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A molecular method to evaluate basidiomycete laccase gene expression in forest soils

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Abstract

Fungal laccases, exo-enzymes lacking high substrate specificity, play a central role in cycling of soil organic matter. In a precedent work based on a PCR technique on soil DNA extracts, we showed that the highest diversity of laccase genes occurred in the most organic horizon (i.e., O_h) of a brown forest soil. In the present article, we develop a method of RNA extraction, RT-PCR, and semi-quantitative PCR to analyze the expression of Basidiomycete laccase genes. We performed a very first assessment of the methodological approach on five cores of the O_h horizon of the same brown forest soil. The level of laccase transcripts was heterogeneous amongst the soil cores. Two samples gave strong expression levels, two showed very faint ones, and the last replicate presented no detectable laccase transcripts. A control with a transcript analysis of the actin gene, which is constitutively expressed in fungi, allowed to rule out that the differences in the transcript level of laccases were due to experimental failures or inhibiting substances in the RNA extracts. A cladistic analysis showed that most laccase transcript sequences detected were grouped in two clades closely related to the ectomycorrhizal fungus *Xerocomus chrysenteron*. Comparison between DNA laccase sequences found in our previous study and RNA laccase sequence profiles found here showed that less than 30% of the laccases genes detected in a soil core were expressed. This preliminary study demonstrates the potential of RT-PCR for gene expression profiling in forest soils. The number of analyzed samples cannot allow us to draw definitive ecological conclusions, but there are some indications that differences between rhizospheric and bulk soil samples (polyphenol abundances, microbial densities, etc.) might be a potential explanation for the variable laccase expression observed.
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Keywords: Laccases; Soil RNA extraction; RT-PCR; Semi-quantitative PCR; Basidiomycetes; Brown forest soil

1. Introduction

A great part of the terrestrial carbon (C) is bound as organic residues in soils (Killham, 1994). This C-pool is maintained quite constant by several processes, such as humification, recycling, and mineralization. Soil microorganisms have been shown to

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be largely involved in these processes, and their high biodiversity contributes to the soil function in ecosystems (Emmerling et al., 2002). As contribution to the present *Geoderma* special issue about the international conference "Mechanisms and Regulation of Organic Matter Stabilization in Soils," this article deals with a methodological development to characterize the expression in soils of fungal laccases, key enzymes involved in both mineralization and humification.

Fungi are one of the most important soil microorganism groups involved in the carbon cycle in many regards. With 40–200 g of mycelial dry matter per square meter of soil, they represent a dominant part of the soil biomass (Thorn, 1997; Dighton and Kooistra, 1993). Due to their mycelial structure, they can explore soil compartments and reach organic matter fractions not accessible to many other organisms (Read and Perez-Moreno, 2003). As partners of mycorrhizal symbioses, they contribute to the transfer of 10–20% of the global photo-assimilates into soils (Smith and Read, 1997). Based on their nutritional mode, fungi can be divided in three functional groups (saprophytes, symbionts, and parasites), in each of which many species are able to produce oxidative exo-enzymes playing an important role in the formation and decomposition of soil organic matter (SOM) (Chefetz et al., 1998; Read and Perez-Moreno, 2003).

Laccases, lignin, and manganese peroxidases are the main oxidative exo-enzymes produced by fungi (Gramss et al., 1998; Chen et al., 2003). Apart from a lack of substrate specificity common to other exo-enzymes, laccases have the widest distribution among fungi (Gramss et al., 1998). Both features allow them to be involved in many biosynthetic processes such as formation of humic acids (Chefetz et al., 1998) and biodegradation of complex organic and aromatic substances (Eggert et al., 1997). It was also shown that laccases can oxidize nonphenolic components in the presence of appropriate radical mediators (Bourbonnais et al., 1995, 1998) and so completely degrade lignin (Eggert et al., 1996).

The development of molecular methods opened new perspectives for investigating and understanding the function of microorganisms in soils. They in particular allow determining the respective role of target groups of microorganisms by characterizing more relevant species and by assessing whether a loss of diversity might disturb soil functions (Emmerling et al., 2002). Direct DNA extraction from soils and its use for PCR amplifications with specific primers has been developed from years for bacteria (Holben et al., 1988; Wintzingerode et al., 1997). Application of this method to fungi mainly focus on polymorphic regions of the ribosomal DNA to assess the diversity and spatial distribution of fungal communities in soils (Dickie et al., 2002; Tedersoo et al., 2003), but studies aiming to analyze protein-coding genes are scarce (Lyons et al., 2003).

In a precedent work (Luis et al., 2004), we optimized a PCR-based method on DNA extracted from different soil horizons of a brown forest soil to analyze the diversity of Basidiomycete laccase genes. The gene diversity profiling provided a glimpse of the oxidative potential of fungi with laccase genes. But, as all genes might not be continually expressed, their distribution gave no information on the real fungal laccase activity in the soil. The aim of the present study was to develop a procedure to analyze the expression of Basidiomycete laccase genes. This procedure was assessed on five soil samples on which the gene diversity had been characterized previously (Luis et al., 2004). This allowed comparing the already known diversity with the expression pattern revealed here. To our knowledge this kind of comparison was never reported for soil fungi previously. We also assessed the respective part of the ectomycorrhizal and saprophytic Basidiomycetes in the revealed expression pattern using a cladistic analysis of detected sequences.

2. Materials and methods

2.1. Samples of soils

Five cores (S1–S5) with 12 cm diameter and 35 cm height of a brown forest soil under a mixed oak beech forest of the "Steigerwald" (Northern Bavaria, 49°52′26″ N, 10°27′54″ E) that belongs to an experimental station of the Institute of Ecosystem Research (BITÖK) from the University of Bayreuth were randomly collected in September 2002 within a plot of 6×6 m. The position of the cores and their contact to the tree vegetation were noted. Two of the cores (S2

and S3) were densely colonized by tree roots indicating a high amount of rhizospheric soil, whereas the other three cores were more reflecting of a bulk soil fraction. In each soil core, the O_h was separated from the A_h and B_v horizons, transported in ice chests, and stored before use at −80 °C.

2.2. RNA extraction from soil samples and cDNA synthesis

For each of the five cores, the O_h horizon was homogenized and the total RNA was extracted from 1 g fractions. Each fraction was divided into four 0.25 g aliquots, which were suspended in 250 μl of DEPC (diethyl pyrocarbonate) treated deionized water. The tubes containing the aqueous aliquots were incubated for 1 h at −80 °C. Then, 0.5 g of glass beads (106 μm; Sigma), 33.3 μl of 20% SDS, 167 μl of 3% diatomaceous earth (Sigma, Taufkirchen, Germany), and 583 μl of Tris-buffered phenol solution (250 g of phenol in 75 ml of 0.5 M Tris–HCl, pH 8, 0.1% (wt/vol) 8-hydroxychinolin, and 0.2% (vol/vol) 2-mercaptoethanol) were added to the frozen aliquots before shaking 2.5 min at 2000 rpm s^{-1} on a Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany). After a centrifugation of 15 min at 14,000×*g* and 4 °C, the supernatants transferred into new tubes were mixed with 36.7 μl of 3 M sodium acetate and 478 μl of 100% ethanol solutions and incubated overnight at −20 °C. The nucleic acid pellets, obtained after centrifugation (15 min at 14,000×*g* at 4°C), were washed with a 70% ethanol solution, dried at room temperature, and dissolved in 25 μl of DEPC treated deionized water. The nucleic acid solutions were pooled and the total RNA was separated from the DNA using the RNA/DNA Midi kit (10) (Qiagen, Hilden, Germany) as recommended by the manufacturer. Before starting the purification of the extracted RNA with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manual instructions, active charcoal (Merck, Darmstadt, Germany) was added to the RLC buffer supplied (2.5 mg ml^{-1}). Moreover, during this RNeasy purification a DNAse step was added as recommended by the manufacturer. The purified DNA-free RNA was used as template in a RT-PCR to synthesized cDNAs. This reaction was performed with 3 μl of total RNA extract (0.18 μg μl^{-1}) by using the SMART™ PCR cDNA Synthesis Kit (Clontech, Heidelberg, Germany). As recommended by the manufacturer, the optimal number of RT-PCR cycles to strike a balance between maintaining transcript representation and reducing nonspecific background amplification was determined. Gel quantifications were done with the software ImageQuant version 5.0 (Molecular Dynamics, Amersham Biosciences).

2.3. Semi-quantitative PCR analyses

cDNAs corresponding to mRNA (transcripts) extracted from the O_h horizons were used as template in a PCR. Fragments of cDNA corresponding to transcripts of Basidiomycete laccase genes were amplified with the degenerate primers Cu1F (5′-CAT(C) TGG CAT(C) GGN TTT(C) TTT(C) CA-3′) and Cu2R (5′-G G(A)CT GTG GTA CCA GAA NGT NCC -3′) optimized previously (Luis et al., 2004). For PCR amplification, 1 μl of the cDNA was added to a 50 μl reaction mixture containing 5 μl of 10× *Taq* buffer with $MgCl_2$ (Q-BIOgene, Heidelberg, Germany), 4 μl of dNTPs (10 mM each) (MBI Fermentas, St. Leon-Rot, Germany), 1 μl of each primer (60 μM), and 0.2 μl of *Taq* DNA polymerase (Q-BIOgene, Heidelberg, Germany). All reaction mixtures were overlaid with two drops of sterile oil and run on a Master cycler gradient system (Eppendorf, Hamburg, Germany) with an initial cycle of denaturation (3 min at 94 °C) followed by 35 cycles with denaturation (30 s at 94 °C), annealing (30 s at 50 °C), and elongation (2 min at 72 °C), and by a final elongation (10 min at 72 °C). A control reaction without template was systematically run to rule out the presence of contaminant DNA.

Additionally to the expression analysis of the laccase genes, the synthesized cDNA was used in a PCR to detect fungal actin, a constitutively expressed gene. This control reaction was performed to rule out that the lack of detection of a laccase gene expression in some samples did not result from a reaction inhibition. For this control PCR, the degenerate primers Act1 (5′-GT(C,A)C ACC AGG GTG TNA TGG TCG-3′) and Act2 (5′-GCC AGA TCT TCT CCA TGT CA(G)T CCC-3′) were designed. *Collybia fusipes* DNA was also used as a positive control to rule out technical mistakes during the laccase and actin PCRs.

For each amplification product, 7 μl was loaded onto a 2% agarose gel (Applichem, Darmstadt, Germany) and electrophoresed in Tris–acetate–EDTA, pH 8, buffer for 45 min at 80 V cm^{-1}. The 100 bp DNA size ladder mix (MBI Fermentas, St. Leon-Rot, Germany) was run in a separate lane. The gels were stained with ethidium bromide and the DNA bands were visualized and photographed under UV light.

2.4. Sequence analysis

PCR products were directly cloned into the pCR 4-TOPO vector, using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies, Karlsruhe, Germany) as described by the manufacturer. The plasmid DNA, containing the PCR product, was extracted from 1.5 ml of *Escherichia coli* TOP10 chemically competent cells by using the Perfectprep plasmid mini kit (Eppendorf, Hamburg, Germany). Cloned products were then sequenced in both directions with M13 reverse (5′-CAGGAAACAGC-TATGAC-3′) and M13 forward (5′-GTAAAAC-GACGGCCAG-3′) fluorescent primers and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK) on a DNA sequencer Long Reader 4200 (LI-COR, Nebraska, USA).

The cDNA sequences obtained for the soil RNA extracts have been deposited at EMBL under accession nos. AJ580828–AJ580837. These transcript sequences were compared in a cladistic analyses to reference sequences of three sources: (1) the deduced coding regions of the laccase gene fragments previously obtained from our DNA investigation on the same soil (Luis et al., 2004); (2) the deduced coding regions of the laccase gene fragments amplified by ourselves from both fruit bodies collected at the site and derived cultures or from a culture collection; and (3) the deduced coding regions of the laccase gene sequences retrieved from GenBank database.

Search for sequence identity in the GenBank DNA database was conducted by Gapped BlastN (NCBI) (Altschul et al., 1997). The 14 cDNA sequences from the present survey were aligned with the deduced coding regions of the DNA sequences obtained from the soil samples (44 sequences), the fruit body and culture collection (51 sequences), and GenBank (13 sequences), using the program MultAlin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalinan.html) (IBCP, Lyon, France) (Corpet, 1988). The resulting alignment was optimized visually. Only unambiguous alignment was used in the phylogenetic and distance analyses. The aligned sequences were exported to a NEXUS file and analyzed using PAUP*4.0b10 (Swofford, 1998). The final alignment of the laccase coding regions has a total of 100 unambiguously aligned sites and 94 characters were parsimony-informative. The final alignment of laccase sequences is available on request. The final tree was constructed using the neighbor-joining (NJ) method. The approach selected for the analysis of the molecular data set was the Kimura two parameters (Kimura, 1980). All characters were treated with equal weight and the bootstrapping was performed with 1000 replications. The final result was compared to the 50% majority-rule consensus tree using maximum parsimony (MP) analysis. The MP tree was performed using heuristic search option with 50 random sequence additions and tree bisection reconnection (TBR) branch-swapping (Swofford, 1998). The tree was drawn with CorelDraw 11. Laccase sequences of the ascomycetes *Colletotrichum lagenarium* (GenBank accession no. AB055709), *Gaeumannomyces graminis* (GenBank accession no. AJ437319), and *Botryotinia fuckeliana* (GenBank accession no. AF243855) were used as outgroup to root the tree.

3. Results

3.1. Semi-quantitative PCR analysis on the soil cDNAs

The cDNAs were synthesized by RT-PCR on the total RNA extracted from the O_h horizon of each soil core. In two independent repeats, the exponential phase of cDNA synthesis allowing a positive correlation between transcript quantity in soil extracts and signal intensity on agarose electrophoresis gels was shown to be obtained for 21 RT-PCR cycles (Fig. 1). The two primer pairs Cu1F/Cu2R and Act1/Act2 were used to, respectively, amplify Basidiomycete laccase and fungal actin fragments from these cDNAs. PCR fragments of 142 and 144 bp were obtained for laccase and actin, respectively (Fig. 2). As many

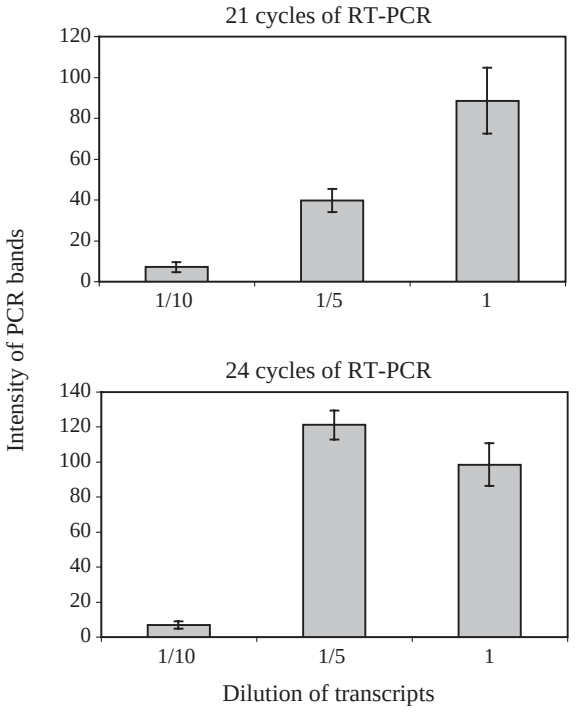


Fig. 1. Correspondence between the potential transcript quantity and the PCR fragment intensity. PCR was performed with the cDNAs, respectively, synthesized by 21 and 24 cycles of RT-PCR on the same soil RNA extracts. The PCR products were separated on 2% agarose gels and their intensity quantified using ImageQuant software. Number (1) was given to the cDNA extracts not diluted; (1/5) and (1/10) correspond to the dilution factors tested. A value of 100 was given to the PCR product intensity obtained for the first undiluted extract of the two independent repetitions. Histograms represent the mean values, and bars standard deviations.

laccase gene fragments contain introns in their coding region, which would have given longer PCR products, the absence of additional larger bands on the gels allowed us to rule out any contamination with residual genomic DNA in both reactions. Under the optimized semi-quantitative PCR, the Cu1F/Cu2R primers gave bands of different intensities for the five compared soil RNA extracts (Fig. 2a). The extracts from samples S2 and S3 gave intensive bands, whereas those from samples S4 and S5 were very faint. No PCR product was detected with the RNA extract from sample S1 (Fig. 2a). By making additional PCRs with the Cu1PCR/Cu2PCR primers under less stringent conditions (increasing the number of cycles to 40 and the annealing time to 2 min), intensive 142 bp bands could be obtained for the RNA extracts S4 and S5. However, additional fragments having no compatible size with laccase transcripts indicated a loss of specificity in the PCR (data not shown).

Under the stringent conditions, the semi-quantitative PCR of actin transcripts gave bands of equal intensity for the five soil samples (Fig. 2b). This result allows to exclude that the variable band intensities for the expression analysis of the laccase genes were due to factors inhibiting the cDNA synthesis in some of the replicates. The variable band intensities likely reflected differences in the expression level of the laccase genes in the five soil cores, indicating a spatial heterogeneity of the gene expression in the analyzed forest soil.

3.2. Comparison between the distribution of laccase genes and laccase transcripts

For samples S2 and S3, the intensive fragments obtained by PCR on the transcripts (soil mRNA) were

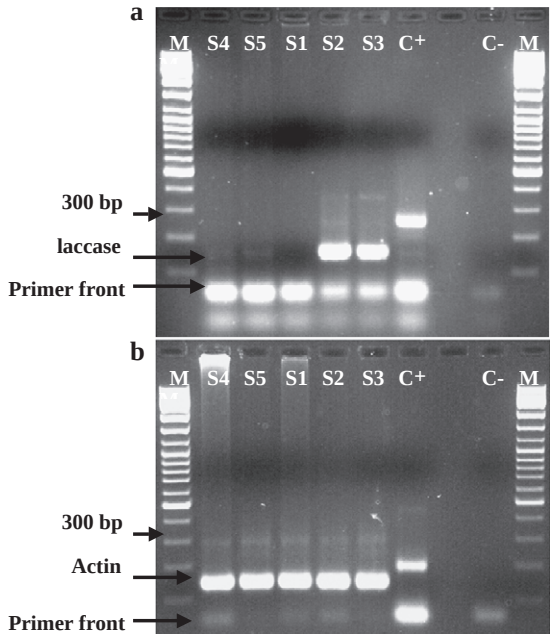


Fig. 2. Semi-quantitative PCR on laccase (a) and actin (b) cDNAs synthesized from total RNA isolated directly from five samples (S1–S5). PCR products were separated using 2% agarose gel electrophoresis. (C+) and (C−) correspond to a 250 bp fragment obtained by PCR on the genomic DNA of *Collybia fusipes* and a negative control without genomic DNA or cDNA, respectively. The 100 bp DNA ladder mix (MBI Fermentas) is represented by the letter (M).

cloned into bacteria, and 25 clones per sample were sequenced. A total of 14 different laccase transcript sequences were revealed. Respectively, six sequences were specific to each core S2 and S3, which shared two additional sequences. From this set of 14 sequences, the two shared transcripts corresponded to genes also detected in the O_h horizon of S2 and S3 in the analyses on DNA soil extracts (Luis et al., 2004): the first transcript to a gene sequence deposited on GenBank under AJ540285 and matching closely to *Xerocomus chrysenteron* (see Fig. 3); the second transcript to a deposited sequence (AJ540297) included in a clade without identified fungus but related to boletaceae (see Fig. 3). Additionally, one transcript sequence of S3 (AJ540255) also closely matched to a *X. chrysenteron*. This laccase had already been detected in this sample at the genomic level (Luis et al., 2004). In S2, one transcript fragment (AJ420347) belonged to the saprophytic fungus *Lepista nuda*. The remaining five different transcript fragments in each soil sample corresponded to new sequences not detected in our previous analysis on DNA extracts at the site.

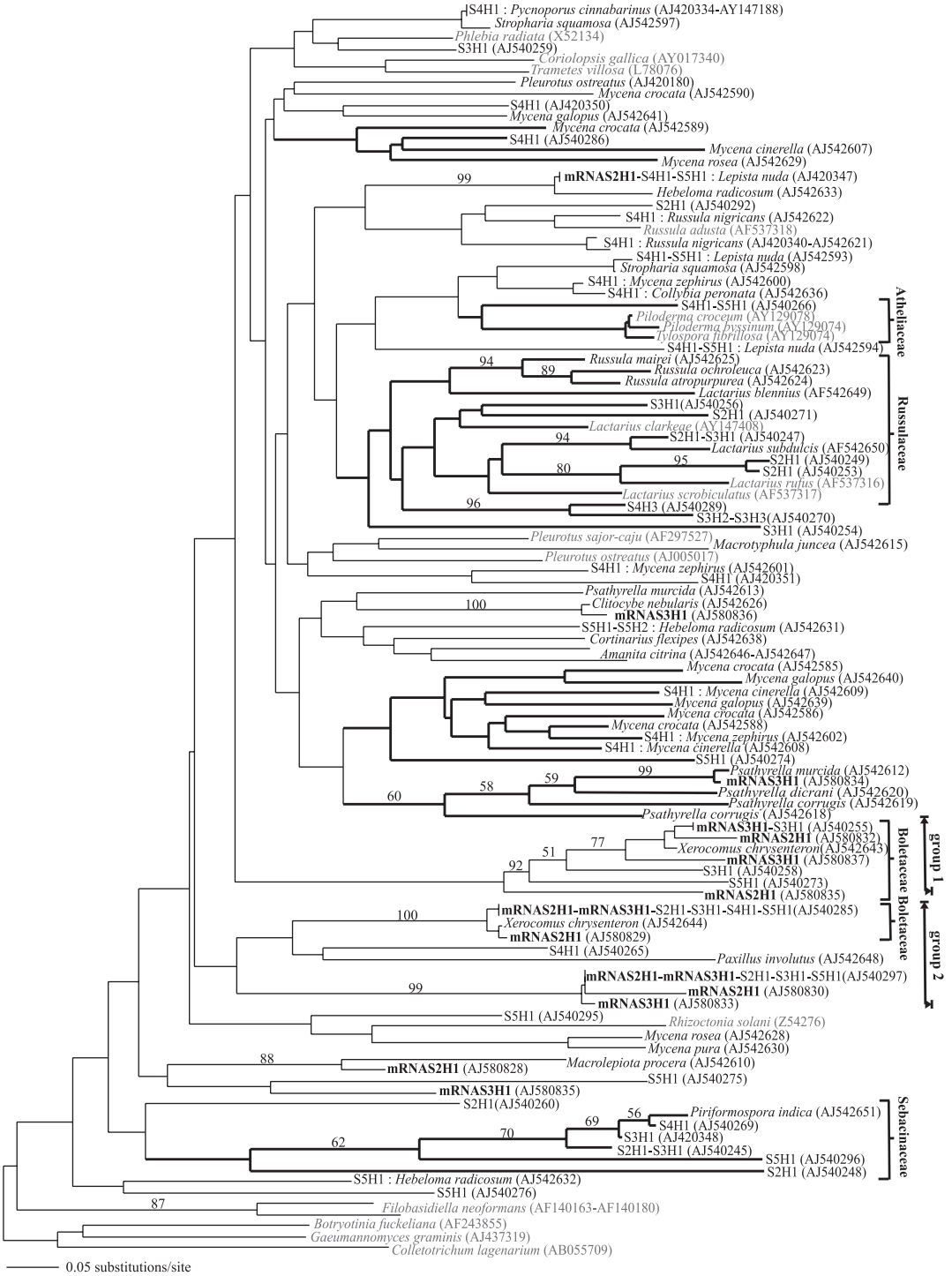
As 11 different laccase genes had been found with the DNA extracted from the O_h horizon of each sample S2 and S3 (Luis et al., 2004), the expression ratios revealed by the RNA analyses were of 18.2 (2/11) and 27.3% (3/11), respectively.

The 14 transcript sequences were included in a NJ analysis with the closely related deduced coding regions of laccase gene fragments obtained from the DNA extracts of identified fungi or soil samples and from the GenBank database. All transcript fragments sequenced appeared to be closely related to Basidiomycete laccase gene sequences, confirming the specificity of the primer pair Cu1F and Cu2R for Basidiomycetes. Six of the 10 new genes, revealed by the expression study, clustered in two groups (Fig. 3) containing a laccase gene sequence from *X. chrysenteron*, an ectomycorrhizal fungus collected at the field site. Among the four remaining new transcript sequences, three were closely related to the *Clitocybe*, *Psathyrella*, and *Macrolepiota* laccase sequences, respectively—three taxa for which no fruiting bodies were found at the site in the investigation period reported here, which did not allow assessing definitely the identity of those genes. The last transcript was related to no known sequence (Fig. 3).

4. Discussion

The aim of this study was to optimize a method based on RT-PCR on soil mRNA extracts followed by semi-quantitative PCR to characterize the expression of Basidiomycete laccase genes in soils. In a very first assessment, the method was used in five soil cores of a brown forest soil to compare such expression profiles with the gene diversity profiles that had been found in the same cores previously (Luis et al., 2004). The brown forest soil of the experimental station of Steigerwald has a fine moder humus layer characterized by a rapid turnover of the organic matter (OM), which sparsely accumulates in the lower horizons. The thin organic horizon O_h (1–0 cm) corresponding to the fine humus layer contains about 19.0% of organic carbon (C_{org}), while the organo-mineral A_h (0–6 cm) and the mineral B_v (6–34 cm) horizons present, respectively, 3.38% and 1.19% of C_{org} (Seiler, 1995). Vertical distribution of laccase nucleotide sequences previously determined for the five cores had showed higher laccase gene diversity in the organic O_h horizons (Luis et al., 2004). For the preliminary assessment of the expression profiles by RNA analysis, we therefore concentrated our investigation on the O_h horizon.

The levels of the laccase transcripts assessed by semi-quantitative PCR were heterogeneous amongst the five soil cores. The equal amplification of the actin transcripts indicated that the absence or the weakness of laccase semi-quantitative PCR fragments in three of the samples (S1, S4, and S5) likely resulted from the absence and low amount of laccase RNA matrice compared to S2 and S3 samples. Selection of an additional fungal gene as positive control system for the laccase expression followed strict criteria. The gene had to be constitutively expressed in fungi. A number of sequences for a diversity of fungi had to be available and to display a polymorphism compatible with PCR amplification in a wide range of Basidiomycete with a single primer set. The amplified transcript had to display the same length as the ones amplified for laccase genes in order to warrant equal amplification conditions in the semi-quantitative PCR. A research in GenBank showed the actin genes to meet all demands and we developed this control system which to our knowledge was not described previously.


S4H1 : Pycnoporus cinnabarinus (AJ420334-AY147188)
Stropharia squamosa (AJ542597)
Phlebia radiata (X52134)
S3H1 (AJ540259)
Coriolopsis gallica (AY017340)
Trametes villosa (L78076)
Pleurotus ostreatus (AJ420180)
Mycena crocata (AJ542590)
S4H1 (AJ420350)
Mycena galopus (AJ542641)
Mycena crocata (AJ542589)
S4H1 (AJ540286)
Mycena cinerella (AJ542607)
Mycena rosea (AJ542629)
99
mRNAS2H1-S4H1-S5H1 : Lepista nuda (AJ420347)
Hebeloma radicosum (AJ542633)
S2H1 (AJ540292)
S4H1 : Russula nigricans (AJ542622)
Russula adusta (AF537318)
S4H1 : Russula nigricans (AJ420340-AJ542621)
S4H1-S5H1 : Lepista nuda (AJ542593)
Stropharia squamosa (AJ542598)
S4H1 : Mycena zephirus (AJ542600)
S4H1 : Collybia peronata (AJ542636)
S4H1-S5H1 (AJ540266)
Piloderma croceum (AY129078)
Piloderma byssinum (AY129074)
Tylospora fibrillosa (AY129074)
Athéliaceae
S4H1-S5H1 : Lepista nuda (AJ542594)
94
Russula mairei (AJ542625)
89
Russula ochroleuca (AJ542623)
Russula atropurpurea (AJ542624)
Lactarius blennius (AF542649)
S3H1(AJ540256)
S2H1 (AJ540271)
Lactarius clarkeae (AY147408)
94
S2H1-S3H1 (AJ540247)
Lactarius subdulcis (AF542650)
95
S2H1 (AJ540249)
80
S2H1 (AJ540253)
Lactarius rufus (AF537316)
Lactarius scrobiculatus (AF537317)
96
S4H3 (AJ540289)
Russulaceae
S3H2-S3H3(AJ540270)
S3H1 (AJ540254)
Pleurotus sajor-caju (AF297527)
Macrotyphula juncea (AJ542615)
Pleurotus ostreatus (AJ005017)
S4H1 : Mycena zephirus (AJ542601)
S4H1 (AJ420351)
Psathyrella murcida (AJ542613)
100
Clitocybe nebularis (AJ542626)
mRNAS3H1 (AJ580836)
S5H1-S5H2 : Hebeloma radicosum (AJ542631)
Cortinarius flexipes (AJ542638)
Amanita citrina (AJ542646-AJ542647)
Mycena crocata (AJ542585)
Mycena galopus (AJ542640)
S4H1 : Mycena cinerella (AJ542609)
Mycena galopus (AJ542639)
Mycena crocata (AJ542586)
Mycena crocata (AJ542588)
S4H1 : Mycena zephirus (AJ542602)
S4H1 : Mycena cinerella (AJ542608)
S5H1 (AJ540274)
99
Psathyrella murcida (AJ542612)
59
mRNAS3H1 (AJ580834)
58
Psathyrella dicrani (AJ542620)
60
Psathyrella corrugis (AJ542619)
Psathyrella corrugis (AJ542618)
mRNAS3H1-S3H1 (AJ540255)
mRNAS2H1 (AJ580832)
77
Xerocomus chrysenteron(AJ542643)
51
mRNAS3H1 (AJ580837)
92
S3H1 (AJ540258)
S5H1 (AJ540273)
mRNAS2H1 (AJ580835)
Boletaceae Boletaceae
group 1
100
mRNAS2H1-mRNAS3H1-S2H1-S3H1-S4H1-S5H1(AJ540285)
Xerocomus chrysenteron (AJ542644)
mRNAS2H1 (AJ580829)
S4H1 (AJ540265)
Paxillus involutus (AJ542648)
group 2
mRNAS2H1-mRNAS3H1-S2H1-S3H1-S5H1(AJ540297)
99
mRNAS2H1 (AJ580830)
mRNAS3H1 (AJ580833)
S5H1 (AJ540295)
Rhizoctonia solani (Z54276)
Mycena rosea (AJ542628)
Mycena pura (AJ542630)
88
Macrolepiota procera (AJ542610)
mRNAS2H1 (AJ580828)
S5H1 (AJ540275)
mRNAS3H1 (AJ580835)
S2H1(AJ540260)
56
Piriformospora indica (AJ542651)
69
S4H1 (AJ540269)
70
S3H1 (AJ420348)
62
S2H1-S3H1 (AJ540245)
S5H1 (AJ540296)
S2H1 (AJ540248)
Sebacinaceae
S5H1 : Hebeloma radicosum (AJ542632)
S5H1 (AJ540276)
87
Filobasidiella neoformans (AF140163-AF140180)
Botryotinia fuckeliana (AF243855)
Gaeumannomyces graminis (AJ437319)
Colletotrichum lagenarium (AB055709)
0.05 substitutions/site

The primer pair Cu1F and Cu2R is Basidiomycete specific and gives fragments around 200 bp for the laccase genes and 142 bp for the corresponding coding regions (Luis et al., 2004). For most laccase transcripts revealed in samples S2 and S3, the corresponding genomic fragment had not been detected during the previous DNA analysis of the soil cores (Luis et al., 2004). This discrepancy could result from (1) the occurrence of introns in the primer annealing sites precluding PCR amplification (Giardina et al., 1995); (2) an excess of some DNA matrices in the soil extracts resulting in a lack of amplification of weakly present genes however displaying expression; and (3) the sequencing of a limited number of clones. Concerning the first hypothesis, it is to underline that Cu1F and Cu2R primers were optimized in comparison to a set of different primers on a wide range of Basidiomycetes and that they appeared to be the most universal in this group (Luis et al., 2004). The copper binding sites are the most conserved region of laccase genes, but occurrence of introns in this region cannot be ruled out. Both last reasons can also basically explain the obtaining of *L. nuda* sequence from the RNA extract and not from the DNA extract of the soil sample S2. However, as a preliminary saturation experiment at the genomic DNA level had revealed that the sequencing of 25 clones allowed to characterize almost thoroughly the diversity of the laccase gene fragments in the Oh horizon (Luis et al., 2004), explanation (3) is less probable.

The NJ analysis showed that a majority of the detected laccase PCR sequences were grouped in two clades closely related to the ectomycorrhizal fungus *X. chrysenteron*(Fig. 3). In ectomycorrhizal fungi, laccase gene sequences tend to form distinct clades within families (Luis et al., 2004). Therefore the new transcript sequences related to *Xerocomus chrysodendron* might correspond to fungi in the Boletaceae (Fig. 3). The remaining transcript fragments clustered closely to sequences of saprophytic species, such as *Clitocybe*, *Lepista*, *Psathyrella*, and *Macrolepiota* species to which they could be related. As we did not find fruiting bodies of these species at the site in the investigation time, this relation could not be assessed. However, these taxa are not uncommon in the forest type examined.

The clear detection of laccase gene expression in S2 and S3 could correspond to the fact that these samples were densely colonized by tree rootlets. Rhizospheric soil compartments are highly colonized by mycorrhizal fungi, many of which were shown to have laccase genes (Chen et al., 2003; Read and Perez-Moreno, 2003). In rhizospheric soils, root exudates (rich in carbon) maintain a broad community of microorganisms including producers of oxidative enzymes involved in the degradation of complex soil components (Landeweert et al., 2003). This diversity is even larger in organic horizons densely colonized by roots due to the abundance of dead rootlets and polyphenols from the litter (McDowell, 2003). In such soil compartments, the high competition for resources (i.e., carbon, nitrogen, and phosphate) in diverse forms might stimulate the production of fungal exo-enzymes by both mycorrhizal and saprophytic fungi. The lack or weak detection of laccase expression in S1, S4, and S5 indicates that the concentration of the corresponding RNA matrices for the RT-PCR was lower in this bulk soil samples. This could reflect either a lower concentration of mycelia per volume unit or, as fungal laccases are known to be actively produced in presence of their substrates (Thurston, 1994; Smirnov et al., 2001), a lack of laccase substrates compared to the rhizospheric soil. These hypotheses should however be considered cautiously, as only one fungal group (Basidiomycetes) and one oxidative enzyme were analyzed. Furthermore, the limited sampling in this study aiming to demonstrate the potential of RT-PCR and semi-quantitative PCR for gene expression

Fig. 3. Neighbor-joining tree representing the genetic distance between the 14 laccase transcript sequences (present study, in bold), the deduced coding regions of 44 laccase genomic fragments found in the same soil cores (Luis et al., 2004) (in black), 51 laccase genomic fragments amplified from fruit body and culture collections (in black), and 13 laccase sequences retrieved from GenBank database (in gray). S1–S5 correspond to the replicate numbers and H1 to the O_h horizon. The NJ tree was rooted using sequences of the ascomycetes *Colletotrichum lagenarium* (GenBank accession no. AB055709), *Gaeumannomyces graminis* (GenBank accession no. AJ437319), and *Botryotinia fuckeliana* (GenBank accession no. AF243855). Bootstrapping was performed with 1000 replications in *p*-distance analysis. The potential mycorrhizal specific clades present bold branches.

profiling in forest soils cannot allow us to draw definitive ecological conclusions.

As less than 30% of the laccases was found to be expressed in soil cores, simultaneous DNA and RNA analyses should be performed to have a representative assessment of the laccase genomic diversity versus laccase expression profiles. The approach developed here could be used in short- to long-time monitoring of the diversity and expression of the Basidiomycete with laccases genes.

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