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Trametes villosa Lignin Peroxidase (TvLiP): Genetic and Molecular Characterization

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White-rot basidiomycetes are the organisms that decompose lignin most efficiently, and *Trametes villosa* is a promising species for ligninolytic enzyme production. There are several publications on *T. villosa* applications for lignin degradation regarding the expression and secretion of laccase and manganese peroxidase (MnP) but no reports on the identification and characterization of lignin peroxidase (LiP), a relevant enzyme for the efficient breakdown of lignin. The object of this study was to identify and partially characterize, for the first time, gDNA, mRNA, and the corresponding lignin peroxidase (TvLiP) protein from *T. villosa* strain CCMB561 from the Brazilian semiarid region. The presence of ligninolytic enzymes produced by this strain grown in inducer media was qualitatively and quantitatively analyzed by spectrophotometry, qPCR, and dye fading using Remazol Brilliant Blue R. The spectrophotometric analysis showed that LiP activity was higher than that of MnP. The greatest LiP expression as measured by qPCR occurred on the 7th day, and the ABSA medium (agar, sugarcane bagasse, and ammonium sulfate) was the best that favored LiP expression. The amplification of the *TvLiP* gene median region covering approximately 50% of the *T. versicolor LPG*IV gene (87% identity); the presence of Trp199, Leu115, Asp193, Trp199, and Ala203 in the translated amplicon of the *T. villosa* mRNA; and the close phylogenetic relationship between TvLiP and *T. versicolor* LiP all indicate that the target enzyme is a lignin peroxidase. Therefore, *T. villosa* CCMB561 has great potential for use as a LiP, MnP, and Lac producer for industrial applications.

Keywords: Lignin, ligninolytic enzymes, white-rot basidiomycetes.

Introduction

Plant cell walls are mainly composed of lignocellulose, which is also a major component in the biogeochemical cycle of carbon. In addition to its traditional use in textile and paper production, lignocellulose has wide biotechnological applications, including uses in civil engineering (reinforcing polymer and thermoplastic matrices), the automotive industry (in manufacturing lighter and safer parts for motor vehicles), and, more recently, in the growing biofuel industry [1–4].

Lignin, one of the most abundant organic polymers in the terrestrial ecosystem, is widely distributed in the layers of the secondary wall of plant cells. Lignocellulosic materials

are usually composed of 20–30% lignin [5]. Comprising approximately 10–20% phenolic and 80-90% non-phenolic units, lignin is difficult to degrade chemically under natural conditions. This recalcitrant feature reflects the molecular synthesis, which is derived from the polymerization of three cinnamyl alcohols (coniferyl, ρ-coumaryl, and sinapyl alcohol). Moreover, its complex structure involves hyperbranched units (guaiacyl (G), ρ-hydroxyphenyl (H), and syringyl (S)), whose carbons are interconnected through ether β-O-4, β-5, β-β, 5-5, 4-O-5, and β-1 bonds [6–9].

Carbohydrate-active enzymes (CAZymes) are involved in glycoconjugate, and oligo- and polysaccharide degradation, biosynthesis, and modification. CAZymes from phytoparasites are significantly important in the synthesis and degradation of plant cell walls besides host-pathogen interactions [10, 11]. The CAZyme classes (glycoside hydrolases, carbohydrate esterases, and polysaccharide lyases) are cell wall-degrading enzymes well known by their central roles in plant biomass decomposition by bacteria and fungi [12].

Comparative genomics of Basidiomycota has been focused on the distinct lineages of wood decay fungi [13–15]. For several years, two broad categories have been described: brown-rot and white-rot fungi [16]. During brown rot, a quick cellulose depolymerization occurs via oxidative mechanisms, where modified lignin stays as a polymeric residue [17, 18]. On the other hand, lignin is completely mineralized by white-rot fungi. The first sequenced genomes of brown-rot and white-rot fungi (*Postia placenta* and *Phanerochaete chrysosporium*, respectively) exhibited a gene complement consistent with their respective wood decay behavior [13, 19].

Lignin decomposition involves high oxidation potential class II peroxidases, classified as lignin peroxidase (LiP), manganese peroxidase (MnP), or versatile peroxidase (VP), based on their conserved catalytic and Mn-binding sites [20–22]. The organisms that most efficiently decompose lignin are white-rot basidiomycetes [23–25]. The lignin-degrading capability of these fungi is due to the expression of a group of extracellular and low-molecular-weight glycoproteins that oxidize the α and β carbons in the structure of aromatic compounds such as lignocellulose [26]. Lignin peroxidase (E.C. 1.11.1.14) stands out from other enzymes for its high redox potential, and it does not require mediators during the catalysis of non-phenolic compounds within the lignin structure [9, 26–29]. Despite their importance in the degradation of lignin, LiP-encoding genes appear to be restricted to the order Polyporales, which includes the genus *Trametes* [30].

Trametes villosa (Sw.) Kreisel, a tropical species that is commonly found in the semiarid region of Brazil [31], is promising for its production of ligninolytic enzymes. There are several works about its application in the decomposition of lignin related to the expression and secretion of its laccase (Lac) enzymes [32–34] and MnP's [35–37], but there have been no reports on the identification and characterization of LiP, an unusual enzyme detected in most white-rot Basidiomycota that has a central role in lignin degradation [26, 29].

In our study, we have, for the first time, identified and partially characterized, at the gDNA, mRNA, and protein levels, a LiP from a strain of *T. villosa* (TvLiP) from the Brazilian semiarid region; furthermore, this strain is also a high MnP and Lac producer.

Materials and Methods

Collection, Isolation, and Identification of the Fungal Strain

Basidiomata of *T. villosa* in decaying wood (fallen branch) of an unidentified angiosperm were collected from a Brazilian semiarid region (Serra das Candeias, Quijingue, Bahia, Brazil; 39°04'30''W and 10°55'16''S). Identification was performed by morphological (macro and microscopic analyses of basidiomata, spores, and hyphal system) and molecular taxonomy ((nrLSU sequencing, defined by the primers LR0R (5'–ACCCgCTgAACTTAAgC-3') and LR5 (5'-TCCTgAgggAAACTTCg-3')). After dehydration, the *T. villosa* basidiomata were deposited into the Herbarium of Feira de Santana State University (HUEFS108280), and the polysporic culture derived from the basidiomata tissue was preserved in sterile distilled water and deposited into the Culture Collection of Microorganisms of Bahia (CCMB561).

Fungal Strain Culture

For isolation and gDNA characterization, *T. villosa* CCMB561 mycelium was grown in Petri dishes on MEA (2% agar-agar, 0.05% yeast extract, 0.2% malt extract) and incubated at 28°C for 7 days.

To induce the ligninolytic enzyme expression and subsequently analyze the enzymatic activity and mRNA expression, *T. villosa* CCMB561 was cultivated at 28°C (± 2°C) for 7, 14, and 21 days in the dark in distinct inducing media. Five different compositions of liquid culture media were used (ABc, ABG, ABM, ABSA, and MEA.B; Table 1).

To qualitatively identify the activity of the ligninolytic enzymes, *T. villosa* CCMB561 was inoculated in Petri dishes in the presence of 0.02% Remazol Brilliant Blue dye R (RBBR; Sigma, USA) in all media. All inoculations were performed in triplicates.

Enzyme Extracts and LiP and MnP Activity Analyses

The enzyme extract (supernatant) was obtained by maceration in sterile cold distilled water of *T. villosa* CCMB561 mycelia grown in different inducing media, followed by vacuum filtration in a

Table 1. Composition of media used to induce the expression of ligninolytic enzymes in the cultivation of *T. villosa* CCMB561.

Inducing media	Composition
ABc	8 g of agar-agar I (HiMedia); 8 g of sugarcane bagasse; 400 ml of distilled water
ABG	8 g of agar-agar I (HiMedia); 8 g of sugarcane bagasse; 2 g of glucose; 400 ml of distilled water
ABM	8 g of agar-agar I (HiMedia); 8 g of sugarcane bagasse; 10.16 g of manganese sulfate; 400 ml of distilled water
ABSA	8 g of agar-agar I (HiMedia); 8 g of sugarcane bagasse; 2 g of ammonium sulfate; 400 ml of distilled water
MEA.B	8 g of agar-agar I (HiMedia); 8 g of sugarcane bagasse; 0.8 g of malt extract; 0.2 g of yeast extract; 400 ml of distilled water
Non-inducing medium	**Composition**
MEA	8 g of agar-agar (HiMedia), 0.2 g of yeast extract, 0.8 g of malt extract; 400 ml of distilled water

Buchner funnel and centrifugation at 3,000 ×*g*. Subsequently, the enzyme extract was preserved on an ice bath.

The analyses of MnP and LiP activities were carried out as previously described in the literature [38, 39]. In the LiP activity assay, an initial analysis was performed without H_2O_2, followed by a subsequent analysis with H_2O_2 after 5 min. All assays with fungal enzyme extracts were performed in triplicates and compared with the corresponding boiled fungal extract, as previously described [35]. The resulting LiP and MnP activities (expressed in U/g) were subjected to analysis using two-way ANOVA followed by the Bonferroni test at 95% confidence.

Primer Design for gDNA and mRNA

The conserved gene regions related to ligninolytic enzyme sequences (LiP and MnP) were used as the basis for the design of the degenerate primer pairs 5′-CCACGACGCYATGGYATCTC-3′ and 5′-CGGACTGGAGACGGATCTCHC-3′ (LiP Id and LiP Ir, respectively), after multiple sequence alignment using ClustalW2 (European Bioinformatics Institute). The primers were later reviewed to determine the %GC content and their theoretical annealing temperatures.

For qRT-PCR analysis, the primers were designed using the PrimerTest tool (IDT http://www.idt.com) to obtain an amplicon of the cDNA sequence from the transcribed mRNA of the corresponding gDNA to be analyzed.

Nucleic Acid Extraction (gDNA and RNA) and cDNA First Strand Synthesis

The extraction of gDNA was performed according to described [40] and modified methods [41]. Lyophilized samples of the fungal mycelia grown in two different culture media (non-inducing medium MEA and best inducing medium ABSA) for different periods of time (7, 14, 21, and 28 days) were homogenized in liquid nitrogen. Equal amounts of each sample were used (100 mg) for the total RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Total RNA (1 µg) was treated with RNAse-free DNAse I (Fermentas, USA) for the removal of possible gDNA contamination.

The extracted nucleic acids were quantitatively analyzed using UV spectrophotometry (NanoDrop2000; Thermo Scientific, USA) and qualitatively analyzed in a 1% agarose gel stained with 4 µl of ethidium bromide (Invitrogen, USA).

The synthesis of first-strand cDNA was performed with the RevertAid First H Minus Strand cDNA Synthesis Kit (Fermentas, USA) using random primers according to the manufacturer's recommendations. The obtained cDNA was quantified and diluted (10 ng/µl) for gene expression analysis.

gDNA Amplification and Sequencing

The reaction mix contained 1 µl of gDNA extracted from *T. villosa* (52 ng/µl), 2 µl of 10 mM dNTP mix, 1 µl of each primer (LiP Id and LiP Ir) (5 ρmol), 1 µl of $MgCl_2$ (25 mM), 5 µl of 10× buffer, and 0.6 µl of Taq DNA polymerase (0.02 U/µl) (Phoneutria, Brazil), brought up to 50 µl with sterile ultrapure water. The annealing temperature used in the amplification reactions was 55°C for 45 sec. Then, the amplicons were sequenced in an automated DNA sequencer (ABI3100; Life Technologies, USA).

Analysis of LiP Gene Expression by Quantitative Real-Time PCR (qPCR)

The LiP expression level (TvLipF: 5′-ATTGCCATCTTCGCC GACATTGAG-3′/TvLipR: 5′-AGTCGGCATGAGAAATGTTGTGGC-3′) was determined using the Stratagene Mx3005P qPCR System with MxPro QPCR software (Agilent Technologies, USA). Experimental data were normalized based on the expression values of 18S ribosomal protein gene (TvRibo; JN164969.1), which was used as the calibration reference and specifically amplified with primers (TvRiboF: 5′-CTTGGATTTGGAGGCTTGCT-3′/ TvRiboR: 5′-CCGCACGGAATCAAGCTAA-3′) designed using Primer Express Software 3.0 (Applied Biosystems, USA). Relative TvLiP expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [42] using samples cultured in non-inducing medium as the calibration data (MEA, 7 days). Reactions performed contained 100 ng of cDNA, 10 µM of each primer pair (forward/reverse) for each gene (LiP and TvRibo), and 1× SYBR Green PCR Master Mix (Applied Biosystems) for a final volume of 22 µl, done in triplicates.

The qPCR program was performed at 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. The specificity of the primers used was verified by construction of the dissociation curve of amplicon products (95°C–55°C). Reactions

without cDNA template were included as negative controls for each primer pair. Statistical comparison of the levels of LiP expression was performed using the ΔCt value of each sample and applying the Dunnett test (ANOVA) with Statistica (6.0). A p value < 0.05 was considered to indicate significance.

DNA Sequence Edition and Contigs Assembly

The STADEN (ver. 4.10) package was used to edit DNA chromatograms obtained from amplicon sequencing (gDNA). Contig assemblies were generated from their respective consensus sequences by global pairwise alignment (EBI EMBOSS Needle & Stretcher; http://www.ebi.ac.uk/Tools/psa/).

Identification of Conserved and Variable Regions in gDNA

The nucleotide sequence of the middle portion of the *T. villosa* CCMB561 gene was aligned and subsequently compared with the most similar sequences in public databases to identify all of the conserved and variable regions.

The nucleotide sequence of the LiP coding sequence (CDS) isolated from *T. villosa* CCMB561, which was manually curated and assembled by comparison with the LiP CDS of *T. versicolor* (NCBI: Z31011.1), was subjected to translation in all six open reading frames (Emboss Transeq EMLB, EBI). The deduced protein sequence of *T. villosa* LiP CCMB561 was aligned to all the NCBI GenPept sequences using BLASTx, tBLASTx, and tBLASTp. Sequences with the best hits were realigned with the *T. villosa* CCMB561 LiP sequence to identify conserved and variable regions.

Subsequently, the three amino acid sequences of *T. versicolor* that were more similar to those of *T. villosa* CCMB561 were realigned, and conserved domains were determined using the Conserved Domains and Protein Classification NCBI tool.

Phylogenetic Analysis

A total of 48 protein sequences with 187 amino acid residues similar to the *T. villosa* contig sequence were aligned (excluding the initial and final regions of the sequences bigger than the fragment in study) using Bioedit ver. 7.0. The result of this analysis was used to generate a symmetric matrix, which was subjected to distance analysis using the program ProtDist (Phylip). A rooted tree was generated by the Fitch-Margoliash sum of squares method [43], using two sequences of distinct species of Pezizomycotina (Ascomycota) as the outgroup.

Results

Fungal Strain Cultivation and LiP/MnP Activity Detection

The use of MEA medium was considered satisfactory in terms of mycelial biomass production and was suitable for the gDNA isolation. However, no enzyme activity was detected in the extracts obtained from the specimens cultured in this medium. The best results for ligninase activity analysis were obtained from *T. villosa* CCMB561 cultivated in ABSA and MEA.B media, supporting the idea that these media could be used for cDNA characterization.

The presence of ligninase activity produced by *T. villosa* CCMB561 grown in media with ligninolytic enzyme inductors was detected visually by the fading of RBBR dye (blue → red → yellow) during radial growth on Petri dishes. The discoloration of RBBR added to the inducing media resulted from the production of mycelial biomass and synthesis of ligninases expressed by *T. villosa*. The diameters of the RBBR discoloration halos during the cultivation of *T. villosa* are shown in Fig. 1.

The quantitative analysis of the activity of each specific ligninase (MnP and LiP) showed that LiP activity was higher than MnP activity (except for cultures in ABM medium, which was supplemented with Mn sulfate) (Fig. 2).

Partial Gene Amplification and Expression Level of LiP

The primer pairs LiP Id and LiP Ir used in the amplification of the intermediate region of the gene of interest (from gDNA or cDNA) flanked an amplified fragment of approximately 630 bp as produced by conventional PCR. The gDNA and mRNA sequences of *T. villosa* were deposited in GenBank (Accession No. KT736150). The BLASTn alignment of the gDNA amplicon with the nucleotide sequence exhibited rates of 89% coverage and 85% similarity with the LPG IV gene encoding the lignin peroxidase isoenzyme LP12 isolated from *T. versicolor* (Coriolus) PRL 572 (Z31011).

Based on the qPCR data (Fig. 3), it was possible to compare LiP gene expression between fungal samples grown in two different culture media (MEA and ABSA). These data showed that LiP expression is significantly different ($p < 0.05$) at distinct culture times in the two media analyzed. The LiP expression in the MEA culture on the 7th day was used as the reference for the $2^{-\Delta\Delta Ct}$ method.

The highest levels of LiP expression were observed in samples grown in ABSA. ABSA samples on the 7th day showed the highest relative expression (136×) compared with the MEA samples on the 7th day. For the ABSA samples on the 14th day, the LiP expression levels were less significant (2.8×) but still higher than those for the sample grown for the same amount of time in MEA medium. ABSA samples on the 21st day and 28th day showed 5.1× and 3.4× increases, respectively, compared with the MEA sample on the 7th day (Fig. 3).

Bioinformatics and Phylogenetic Analyses

The amplicon corresponding to the median region of the *T. villosa* LiP gene characterized in this study, after alignment

with the LiP gene sequence from *T. versicolor*, corresponded to the end of intron 2, all of intron 3, all of exon 3, and part of exon 4 of the *Trametes versicolor* LiP gene.

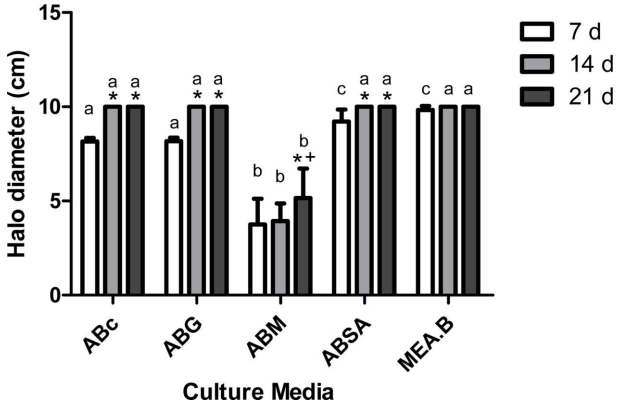

Fig. 1. Average values of the Remazol Brilliant Blue R discoloration halo measurements resulting in the production of ligninolytic enzymes synthesized by *T. villosa* CCMB561 grown in five different culture media after 7, 14, and 21 days at 28°C in Biochemical Oxygen Demand incubator (BOD).

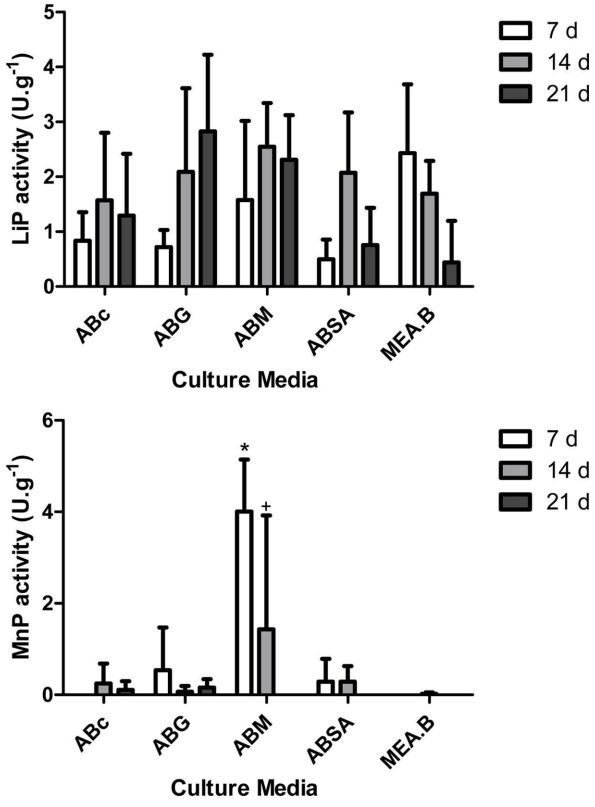

Fig. 2. Average values of lignin peroxidase (LiP) and manganese peroxidase (MnP) activity (U/g) of enzymes produced by *T. villosa* CCMB561 grown in inducing media.

The translation of *T. villosa* LiP coding sequence (CDS) compared with the *T. versicolor* LiP generated a sequence of 170 aa. The protein sequence alignment suggested that amino acids 7–170 correspond to the LiP protein sequence synthesized by *T. villosa* CCMB561. The alignment of TvLiP with the most similar *T. versicolor* LiP protein sequences indicated a sequence similarity and identity of 97% and 93%, respectively (Fig. 4). Conserved domain tool analysis corroborated that the characterized enzyme is classified as a class II peroxidase. Phylogenetic analysis indicated that TvLiP (*T. villosa* CCMB561) is closer to the *T. versicolor* LiP sequence than to any other basidiomycete LiP, forming a distinct clade with other LiPs from *Trametes* and *Pycnoporus* (Fig. 5).

The partial nucleotide and amino acid sequences that were determined in this work were deposited under GenBank accession numbers KT736150 and ALR87300, respectively.

Discussion

LiP/MnP Activity Detection, Expression Levels of LiP, and Partial Gene Amplification

The regulation of LiP gene expression and other ligninases (resulting in different amounts of isoenzymes produced by white-rot fungi) is linked to a variety of environmental conditions, such as carbon and nitrogen sources availability, presence of metal ions and organic inducers, temperature, and exposure to light [44]. As a general rule, these enzymes are usually produced when carbon and/or nitrogen depletion in the medium induces the establishment of secondary

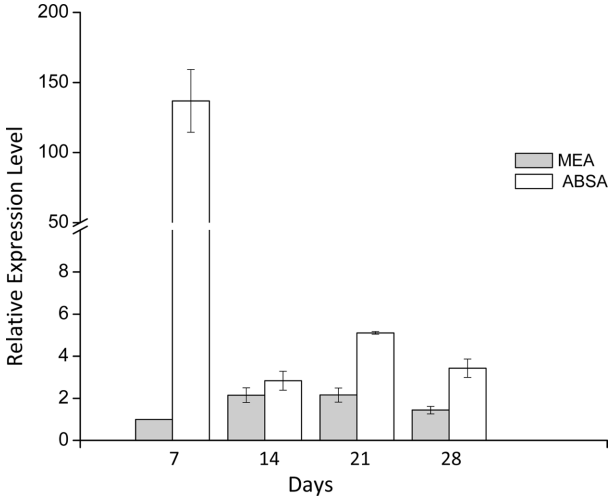

Fig. 3. Expression of lignin peroxidase produced by *T. villosa* grown for 7, 14, 21, and 28 days in either MEA or ABSA medium.

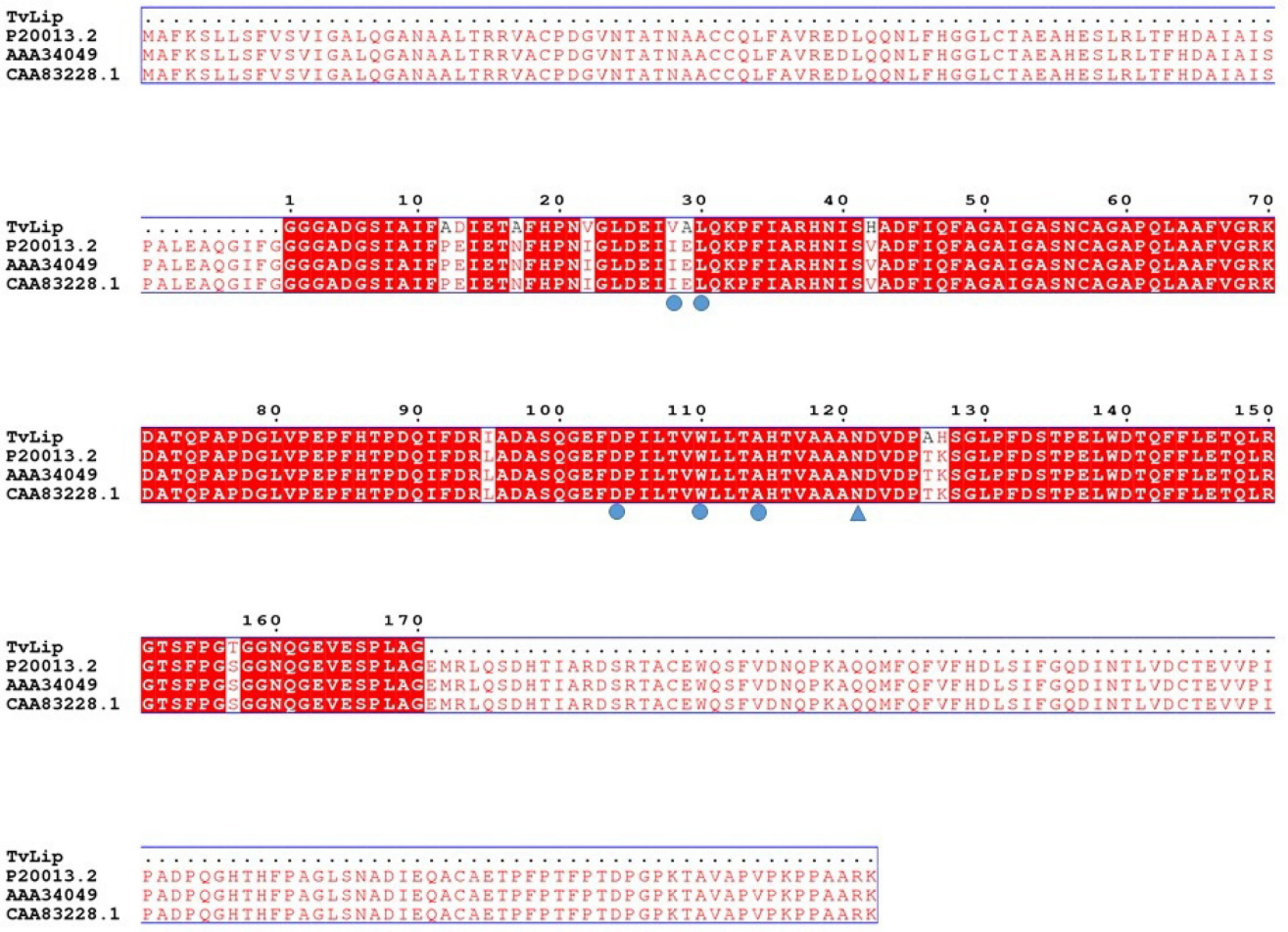

Fig. 4. Alignment sequence in the ESPript program with the amino acid sequence corresponding to *T. villosa* CCMB561 lignin peroxidase (LiP) and the *T. versicolor* LiP sequences (Accession Nos. P20013.2, AAA34049, and CAA83228.1) considered the most similar to *T. villosa* LiP.

Circles, active amino acid residues in the LRet I, II, and III pathways; triangle, conserved amino acid residues in versatile peroxidase and manganese peroxidase.

metabolism [45].

Discoloration of dyes (such as RBBR) added to a medium in which ligninolytic fungi are grown occurs as a result of the expression of lignin-modifying peroxidase enzymes [46]. The RBBR assay was used as a qualitative test to eliminate culture media that apparently did not induce the ligninolytic enzymes expression. Since MnP activity has already been described for *T. villosa* [35], we investigated the presence and the contribution of LiP activity described in phylogenetically more closely related species [20, 26]. Thus, positive RBBR assays for *T. villosa* CCMB561 were considered preliminary evidence of the production of these enzymes by the strain of interest (Fig. 1). The best results for ligninase production were obtained from *T. villosa* CCMB561 cultivation in ABSA and MEA.B media. The low discoloration rate of RBBR in the ABM medium could be attributed to low biomass production, since this medium was clearly suitable for peroxidase induction (especially MnP) after 7 days of cultivation (Fig. 2). A similar pattern of MnP and LiP production was also detected in *T. versicolor* using a sugarcane bagasse medium, ammonium sulfate, and a Mn source [47].

The result obtained in the ABSA medium is in accordance to the work of Guerra *et al.* [48], who observed MnP activity in *Earliella scabrosa* and *Trametes maximum* species grown in medium containing sugarcane bagasse and ammonium sulfate. The activity detected in the extracts grown in ABSA was constant for the first 2 weeks but eventually reduced to

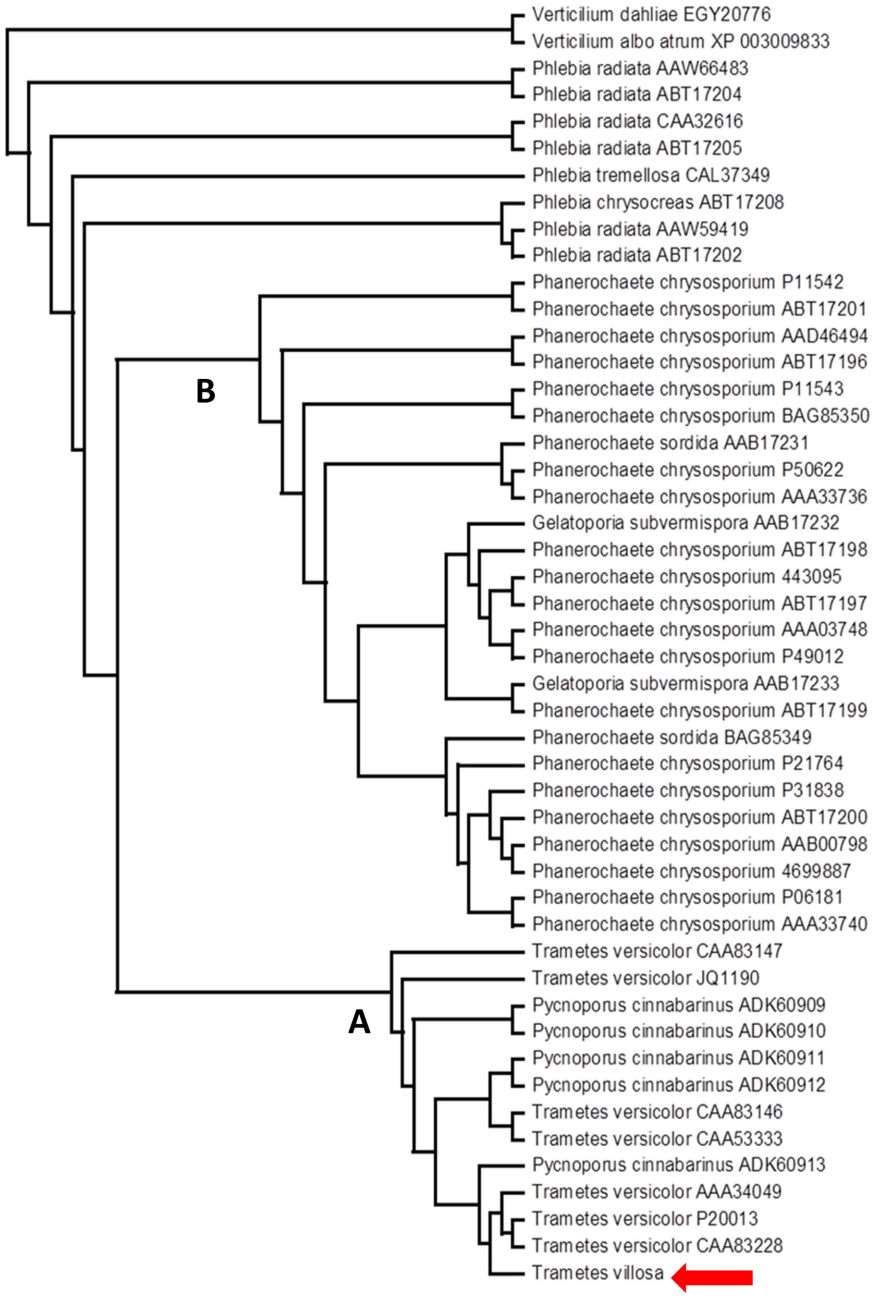

Fig. 5. Phylogenetic relationships between *T. villosa* CCMB561 lignin peroxidase (LiP) and other basidiomycete LiP protein sequences.

zero on the 21st day, which can be explained by the stationary phase of fungal development or enzyme inhibition (by physiological or pH change); however, the exact mechanism requires a more thorough investigation [49].

The highest LiP activity occurred on the 14th day of culturing, and ABSA was the best medium for expression, as was previously reported by Wang *et al.* [25] and Sarnthina *et al.* [50], who also observed LiP expression using additional inorganic or organic nitrogen sources in the culture medium, respectively. In spite of changes in the enzymatic profiles, the LiP and MnP activity values suggested that *T. villosa* CCMB561 showed the expected response for inducing enzymatic synthesis in the presence of lignocellulosic substrate (Fig. 2). The difference between the low value of the LiP activity in ABSA-cultured samples on the 7th day (Fig. 2) and the high expression level (qPCR) of LiP in ABSA-

cultured samples on the 7th day (Fig. 3) can be explained by the different scales of mRNA production, since the increase in mRNA is followed by elevated enzyme production levels in fungal metabolism. The data show that the maximum expression level of mRNA likely precedes the subsequent increase of enzyme activity that occurs between the 7th and 14th days. Moreover, for several LiP and MnP fungal producers submitted to nutrient-limited conditions, ligninolytic enzyme activities can be suppressed in a medium containing high nitrogen concentrations [44, 51].

Bioinformatics and Phylogenetic Analyses

The characterization and analysis of the partial nucleotide and its translated amino acid sequences allowed for the first time, at both the gDNA and mRNA levels, the description and identification of a LiP from a strain of *T. villosa* (TvLiP). The partial *TvLiP* gDNA sequence in the present work exhibited a high identity with *LPGIV*, which is one of the two lignin peroxidase gene products from *T. versicolor* characterized by Johasson and Nyman [52].

Cullen [53] characterized a LiP isoform, isolated from *P. chrysosporium*, and identified the amino acids Ile113, Leu115, Asp193, Trp199, and Ala203 (the original positions in *T. versicolor*) in the adjacent region to the proximal histidine of this enzyme. These five amino acids are related to all three pathways of electron transfer in long distance LRet (I, II, and III) systems [54]. The critical tryptophan (Trp199) is essential for the oxidation of aromatic compounds via the LRet II pathway. Moreover, Morgenstern *et al.* [30] also stated that those same amino acids are present only in LiP enzymes because VP and MnP can be recognized by the presence of aspartic acid (D) in the Mn^{2+} binding site [30]. These same five amino acids were found in TvLiP (Fig. 4), corroborating its identification as a typical LiP. In addition, another intrinsic feature of LiPs is the substitution of the typical VP and MnP aspartic acid (D) for another amino acid; Asn121 is the substituted residue in TvLiP.

Morgenstern *et al.* [30] proposed a clade that phylogenetically grouped all LiPs secreted by Polyporales (termed as 'F' in their original work) and identified by the presence of the critical tryptophan (Trp199). Therefore, comparing our results with those of Pérez-Boada *et al.* [54] and Morgenstern [30], TvLiP is unequivocally the gene product of a class II peroxidase LiP of the same clade F of white-rot Basidiomycota (Fig. 5).

White-rot basidiomycete fungi are the main producers of ligninases that substantially contribute to lignin decay in wood [26, 55]. The *Trametes* species are among the most efficient wood decomposers, with a lignocellulolytic enzyme system mainly comprising laccases and Mn-dependent peroxidases as well as a series of CAZymes [56]. LiPs have already been detected in seven distinct *Trametes* species in public protein databases, but currently, there is no experimental evidence of LiP production in *T. villosa*.

Ligninolytic enzymes of white-rot fungi have been broadly studied for their potential applications in a wide range of industrial bioprocesses such as decolorization of industrial dyes, the pulp bleaching of paper and textiles industry, and the degradation of organopollutants [44]. There are many white-rot fungi species and strains of which some, such as *T. versicolor*, simultaneously produce LiP, MnP, and Lac [57], whereas others produce only one or two of these ligninolytic enzymes [58, 59].

An efficient production system is necessary for the biotechnological applications of these enzymes; thus, our study of *T. villosa* CCMB561 as a good production strain for LiP, MnP, and Lac, which are the key enzymes of lignin degradation, provides a major advantage for its use in industrial processes.

To summarize, the results from our genetic, transcriptional, and biochemical analyses lead to the first characterization of a LiP gene that is actively transcribed into functional mature mRNA and subsequently translated to its corresponding functional enzyme in a *T. villosa* strain (CCMB561) adapted to a semiarid climate and that is also a prolific producer of MnP [35] and Lac [55]. Therefore, *T. villosa* CCMB561 has great potential to be used to produce ligninolytic enzymes for industrial applications.

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