coordinatively unsaturated [54]. The anionic charges in the vicinity of the cluster appear to stabilize its high positive charge and tune

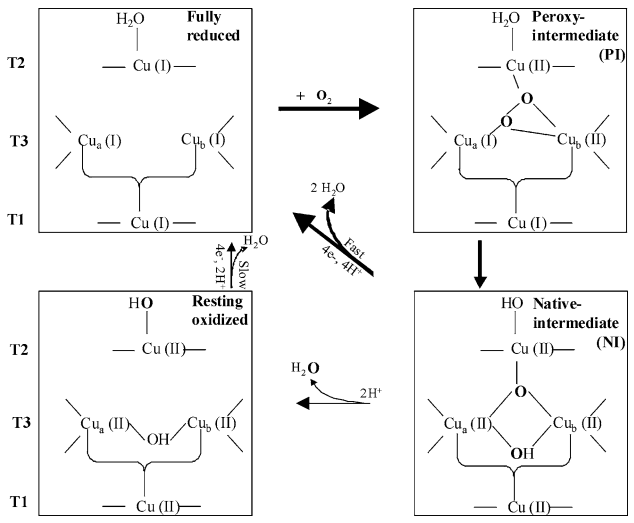

Fig. 5 Mechanism of O_2 reduction to water by the MCOs. *Broad arrows* indicate the steps that take place in the catalytic cycle of the MCOs; *thin arrows* indicate steps that can be experimentally observed but are not part of the catalytic cycle. The peroxy intermediate is a $2e^-$-reduced species, and the native intermediate is a $4e^-$-reduced species. Adapted from [34] with the author's permission

its coordination unsaturation and redox properties, contributing to the O_2 reactivity of the cluster. In particular the presence of a highly conserved anionic residue near the Cu2 (corresponding to D77 in TvL1KYA) plays a critical role in the reaction of the reduced TNC with O_2 [34, 54].

NI is a fully oxidized species with O_2 fully reduced to water-level products that remain bound to the trinuclear site as μ_3-oxo and μ_2-hydroxo bridging ligands [33, 49]. The all-bridged structure of NI is consistent with the rapid $4e^-$ reduction of NI to the fully reduced enzyme because the μ_3-oxo bridge would allow electron delocalization over the three Cu centers for facile electron transfer (ET) through the cluster [34]. In contrast, the resting form, a fully oxidized form as NI, has the Cu2 isolated from the other atoms of the clusters. In the absence of reducing substrate, the NI decays to the resting form at a rate that is too slow to be relevant in the catalytic cycle, thus the NI appears to be the catalytically fully oxidized form of the enzyme [33]. Indeed the decay of NI proceeds via successive proton-assisted steps and involves large structural rearrangement of the μ_3-oxo-bridge ligand from inside to outside the cluster leading to the resting enzyme. In this form, the one remaining O atom of the O_2 is bound as OH^- to Cu2 outside the cluster, and the two Cu3 centers are bridged by a OH^- ligand [49].

The three Cu centers in the TNC are sequentially reduced by reducing substrate via the Cu1. A reasonable mechanism for the reduction of NI has been proposed by Yoon and coworkers [49]. According to the authors, Cu3a is expected to reduce first because the negatively charged residue (D77 in TvL1KYA) near the Cu2 and Cu3b centers significantly lowers the reduction potentials of these Cu centers below that of Cu3a. Then, Cu2 would likely reduce before Cu3b; further reduction of the remaining Cu3b center would be fast via the Cys–His pathway between Cu1 and Cu3 centers and would be accompanied by dissociation of two water molecules from the TNC. On the other hand, reduction of Cu3b before Cu2 would result in protonation of the OH^- bridge, leading to the loss of the Cu2–Cu3b electronic coupling for rapid ET to Cu2.

On the basis of X-ray structural studies on the bacterial laccase CotA and other MCOs, Bento and coworkers [55] have suggested a different mechanism for the dioxygen reduction. One of the main differences is linked to the role of the resting state as one of the intermediates within the catalytic cycle. As recently assessed by Ferraroni and coworkers [26] and Matera and coworkers [27], structural data of MCOs obtained to date and acquired using synchrotron radiation are hard to interpret as far as the coordination geometries, the bond distances, and the oxidation states of the Cu1 and Cu2/Cu3 active sites are concerned. Metalloproteins are subjected to progressive reduction by exposure of crystals to high-intensity X-ray synchrotron beams. The X-ray synchrotron radiation

reduces copper centers during data collection, and the enzyme undergoes redox cycling. The crystals are exposed to air, the molecular oxygen can be reduced into water molecules, and the catalytic cycle repeated additional times resulting in collection of data generating electron density maps which represent an average of the several catalytic steps. Therefore, a strategy, newly applied to MCOs, was used under aerobic conditions and high pH in order to trap the intermediate states, and the authors observed the progressive reduction of the copper ions. The structure of the two sequential intermediates of the LtL2QT6 in the molecular oxygen reduction pathway was detected—the PI and NI resulting from two- and four-electron reductions of molecular oxygen, respectively [26, 27]. In contrast to what was suggested by spectroscopic studies, the peroxide in the PI appears to bridge only the Cu3 pair and not to connect the reduced Cu2. PI is further reduced in a second step, thus oxidizing the Cu2 and the distant Cu1 centers to generate the NI [26]. An oxo ion bridging the three coppers of the TNC (μ_3-oxo bridge) together with an hydroxide ion externally bridging the two Cu3 ions has been modelled in the NI, thus confirming the results of the spectroscopic studies.

As far as Cu3a and Cu3b ions are concerned, they are asymmetrically coordinated to an hydroxide/water moiety in the crystal structures of R1G1V10 [25], LtL2QT6 [26], and TtL2HRG [27]. In R1G1V10 structure [25], the asymmetric binding of the hydroxide ion bridging the Cu3 ions (with the Cu3a–OH bond shorter than the Cu3b–OH bond) has been explained by assuming a reduced oxidation state for Cu2 and Cu3a ions. This asymmetry could have a crucial role in the progression of the dioxygen splitting. In the structures of LtL2QT6 [26] and TtL2HRG [27], the Cu3a ends up closer to the Cu1 site than the Cu3b. According to the theoretical calculations performed by Kyritsis and coworkers [56] for ascorbate oxidase, the Cu1–Cu3a pathway should be up to three times more efficient than the Cu1–Cu3b, thus a differential reduction progress in TNC would result in the observed asymmetries.

Two solvent channels provide access to TNC, located in the interior of the protein structure. The first channel points towards the two Cu3 ions on one side of the TNC allowing the molecular oxygen to enter and to bind to it. The second channel pointing towards the Cu2 ion on the other side of the cluster permits water molecules produced in the O_2 reduction to move to the bulk solvent [23, 26, 27, 41].

Role of the C-terminal tail

A C-terminal protruding tail (13–14 amino acids long) has been found in the deduced amino acidic sequences of laccases from the ascomycetes *Podospora anserina* [57], *Neurospora crassa* [58], *M. albomyces* [59], and *M. thermophila* [60, 61]. This tail is generally cleaved off by proteolysis at a conserved cleavage site to produce the active form of the enzyme. Analysis of the 3D structure of the laccase from the fungus *M. albomyces* has shown this C-terminal extension as a plug obstructing the solvent channel [28], thus leading to the hypothesis that its cleavage is required to favor the entrance of oxygen and the subsequent exit of water molecules.

When Kiiskinen and Saloheimo [59] studied the expression of MaL1GWO in *S. cerevisiae*, they suggested that yeast apparatus is not able to process the C terminus correctly, whilst the introduction of a stop codon after the native processing site at the C terminus gives rise to an increase in laccase activity. Furthermore, Bulter and coworkers [60] reported the correct processing of the C terminus of *M. thermophila* laccase in *S. cerevisiae*, after the introduction by directed evolution experiments of a Kex2 protease site in the laccase sequence. Thus, it seems to be clear that carboxyl-terminal processing may play a role in the activation of ascomycete enzymes.

Zumarraga and coworkers [61] selected a *M. thermophila* laccase variant with two consecutive mutations in the C-terminal tail exhibiting an increase in the p*I* of the C terminus, while keeping constant the p*I* of the mature protein. The authors assumed that these mutations might contribute to a higher grade of tightness between the C-terminal extension and the main enzymatic core, which would affect the protein folding and therefore the final mature enzyme. Whether a similar role of the C-terminal tail is possible among basidiomycete laccases too is not yet known. Gelo-Pujic and coworkers [62] reported that the redox potential of the TvL1GYC basidiomycete laccase changes when its C terminus is truncated by 11 amino acids, thus suggesting that C-terminal amino acids can also affect the function of fungal laccases from basidiomycetes. An unusual C-terminal extension of 16 amino acids has been found in the POXA1b laccase from *Pleurotus ostreatus* [63], although a C-terminal processing of two to four amino acids has been observed for both the native and the heterologously expressed protein in *Kluyveromyces lactis* [64]. The POXA1b C-terminal tail has been found mutated in one of the selected random mutants from directed evolution experiments [65]. The P494T mutation is located in a variable and mobile loop at the C terminus. Molecular dynamic simulations on 3D model structure of this mutant have shown the mutation affects the flexibility of some regions of the protein, thus leading to an improved stability and activity of the enzyme.

Laccase reactivity

Phenols are typical laccase substrates because their redox potentials (ranging from 0.5 to 1.0 V vs. NHE) are low

enough to allow electron abstraction by the Cu1. Commonly used phenolic substrates are syringaldazine, DMP and guaiacol; however, laccases are also able to oxidize electron donor substrates such as ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] or ferrocyanide [$K_4Fe(CN)_6$] [14, 15, 63]. Phenolic substrates are oxidized to phenoxy radicals, which, depending on reaction conditions, can spontaneously polymerize via radical coupling or rearrange themselves leading to quinones (through disproportionation), alkyl–aryl cleavage, Cα oxidation, cleavage of Cα–Cβ bond or aromatic ring [66].

Xu performed a comparative study with several fungal laccases for the oxidation of a series of phenols, anilines, and benzenethiols [67]. The observed catalytic efficiencies were found to be correlated to the structure of the substrate and to the one-electron redox potential difference between the laccase Cu1 and substrate. Garzillo et al. [68] observed an increase in the oxidation rate of different substrates by a laccase from *T. trogii* when two *o*- and *p*-oriented groups were present simultaneously. As reported above, the relative contribution of steric and redox properties of a substrate in determining its susceptibility to laccase oxidation has been recently assessed by Tadesse and coworkers [46].

Because of their high nonspecific oxidation capacity, and because they use readily available molecular oxygen as an electron acceptor, laccases are useful biocatalysts for a wide range of biotechnological applications. Thus, they find potential applications in pulp delignification and biobleaching [69], treatment of industrial plant wastewater [70], enzymatic modification of fibers and dye-bleaching in the textile and dye industries [71], enzymatic removal of phenolic compounds in beverages, fruit juice processing [72], and biosensor and biofuel cell construction [73]. Several recent studies have been focused on dye degradation by laccases. Most of these works used commercially available dyes as model pollutants [74]. Laccase potential in the decolorization of recalcitrant azo dyes, such as those commonly used in the leather industry, has been assessed [75]. A crude laccase mixture preparation from *P. ostreatus* is able to decolorize the Remazol Brilliant Blue R (RBBR) anthraquinonic dye [76] achieving a maximum of 70% decolorization. Moreover, the same preparation can be re-used several times when immobilized in copper alginate beads [77]. The role of laccase in cytotoxicity reduction during dye decolorization has also been demonstrated [78]. Nevertheless, real industrial effluents usually include mixtures of several dyes, and only limited data are now available on mixed dye degradation. The degradation of a mixture of reactive dyes, simulating a real textile effluent, has been successfully tested in the framework of the SOPHIED EU project (NMP2-CT-2004-505899, 6FP), indicating the possibility of implementing this technique for the treatment of textile-dyeing wastewaters. Real effluents have also been treated and the removal of the mutagenic character of the effluent has been achieved [79].

Laccases might also be useful in synthetic chemistry [80]. For instance, laccase-catalyzed reactions can be used for the polymerization of catechol monomers for polycatechol synthesis [81] or for the production of inert phenolic polymers, such as poly(1-napthol) [82]. These polymers have application in wood composites, fiber bonding, laminates, coatings, and adhesives. A novel system of enzymatic polymerization for the preparation of "artificial urushi" polymeric films (Japanese traditional coating) has been demonstrated, using a laccase-catalyzed cross-linking reaction [83]. Laccases have been used to synthesize dyes [84] and products of pharmaceutical importance, such as the anticancer drug actinocin [85] or the phytoalexin resveratrol [86].

Substrates characterized by high redox potential cannot be oxidized directly by laccases. Nevertheless, laccases are considered to play a major role in the lignin degradation by some white-rot fungi [4]. Suitable compounds, the so-called mediators, acting as intermediate substrates for laccases, enable laccase to indirectly oxidize large molecules and even nonphenolic substrates, such as nonphenolic β-1 lignin model dimer [87, 88]. The first mediator used in the laccase-mediator system (LMS) for pulp delignification was ABTS [88]. Since then about 100 molecules have been tested for their ability to oxidize lignin or lignin models [89, 90]. An effective redox mediator should be a good laccase substrate, its oxidized radical form should have a half-life long enough to permit its diffusion towards the substrate, and possess high oxidation potential to effectively oxidize it. Xu and coworkers [91] have shown that the activity and stability of N–O· radicals seem to be better balanced in comparison with those of phenoxy radicals. Indeed the most effective mediators for lignin degradation have proved to be the *N*-heterocycle-bearing N–OH groups and in particular 1-hydroxybenzotriazole (HBT).

Laccase/mediator-catalyzed oxidations of nonphenolic substrates can proceed via two different mechanisms. ABTS-mediated reactions follow an ET route (Fig. 6a), whereas the >N–OH-type mediators, such as HBT, follow a hydrogen atom transfer (HAT) route by abstraction of a hydrogen atom from the >N–OH-type mediators, producing a >N–O· radical species (Fig. 6b). The former mechanism requires substrates with a low oxidation potential; the latter mechanism requires substrates with relatively weak C–H bonds [92]. The characterization of the radical intermediates (neutral or cationic) formed from both mediators after the enzymatic oxidation has been recently obtained through a multifrequency EPR approach [93].

The LMS has been demonstrated to be efficient for degradation of aromatic contaminants [94], paper pulp

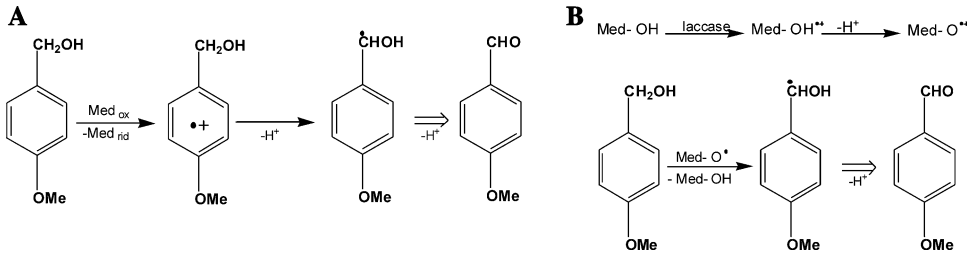

Fig. 6 Laccase/mediator-catalyzed oxidations of nonphenolic substrates via ET route (**a**) and radical HAT route (**b**), adapted from [92] with the author's permission

bleaching [95], pitch control [96], and dye decolorization [97]. An LMS process has been introduced in the textile industry for indigo oxidation on denim garments in industrial laundries—the dye chromophore is split to yield two molecules of uncolored compounds [98].

Utilization of synthetic mediators in industrial processes is hindered by their high cost and the possible generation of toxic species. The availability of low cost and environmentally friendly natural mediators could facilitate their application in white-biotechnological processes. Indeed, the ability of some lignin-derived phenols to mediate laccase decolorization of dyes [99], removal of lignin from paper pulps [100], and oxidation of PAHs [99, 101] has been proved. As a matter of fact, the efficiency of *p*-coumaric acid as a natural laccase mediator has been higher or similar to that of synthetic mediators in benzo[a]pyrene degradation [101].

Jeon and coworkers [102] compared the mediating capabilities of individual mediators with those of dual-agent mediator cocktails containing ABTS, vanillin, and/or acetovanillone in the oxidation of pentachlorophenol (PCP) by *Ganoderma lucidum* laccase. Cocktails strongly promoted PCP removal compared to the use of each of the mediators alone. *G. lucidum* laccase was very prone to react with ABTS rather than vanillin and acetovanillone in the cocktails. Moreover, the presence of the ABTS radical ($ABTS^{+\cdot}$) and vanillin or acetovanillone significantly enhanced PCP removal concomitantly with ET from vanillin or acetovanillone to $ABTS^{+\cdot}$. These results strongly suggest that vanillin and acetovanillone mediate the reaction between ABTS and PCP via multiple sequential ET among laccase and its mediators.

Atypical laccases

Laccases with unusual molecular weight

Laccases with regular three domains usually have a molecular mass of 50–70 kDa or larger, whereas a two-domain MCO weighs between 30 and 40 kDa [103]. Two-domain laccases have been isolated from *Botrytis cinerea* [104] and from fresh fruiting bodies of *Tricholoma giganteum* [105]. The latter enzyme shows an N-terminal sequence with no homology to those of other mushroom laccases and is able to inhibit HIV-1 reverse transcriptase. Because of their small molecular mass, the two-domain laccases could need to assemble into a quaternary architecture to properly work. However, the reported data suggest that these enzymes work as monomers.

Laccases with quaternary structure

While most laccases are built up and act as monomers, oligomeric laccases are also known. Thus, enzymes exhibiting a homodimeric structure composed of two identical subunits have been isolated. This is the case for *T. villosa* [106] and *Phellinus ribis* [107], for the phytopathogenic ascomycetes *Rhizoctonia solani* [108] and *Gaeumannomyces graminis* [109], and for the aquatic ascomycete *Phoma* sp. UHH 5-1-03 [110]. The latter enzyme undergoes a pH-dependent dimerization, with the dimer predominating in a pH range of 5.0–8.0. Homodimeric laccases whose subunits account for two domain laccases have been purified from *Pleurotus pulmonarius* [18], *Pleurotus eryngii* [111], and from the mycorrhizal fungus *Cantharellus cibarius* [112]. These enzymes seem to need dimerization to exploit their function. Moreover, a trimeric arrangement of two-domain chains seems to be essential for some bacterial laccases to ensure a geometry of the active site similar to that of the usual MCOs [113–115]. The quaternary architecture of laccases isolated from the basidiomycetes *Agaricus bisporus* D621 [116] and *Armillaria mellea* [117], and from the ascomycetes *P. anserina* (a tetramer of 390 kDa) [118], *Aspergillus nidulans* [119], and *Monocillium indicum Saxena* [120] has been assumed, even if not deeply investigated.

Lac2 from *A. bisporus* consists of a predominant chain of about 65 kDa and several smaller polypeptides. Perry and coworkers [121] have hypothesized that the enzyme is produced as a dimer of identical polypeptides, one of which is then partially proteolytically cleaved. The heterodimeric laccases POXA3a and POXA3b purified from *P. ostreatus* [122] are comprised of a large (67 kDa) and a small subunit (18 or 16 kDa), whose heterogeneity is due to the presence or absence of a glycosidic moiety [123]. The POXA3 large subunit is clearly homologous to fungal

laccases showing all known consensus sequences involved in copper binding but displaying some peculiarities. The well-conserved Asp206 is replaced by an Arg residue, as already found in the above-mentioned *A. bisporus* Lac2 [116]. It is worth noting that phylogenetic analysis of laccases revealed that heterodimeric POXA3 and Lac2 fall in the same cluster [124], in contrast to all the other members of the *P. ostreatus* laccase family [125]. On the other hand, the sequence of the small subunit does not show significant homology with any sequence in data banks, therefore no indication of the function of this subunit can be inferred from its primary structure. A potential role of the POXA3 small subunit in the stabilization of the heterodimer has been assumed [126]. The same role of quaternary structure in stabilizing laccases from *T. villosa* and *R. solani* has been briefly mentioned by Xu and coworkers [36]. Moreover, studies on ascorbate oxidase have allowed a stabilizing and functional role to be ascribed to the homodimer formation, as well as a role in driving the final folding to the native conformation [127, 128].

Laccases with unusual spectral properties

Although laccases usually contain four Cu atoms and show a UV absorption peak at around 600 nm, some laccases lacking the Cu1 characteristic absorption spectrum have been isolated. These laccases have been called "yellow" or "white," although some authors do not regard them as true laccases. However, considering laccases as enzymes able to oxidize polyphenols, methoxy-substituted phenols, aromatic diamines, and a range of other compounds but not tyrosine, both "yellow" and "white" laccases can be defined as true laccases.

Yellow laccases have been purified from the phytopathogenic ascomycete *G. graminis* (oligomeric) [109] and from the basidiomycetes *A. bisporus* D621 (oligomeric) [116], *Schizophyllum commune* [129], *Panus tigrinus* [130], *Phlebia radiata* [131], *P. ribis* (oligomeric) [107], and *P. ostreatus* D1 [132, 133]. Yellow laccases from *P. tigrinus* and *P. ostreatus* D1 are able to oxidize nonphenolic aromatic substrates in the absence of mediators, contrary to their blue counterparts [130, 132, 133]. Leontievsky and colleagues [130] have supposed that yellow laccases are formed as a result of binding of aromatic products of lignin degradation to the blue laccase during fungal growth in solid-state conditions. A consequence of this modification may be the reduction of Cu1 and Cu2 in the active center of the enzyme and the disappearance of the blue color. Apparently, the modifier molecule bound to the apoenzyme of the yellow laccase performs the function of ET mediator in the reaction with nonphenolic substrate.

The first purification of a white laccase was obtained from the basidiomycete *P. ostreatus* [134]. POXA1w is a neutral laccase showing a remarkably high stability with respect to both pH and temperature. The enzyme contains only one copper atom/molecule instead of the usual four, along with two zinc atoms and one iron atom in each protein molecule. Other so-called white laccases were purified from *Pycnoporous sanguineus* [135], from *T. hirsuta* [136], and from *P. radiata* BP-11-2 [137]. The analysis of metal ion contents of the laccase from *T. hirsuta* has shown the presence of copper and manganese in a 3:1 ratio. This laccase is able to oxidize the hydroxy polyaromatic dye alizarin red S and the nonphenolic dye methyl red without mediators.

Other atypical laccases are secreted by the basidiomycete *Steccherinum ochraceum* strain 1833 [138]. Their absorption band around 600 nm shows values of molar extinction coefficients (ranging from 7,200 to 7,800 M^{-1} cm^{-1} at 611 nm) higher than those of other laccases and a lower shoulder of absorption at 330 nm. These features can be the result of slightly different coordination geometries of the Cu1 and Cu3 ions. Both isoforms from *S. ochraceum* 1833 show high optimal temperatures and significant thermostability.

In conclusion, results obtained on these laccases highlight, on the one hand, their ability to oxidize nonphenolic substrates and, on the other hand, their high stability or functionality in extreme conditions. The properties of nonblue laccases give rise to many questions. For example, is it possible to link the peculiarity of these enzymes to an explicit function? If transition metals are simply replacing the positions of copper in laccases, then which ion takes which position? Moreover, if some copper ions of laccases can be replaced with other metals, can copper ions in other MCOs be replaced without losing the function? Nakamura and Go [103] propose that the Cu1 may be replaced with Fe/Mn (losing the blue color) and the Cu3 may be replaced with zinc ions, with the Cu2 remaining.

Laccase gene families

Multiplicity of laccase genes is a common feature in fungi, and the production of several laccase isozymes has been observed in many species. Perry and coworkers [121] described the presence of two laccase genes in the same chromosome of the basidiomycetes *A. bisporus*, thus reporting the first example of a laccase gene family in fungi. Five distinct laccase genes have been characterized from *T. villosa* [106] and *Trametes sanguinea* [139], four from *R. solani* [108], and three from *Trametes* sp. I62 [140], *Trametes* sp. AH28-2 [141], and *G. graminis* [142]. Laccase gene families have also been described in *Pleurotus* genera, with four isolated members in *P. sajior-caju* [143], two in *P. eryngii* [144], and seven in *P. ostreatus*

[125]. Characterization of laccase gene families has been enhanced by the availability of a growing number of fungal genome sequences. In the *Coprinopsis cinerea* genome, 17 nonallelic laccase genes were identified and at least 9 of these members are translated into functional laccase products [145]. Likewise, the occurrence of laccase genes in brown-rot fungi has been identified with the discovery of two laccase gene models in the *Postia placenta* genome [146]. Investigation of the ectomycorrhizal fungus *Laccaria bicolor* genome disclosed a complex MCO family composed of 11 members, 9 of which correspond to laccases *in sensu stricto* [147]. The analysis of the recently released *P. ostreatus* genome has highlighted the presence of previously uncharacterized laccase genes, enriching the panel of laccase genes up to 12 members (unpublished data).

Gene clustering of laccase genes has been observed in many of the above-mentioned examples. The two tandem organized laccase genes from *A. bisporus* have been mapped 1.5 kb apart [121], and the three genes in *R. solani* are linked within a 12-kb fragment [108]. A genomic fragment of 150 kb has been shown to contain seven laccase genes in *P. ostreatus* [125]. The 17 laccase genes of *C. cinerea* are clustered at seven different loci in the genome [145], whereas the nine laccase-encoding genes from *L. bicolor* are randomly distributed throughout the genome, except for three clustered genes, suggesting a more complex story of gene duplication events [147]. Based on intron composition and protein similarity, laccase subfamilies have been identified within several gene families [106, 125, 139, 145].

The occurrence of such complex gene families gives rise to a key question: why should a fungus require more than one laccase? A plausible explanation can be put forward considering the variety of different physiological functions proposed for this enzyme during the fungal life cycle. Fungal laccases have been associated with delignification [124], fruiting body formation [148], pigment formation during asexual development [149], pathogenesis [142, 150], and competitor interactions [151]. Laccases of saprophytic and mycorrhizal fungi have also been implicated in soil organic matter cycling [152]. It can be inferred that the paralogous laccase copies within the same species may have specifically evolved to fulfill a variety of targeted functions. The phylogenetic analysis of basidiomycetous laccases further supports this idea, since clustering of the sequences in the neighbor-joining tree was found to reflect, at least in part, the function of the respective enzymes [124].

Laccase gene transcriptional regulation

Synthesis and secretion of laccases are strictly influenced by nutrient levels, culture conditions, and developmental stage as well as by the addition of different inducers to cultural media. The effect of these factors at the level of laccase gene transcription has been demonstrated in many fungal species. In most of the reported examples, laccase expression is regulated by an array of factors, often acting in a synergistic way [153–155].

Metals

Regulation of laccase expression by metals is widespread in fungi. Copper has been shown to regulate transcript levels in *T. versicolor* [156], *C. subvermispora* [157], *P. ostreatus* [158], *P. sajor-caju* [143], and *Trametes pubescens* [155]. Cd^{+2}, Ag^{+}, and Mn^{+2} ions have also been reported as strong modulators of laccase transcription levels [143, 159]. It is worth noting that the same metal can exert opposite effects in different species [160]. In many cases, the observed effect is associated with the presence of putative metal-responsive elements (MRE) in the laccase promoter regions. However, only a few reports go through the molecular mechanisms underlying laccase regulation by metals. In *P. ostreatus*, the formation of protein complexes on specific MRE elements identified in the promoter regions of two copper-induced laccase genes *poxc* and *poxa1b* [158] has been found to occur only in the absence of copper ions. Thus, the involvement of a negative-acting regulatory factor in metal response has been proposed. Moreover, in *poxa1b* promoter, a GC-rich region homologous to the core binding site for transcription factor Sp1 has been found to decrease the binding affinity of the adjacent MRE, affecting its interactions with fungal protein factors [161].

In silico inspection of some laccase promoters has highlighted the presence of several putative "activation of *cup1* expression" (ACE) responsive elements. First described in *S. cerevisiae*, ACE is the recognition site for the ACE1 copper-responsive transcription factor, which binds its target sequence in a copper-loaded conformation, activating the transcription of several genes [162]. Fungal ACE1-like transcription factors have been isolated in *C. subvermispora* [163]. It is worth noting that copper induction has been observed for laccase genes whose promoters lack ACE or MRE elements, thus a different mechanism, which might involve the generation of reactive oxygen species, has been hypothesized [156].

Aromatic compounds

Aromatic compounds structurally related to lignin, such as xylidine (XYL), ferulic acid (FA), or veratric acid (VA), are routinely added to fungal cultures to increase laccase production [106, 156, 164]. Laccase induction has been also demonstrated in the presence of dyes, the induction

level being highly sensitive to small differences in the chemical structures [165]. Laccase induction by phenolic substances may represent a response developed by fungi against highly reactive aromatic compounds—by catalyzing their polymerization, laccases play a defensive role, reducing the oxidative stress caused by oxygen radicals that originate from these molecules [4]. In many cases, the induction has been found to occur at the transcriptional level, in agreement with the finding of putative xenobiotic response elements (XRE) in the upstream regions of several induced laccase genes, such as those from *T. versicolor* [156], *P. sajor caju* [143], *P. ostreatus* [161], and *Trametes* sp. AH28-2 [141]. White-rot fungi display a wide diversity in their response to aromatic inducers. Transcription of one laccase gene, *lcc1*, from *T. villosa* is induced by the addition of XYL, but a second gene is constitutively expressed under tested conditions [106]. Likewise, *lcc1* transcript titres in *Volvariella volvacea* are differentially tuned by the addition of various aromatic compounds, whereas *lcc4* transcription is not affected by these molecules [148]. Laccase induction in various fungi seems to be specific to certain aromatic compounds [141, 143]. In *T. versicolor*, XYL and HBT effectively activate *lcc* transcription, whereas no induction is observed in the presence of either VA and FA [156]. In the ligninolytic basidiomycete *Trametes* sp. I62, nine structural closely related aromatic compounds appear to have different effects on laccase gene expression. The three laccase genes of this fungus are differentially expressed in response to these compounds, with specific induction patterns being observed for each molecule tested [153].

Nitrogen and carbon sources

Laccase activity has also been shown to be dependent on the concentration and nature of carbon and nitrogen sources as well as on their ratio [166]. As far as nitrogen sources are concerned, change in laccase activity in response to nitrogen concentration is a controversial issue, since examples of activity increases have been described under both limiting and nonlimiting conditions [167]. Laccase induction at the transcriptional level has been reported to occur under nonlimiting nitrogen levels in *T. versicolor* [4] and in the ligninolytic basidiomycete I62 [167]. Expression of *lcc1* in *T. trogii* is preferentially induced by organic nitrogen sources compared with inorganic ones [168]. Individual laccase isoenzyme genes in *P. sajor-caju* are differentially induced at the transcriptional level by nitrogen sources. Positive regulation by nitrogen has been found to occur in *lac2* and *lac4*, whereas *lac1* and *lac3* are largely unaffected [143]. The observed N-regulation may be mediated by a NIT2-like protein (NIT2 is involved in N-regulation in *N. crassa*) since NIT2-binding sites have been found in the upstream region of *lac4* in *P. sajor-caju* and of *lcc1* and *lcc2* in basidiomycete I62 [167]. Laccase expression has also been found to be subjected to catabolite repression. High glucose levels inhibit laccase transcription in basidiomycete I62 [167], *T. pubescens* [155], and *Trametes* sp. AH28-2 [141]. Putative CreA-binding sites have been mapped in the promoter regions of these repressed laccase genes suggesting the existence of a carbon catabolite repressor similar to the CreA isolated in *A. nidulans* [169].

Fungal growth stages

Several reports support the hypothesis that members of the laccase families may play different roles during the life cycle of the organism [154]. Both *lcc1* and *lcc2* transcriptions in *Trametes* sp. I62 are inducible at different growth stages—*lcc1* is expressed in early stages of growth and *lcc2* in the stationary phase [167]. In *A. bisporus* and *L. edodes* growing on solid substrates, laccase expression has been found to be greatest at the fully colonized stage and then declining to very low levels during fruiting [170, 171]. This expression profile is in accordance with a direct involvement of laccase activity in lignin bioconversion, which is expected to be required in the earlier stages of substrate colonization. On the other hand, in *V. volvacea* a key role in the morphogenesis of its fruiting body has been inferred for *lcc1*, since laccase expression is induced later in the substrate colonization phase and at the onset stages of fruiting body morphogenesis (primordium formation, pinhead stage) [148]. It has been proposed that laccase could crosslink hyphal walls into coherent aggregates during primordium formation and may continue to act on the hyphal surfaces throughout fruiting body development. A strong correlation between laccase expression and fungal morphogenesis has been determined by transcript analysis of the MCO gene family in the *L. bicolor* ectomycorrhizal fungus [147]. Transcript profiling has unveiled that *L. bicolor* laccase genes are differentially expressed as a function of the developmental stage. Some transcripts accumulate in the fruiting bodies, others are believed to play a role in the functioning of fungal symbiotic tissues, and some others are greatest in the free-living mycelium grown on agar medium.

Pathogenesis

Expression studies providing evidence for laccase involvement in pathogenesis have been reported. In the root pathogen *G. graminis*, *lac2* has been found to be expressed only in planta or in the presence of plant homogenate, thus providing the first evidence of a fungal laccase whose transcription depends on the presence of a host [142]. The authors have proposed a specific role for

this laccase in plant pathogenesis, suggesting that it could degrade lignin depositions produced by the plant in response to invasion, and/or oxidize/reduce phytoalexins and other toxic compounds.

Laccases have also been described as prominent virulence factors in *Cryptococcus neoformans*, a pathogenic yeast causing meningoencephalitis [150]. The expression of *C. neoformans* laccases has been found to be induced in response to reactive oxygen and nitrogen species produced by macrophages as a defense mechanism. Ssa1, a Hsp70 homologue identified in *C. neoformans*, interacts with a heat shock-factor (HSF) acting as a transcriptional co-activator of fungal laccase in response to the attack encountered in the host [172]. Missal and coworkers [150] have also reported the importance of two *C. neoformans* laccase isoforms in fungal virulence, suggesting that independent regulatory mechanisms may control the expression of these two genes during nitrosative but not oxidative stress.

Laccases are ancient enzymes with a promising future. This class of enzymes still remains of great relevance both as a model for structure/function relationships and as a green tool in many processes of the biotechnology industries in the near future.

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