Molecular characterization of laccase genes from the basidiomycete *Trametes hirsuta* Bm-2 and analysis of the 5' untranslated region (5'UTR)

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Abstract

The aim of this study was to identify and characterize laccase genes produced by *Trametes hirsuta* Bm-2 in a liquid medium, both with and without induction. The amplification of 5' and 3' regions of laccase sequences was obtained by the RACE-PCR method, and these were assembled to obtain a cDNA of total length. Two new laccase genes were isolated from basal medium (*lac-B*) and lignocellulosic grapefruit substrate (*lac-T*), both encoding open reading frames of 2566 bp. Both laccase-predicted proteins consisted of 521 amino acids, four copper-binding regions, a signal peptide, and five potential glycosylation sites (Asn-Xaa-Ser/Tre). Moreover, the deduced amino acid sequences share about 76–85% identity with other laccases of WRF. Sequence comparison showed 47 synonymous point mutations between *lac-B* and *lac-T*. In addition, 5' untranslated regions (UTR) of laccase genes *lac-B* and *lac-T* showed differences in length and number of regulatory elements that may affect transcriptional or translational expression of these genes.

Keywords *Trametes hirsuta* · Laccase genes · RACE-PCR · Untranslated regions

Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper glycoenzymes produced by a large number of plants, fungi, insects, bacteria, and archaea (Givaudan et al. 1993; Brijwani et al. 2010; Uthandi et al. 2010; Kandam et al. 2016). Among these organisms, white rot fungi (WRF) are the only ones capable of efficiently degrading lignin due to the secretion of ligninolytic enzymes such as

laccases. These enzymes catalyze the four-electron reduction of molecular oxygen to H₂O (Thurston 1994).

Many WRF produce multiple laccase enzymes which are encoded by gene families (Wang et al. 2015). For example, from the whole genome of *Flammulina velutipes*, twelve putative laccase genes *fvLac1–12* have been detected (Kim et al. 2014); isoenzyme genes of *Pleurotus ostreatus* include *pox11*, *pox3*, *pox4*, *poxc*, *poxaa*, and *poxa1b* (Park et al. 2014); and Teerapatsakul et al. found five laccases named

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KULac1–5 produced by a new strain *Ganoderma* sp. KU-Alk 4 (Teerapatsakul et al. 2007).

Laccase isoenzymes show different properties related with substrate specificity, optimum pH and temperature. They oxidize a wide range of diverse phenolic compounds including mono-di-phenols, polyphenols, diamines, and recalcitrant compounds, which affect the environment (Kim et al. 2013). Isoenzyme expression is affected by different environmental conditions, i.e., induction by phenols (Terrón et al. 2004), lignocellulosic substrates (Park et al. 2014), and metals such as copper (Vasina et al. 2015). Moreover, posttranscriptional processes (generally glycosylation) could modify each isoenzyme (Morozova et al. 2007). Due to their multiplicity and broad range of specificity, laccases play an important role in several biological functions. They are involved in lignin synthesis (Baldrian 2006), fruiting body and mycelial development (Jin et al. 2018), pigment synthesis, and plant pathogenesis (Feng et al. 2015); however, the biological functions of many laccases remain unknown.

There is great potential concerning the use of laccases, ranging from textile dye colorization, food industry pulp delignification, and removal of phenols from wines, to uses in the pulp and paper industry, synthesis of medical compounds, and the design of biosensors (Kunamneni et al. 2008; Asgher et al. 2014; Viswanath et al. 2014; Palanisamy et al. 2017). The most important obstacles in laccase application are low concentrations of enzyme produced and low stability. In addition, the isoenzyme profile and properties should be considered for particular applications.

Many laccase genes have been isolated and characterized in *Trametes villosa*, *T. versicolor*, *Rhizoctonia solani*, *Pleurotus eryngii*, *Phanerochaete chrysosporium*, *P. flavidobalva*, and *Coriolus hirsute* (Viswanath et al. 2014). Laccase levels have been improved by cloning, expression and secretion in heterologous hosts like *Pichia pastoris* (Wang et al. 2015), *Saccharomyces cerevisiae* (Bulter et al. 2003), *Yarrowia lipolytica* (Kalyani et al. 2015), and *Aspergillus niger* (Téllez-Jurado et al. 2006), but the yields are not high enough yet. Thus, more research of genes including from novel endemic white rot fungi, and the development of advanced expression vectors are necessary.

Trametes hirsuta Bm-2 was isolated from wood decay in the Yucatan Peninsula, Mexico (Tapia-Tussell et al. 2011). This fungus increased extracellular laccase activity eight-fold when it was grown on a medium induced with agro-industrial substrates, compared to the basal medium. Furthermore, three laccases were purified and characterized from this strain. These enzymes showed high resistance to organic solvents, thermostability, and an ability to decolorize synthetic dyes and textile effluents (Zapata-Castillo et al. 2015). The present study was carried out to elucidate the molecular characteristics of laccase genes of *T. hirsuta*

Bm-2, produced on basal medium and media supplemented with grapefruit peel as inducer.

Materials and methods

Fungal strain, media, and culture conditions

Trametes hirsuta strain Bm-2 (GQ280373.1), was isolated from decaying wood in Yucatan, Mexico (Tapia-Tussell et al. 2011), and used throughout this work. The strain was grown in two culture media: (1) Kirk's liquid basal medium (BM) pH 6, which consists of 10 g glucose, 5 g ammonium tartrate, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g KH_2PO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg thiamine, and 10 mL trace compound solution, without Tween 20 or veratryl alcohol (Kirk et al. 1986); and (2) A medium enriched with grapefruit peel (GM) was (g/L): glucose, 2; malt extract, 15; $(\text{NH}_4)_2\text{SO}_4$, 0.9; KH_2PO_4 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; thiamine-HCl, 1 mg/L and 3% (w/v) of grapefruit peel. 250 mL Erlenmeyer flasks containing 50 mL of each medium were inoculated with 1 mL of cell suspensions, produced in accordance with (Zapata-Castillo et al. 2012). Flasks were incubated at 35 °C and shaken at 150 rpm.

Extraction of total RNA

After 7 days, mycelia were harvested from the liquid culture by vacuum filtration, and frozen in liquid nitrogen. The total RNA was extracted in accordance with the TRIzol™ procedure (Invitrogen, Carlsbad, CA, USA). RNA quantification was measured at 260 nm with a UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific™, Waltham, MA, USA). The RNA integrity was visualized in 0.8% (w/v) agarose gel electrophoresis prepared in 1× Tris-Borate-EDTA buffer (0.9 M Tris-borate pH 8; 20 mM EDTA) and stained with ethidium bromide. The analysis was performed with a UVP BioImaging System (UVP, Inc., Upland, CA, USA) using Lab Works 4.0 software.

Cloning of full-length cDNA of laccase genes by 5'/3' RACE

cDNA synthesis was performed using 1 µg of total RNA as a template with SMARTer® RACE 5'/3' Kit (Clontech, CA, USA) following the manufacturer's instructions. Gene-specific primers (GSPs) for 5' and 3' RACE were designed (Table 1). The amplified PCR products were purified from agarose gel using the NucleoSpin Gel and PCR Clean-Up kit (Clontech, EE.UU) and cloned into the pUC19 vector in fusion HD cloning kit (Clontech, EE.UU). DNA inserts were sequenced at MacroGen (Seoul, South Korea).

Table 1 Primers sequences of RACE-PCR for amplification of laccase genes

5' RACE	3' RACE
<i>RACE adopter primer</i>	<i>RACE adopter primer</i>
Outer UPM long: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCACGC AGAGT	UPM long: CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
Inner UPM short: CTAATACGACTCACTATAGGGC	Inner UPM short: CTAATACGACTCACTATAGGGC
<i>Gene-specific primer</i>	<i>Gene-specific primer</i>
lacSP R1: CGCTTGATCGGTCGCGGTGAAGTCGTAG	lacSP F1: CAGAAGGGGACTAACTGGGCTGACGGCC
lacSP R2: GCATTGGTTACGAAGGCGGGGCC	lacSP F2: AACCAATGCCCCGATTGCCACCGGAAAC

Bioinformatics analysis

The predicted amino acid sequences were aligned with the MUSCLE algorithm, (Edgar 2004). The identity percentages of *T. hirsuta* Bm-2 amino acid sequence with other laccase sequences were calculated using the BLAST algorithm, at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul et al. 1997). The phylogenetic tree was built using the Maximum Likelihood Method, implemented in MEGA 7.0 (Kumar et al. 2016). The support for the branches was evaluated using the bootstrapping method with 1000 replicates.

The signal peptide was predicted using SignalP 4.0 (Nielsen 2017) and the glycosylation sites with NetNGlyc 1.0 (Gupta and Brunak 2001). The hairpins and potential IRES sites were identified with the IRESPred webserver (Kolekar et al. 2016). For the G-quadruplex structures, the Q-GRS Mapper program was used (Kikin et al. 2006).

Results and discussion

Laccases are multi-copper enzymes, present in families of genes, mostly in basidiomycete fungi, and various factors, such as the carbon source, regulate their expression (Wang et al. 2015). In this study, we analyzed the structure of two laccase genes expressed in a basal medium and in a medium enriched with a lignocellulosic agro-industrial residue (grapefruit peel).

For isolation of laccase genes from *T. hirsuta* Bm-2, the 5'/3' RACE-PCR technique was utilized, using total RNA as a template, and the specific primers designed from the copper-binding sites I and II (Table 1).

The 5'/3' RACE-PCR amplification products were sequenced and assembled to obtain two cDNA full-length sequences, 2277 bp and 2339 bp, which were named *lac-B* (basal medium) and *lac-T* (grapefruit peel medium), respectively. These genes are in the size range of the laccase genes (1800–2400 bp) reported by Zhou et al. (2014) and Vasina et al. (2015).

The complete sequences for *lac-B* and *lac-T* genes are shown in Figs. 1 and 2, respectively. Both sequences

presented the same open reading frame size (ORF) of 1566 bp, where 47 single nucleotide polymorphisms were identified between *lac-B* and *lac-T* sequences; all these mutations are synonymous, and therefore did not alter the encoded protein.

The ORF sizes reported in our study are in the range of the *lacA-lacE* genes of *T. hirsuta* strain 072 and coincide with the size of *lac C* (Table 2) (Vasina et al. 2015). The putative protein of both sequences of 521 amino acid residues contains a secretion signal of 23 amino acids (MRDLTLLRAATAAFLSFGAPALA). The size of the signal peptide varies in the fungal laccases, as is the case of laccases *lac1-lac8* from *Cerrena* sp. HYB07, which have signal peptides in the range of 18–21 amino acids (Yang et al. 2016). While Wang et al. 2015 reported a signal peptide of 23 amino acids for *lac4*, *lac5*, and *lac11* of *F. velutipes*.

In silico analysis of the potential *N*-glycosylation sites for laccases of *T. hirsuta* Bm-2 showed five sites at positions Asn-74, Asn-204, Asn-240, Asn-356, and Asn-458 (Figs. 1 and 2), unlike the laccase GwLAC1 of *Ganoderma weberianum* TZC-1, which has seven glycosylation sites, three of which coincide with those reported in this work (Asn-75, Asn-355, and Asn-458) (Zhou et al. 2014). Laccases are glycoproteins that have a wide range of glycosylation, between 10 and 25% (Kim et al. 2013). The post-translational modification, by protein glycosylation, is responsible for the structural stability of the enzyme, and protects it from proteolysis and inactivation by free radicals (Vite-Vallejo et al. 2009).

The alignment of the amino acid sequence of the laccase of *T. hirsuta* Bm-2 (Fig. 3) with other fungal laccases revealed that *T. hirsuta* Bm-2 laccase has the four typical conserved copper-binding domains: L1 (HWHGFFQ), L2 (HSHLSTQ), L3 (HPFHLHG), and L4 (HCHIDFHL). We also observed ten conserved residues of histidine and a cysteine residue which are located in the signature sequences specific to fungal laccases L1–L4 (Kumar et al. 2003). It is important to note that Bm-2 laccases share a high degree of identity, between 76 and 85%, with the laccases of *T. villosa*, *T. versicolor* and other strains of *T. hirsuta*.

Phylogenetic relationship analysis between *lac-B* and *lac-T* genes of *T. hirsuta* Bm-2 and other fungal laccases

	ATGGGGGCTCTCCTTCGCTGCACTCCCAATCCGAAATTCGTTGCTCACGCTGATGTGCGGCCAACGCGCGTATCCGGTACAACTATATCT	-503
	GAGTAGGATCGCAATGATTAATGTGGCGGCGCGAGATTACGGCCGACGCGGTCGCGTTCGAATCCTACGTCTACAGAACCGCTGA	-408
	TCTGTGTCTGCGGGTAGGCCGACGAGCATCTGTCTGAGGCCGATCTCGGATCGGACTTCATCTCCGCTGATCACTGCGGTCCCGCGCG	-313
	CTCATTTCCTCTGCGGCTTGCACGTAGCGTTCAGGCTCAACGTATCGGTGGAGGGGTAGAGCACTGCCGCAATGACTGAGTTCCAGCGCG	-218
	AGGTGACAGTCATGCGCGCAGATATAAGGGTGTCCGTCGCCAGAGGCTGACACACAAACCCGTTCTTTCGCCAGCAACTCCCTTCCCCCTT	-123
	CTCTCCATTTCATTTTTCAGCTCAC	-28
1	ATG GAC AGA CTC ACT CTG TTG CGC GCG GCT ACC GCC GCT TTC CTT TCG TTT GGA GCT CCT GCT CTG GCC GCG	72
	<u>M R D L T L L R A A T A A F L S F G A P A L A A</u>	
	GTC GGG CCT GTG GGC GAC CTC ACT ATA TCC AAC GCG GAC GTC TCT CCC GAT GGC TAC ACT CGC GCT GCC GTC	144
25	V G P V A D L T I S N A D V S P D G Y T R A A V	
	GTG GTC AAC GGG GTT TTC CCT GGT CCA CTC ATC ACC GGA AAC ACC GGC GAC AAC TTC CAG CTC AAC GTC ATT	216
49	V V N G V F P G P L I T G N T G D N F Q L N V I	
	GAC AAC CTG ACC AAT GCG ACG ATG TTG AAG TCA ACG ACC GTG CAC TGG CAC GGC TTC TTT CAG AAG GGG ACT	288
73	D <u>N</u> L T N A T M L K S T T V <u>H W H G F F Q</u> K G T	
	AAC TGG GCT GAC GGC CCC GCC TTC GTG AAC CAA TGC CCG ATT GCC ACC GGA AAC TCT TTC CTC TAC GAC TTC	360
97	N W A D G P A F V N Q C P I A T G N S F L Y D F	
	ACT GCG ACC GAT CAA <u>GCA</u> GGC ACG TTC TGG TAC CAC AGC CAC TTG TCG ACG CAA TAT TGC GAT GGC CTG CCG	432
121	T A T D Q A G T F W Y H S H L S T Q Y C D G L R	
	GGC CCG ATG GTC GTT TAC GAC CCC AAC GAC CCG CAT GCG GAC CTT TAC GAC GTG GAC GAG TCT ACC GTG	504
145	G P M V V Y D P N D P H A D L Y D V D D E S T V	
	ATC ACC CTG TCC GAC TGG TAT CAT ACC GCG GCG AAG CTC GGT CCT GCT TTC CCG GTT GGG TCG GAC TCC ACT	576
169	I T L S D W T H T A A K L G P A F P V G S D S T	
	TTG ATG AAC GGG CTG GGC CGT TTC TCT GGT GGC AAC AGC ACC GAC CTT GCG GTC ATC AAC GTC GAG CAG GGC	648
193	L I N G L G R F S G G <u>N</u> S T D L A V I N V E Q G	
	AAG CGC TAT CGT TTC CGC CTC GTC TCG CTC TCC TGC GAT CCC AAC TAT GTC TTC TCT ATC GAC GGC CAT AAC	720
217	K A Y A F A L V S L S C D P N Y V F S I D G H <u>N</u>	
	ATG ACC ATC ATC GAG GTC GAC GCT GTC AAC CAC GAG CCG CTG CTG GTT GAC TCC ATC CAG ATT TTC GCC GGG	792
241	M T I I E V D A V N H E P L L V D S I Q I F A G	
	CAG CGG TAC TCC TTC GTC CTT ACC GCC GAT CAA ACC ATC GAC AAC TAC TTC ATC CGT GCC CTA CCC AAC GCG	864
256	Q R Y S F V L T A D Q T I D N Y F I R A L P N A	
	GGT ACT GTC TCG TTC AGC GGC GGC GTT AAC TCC GCT ATC CTG CGC TAC TCG GGC GCC GCT GAG ATT GAC CCG	936
289	G T V S F S G G V N S A I L R Y S G A A E I D P	
	ACT ACC GAG TTC GTG ACG AAG ACC AAC CCT CTC GTT GAG GCG AAC CTC GTG CCG CTT GAG GAC GCC GCT GCT	1008
313	T T E F V T K T N P L V E A N L V P L E D A A A	
	CCT GGC GAC CCC GTT GCA GGC GGT GTC GAC TAC GCG CTC AAC CTT GAC TTC TCG TTC AAC GGC AGC AAC AAT TTC	1080
337	P G D P V A G G V D Y A L N L D F S F <u>N</u> G S N F	
	TTC ATC AAC GAC GCG ACC TTC GTG CCC CGG AYC GTC CCC GTT CTC CTC CAG ATC CTC AGC GGT GCC TCT TCG	1152
361	F I N D A T F V P P T V P V L L Q I L S G A S S	
	GCC GCC GAC CTC CTC CCC AGT GGC AGC CTC TTC TCG CTC CCC TCC AAC TCG TCC ATT GAG CTC TCC TTC CCC	1224
385	A A D L L P S G S L F S L P S N S S I E L S F P	
	ATC ACC GCR ACG AAC GCG CCC GGA GCG CCG CAC CCC TTC CAC CTG CAC GGT CAC ACG TTC TCC GTC GTC CGC	1296
409	I T A T N A P G A P <u>H P F H L H G H</u> T F S V V R	
	AGC GCG GGC GGC AGC GAC GCC AAC TAC GTG AAC CCG CCC CGC CGC GAC GTC AGC ACC GGC ACC GCC GGC	1368
433	S A G G S D A N Y V N P P A A D V V S T G T A G	
	GAC AAC GTC ACC ATC CGT TTC ACC ACG GAC AAC CCC GGT CCG TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC	1440
457	D <u>N</u> V T I R F T T D T P G P W F L <u>H C H I D F H</u>	
	CTC GAG GCG GGC TTC GCG ATT GTC TTC AGC GAA GAC ACG AGC GAG GTT TCG TCC GTC ACC GAT CCC TCT GCT	1512
481	<u>L E A G</u> F A I V F S E D T S E V S S V T D P S A	
	GCT TGG AGC AAC CTG TGC CCG ACT TAC AAC GCC CTG GAC TCG GGC GAT CTC TGA	1566
505	A W S N L C P T Y N A L D S A D L *	
	TTTACGCCGAGGCGCGTATGCGTCTACTACTGGGCTACACTTATACCTACCTCGGGTACTATGGACTTGACTCCGGGTTATCAGCCGTTATATA	1663
	CGCGCTACTATACAAAGAGCTGCTCTGATGAGGTTGGGTTTTTCAGGGAAACGGACTTGCCAGGATTGGGCGACTCGAAGAAGTCTCAAGAATACC	1760
	CTTGACAGTAGCATGAOCTCCGGCTCTCCTGTGGACTCCTCTTCTTGTGTGGGAATTAACCTGCTTTGCA	1833

Fig. 2 Nucleotide and amino acid sequence of the *lac-T* gene. The secretion peptide is double underlined, the potential sites of *N*-glycosylation are shown in boxes, and the four-conserved laccase domains

are underlined. An asterisk indicates the stop codon. Highlights show the different nucleotides with respect to the sequence of the *lac-B* gene

Table 2 Properties of identified cDNA and predicted protein sequences for laccases from strain Bm-2 of *T. hirsute*

Gene	Full-length cDNA (bp)	5'UTR (bp)	3'UTR (bp)	ORF (bp)	Predicted amino acid sequence Length MW pI	Potential N-glycosylation sites	GenBank accession no.	References
<i>lacB</i>	2277	441	267	1566	521 56.6 6.1	5	MK159302	In this work
<i>lacT</i>	2339	503	267	1566	521 56.6 6.1	5	MK159303	In this work
<i>lacA</i>	1811	63	170	1563	520 55.9 5.06	8	KP027478	Vasina et al. (2015)
<i>lacB</i>	1846	65	192	1563	520 56.2 6.21	6	KP027484	Vasina et al. (2015)
<i>lacC</i>	1858	80	197	1566	521 55.6 4.40	11	KP027479	Vasina et al. (2015)
<i>lacD</i>	1837	45	205	1572	523 57.6 5.72	10	KP027480	Vasina et al. (2015)
<i>lacE</i>	1742	68	99	1560	519 58.7 6.17	6	KP027481	Vasina et al. (2015)

genes is shown in Fig. 4. These results confirm that *lac-B* and *lac-T* genes form an independent clade with a 100% branch support. This clade is grouped together with the sub-clade where laccase genes of *T. versicolor* are located, with a branch support between 99 and 100%.

The 5'/3' non-coding regions have important roles in the regulation of gene expression affecting mRNA stability, localization, and translation efficiency (Bradnam and Korf 2008). In this work, the non-coding 3'UTR region showed the same length and sequence (270 pb) in both genes. The 5'UTR regions showed moderate differences in length, *lac-B* 441 bp and *lac-T* 503 bp; however, there were clear differences in the structure and number of putative regulatory elements. The in silico analysis predicted that the 5'UTR of both genes is folded into structures with several hairpins that irradiate from a central loop region, whose number and location were different between the genes (Figs. 5 and 6).

The structures ΔG were -172.5 kcal/mol for *lac-T* and -135.8 kcal/mol for *lac-B*, suggesting they are stable motifs that could be targeted by proteins that bind to RNA, allowing the migration of the 40S ribosomal subunit, which requires at least -50 kcal/mol (Mignone et al. 2002).

The 5'UTR sequences were analyzed with the RNA fold program (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>), to explore the possible regulatory elements present.

Both genes present a single potential IRES site (internal ribosome entry site) that allows the translational complex with the 40S to function in a cap-independent process. Contrastingly, *lac-T* contains twice the number of motifs than *lac-B* such as hairpins, guanine-rich quadruple G structures (G4) that can fold into a structure very stable to strongly repress translation, and uORF (upstream open reading frame) which can impact on downstream expression by altering the efficiency of translation or initiation at the main ORF

(Table 3). The number and nature of the regulatory elements present in the 5'UTR represent an accessibility factor which impacts the regulation of gene expression, by activating or inhibiting the translation of proteins (Ganapathi et al. 2005; Barrett et al. 2013). Both genes also have a high GC content of approximately 58%.

The analysis of the untranslated sequences has been done in mammals and in cancer cells, but there are very few reports in fungi (Mignone et al. 2002). In eukaryotes, the length of the 5'UTR varies considerably among genes, ranging from a few base pairs to several hundred base pairs (Nagalakshmi et al. 2008). Recent studies in *Saccharomyces cerevisiae* suggest that the 5'UTR length is related to different functions of genes and that it can influence the regulation of gene expression (Bruno et al. 2010; Lin and Li 2011).

It has been suggested that 5'UTRs of genes with low protein production are on average longer (greater than 100 bases) than those of other genes, and possess a higher degree of predicted secondary structure (Pickering and Willis 2005). By in silico comparisons, it was revealed that the short 5'UTRs, with low GC content (less than 30%), are relatively unstructured, do not contain AUG codons upstream, and generate high levels of protein production (Kochetov et al. 1998). This differs from the study by (Dmitriev et al. 2009), who found that short unstructured 5'UTRs, and highly structured lengths, did not modify the efficiency of mRNA translation of mammalian cells. Kozak mentioned that mRNAs that code for proteins involved in the cell development process often contain long 5'UTRs that must be finely regulated (Kozak 1989). In this study, we determined that although the 5'UTRs of *lac-B* and *lac-T* are both long, the expression levels are drastically different. In the medium supplemented with grapefruit, the activity of laccases was 50 times higher with respect to the basal medium (data not shown). The discrepancies in the reports indicate

Fig. 3 Alignment of the amino acid sequence of laccase genes of *T. hirsuta* Bm-2 and other ligninolytic fungi. The identical residuals are shown with asterisks. The four-conserved laccase domains are indicated in boxes

<i>T. hirsuta</i> Bm-2	MRDLTLRAATAAFSLFSGAPALAAVGFVADLTISNADVSPDGYTRAAVVVNGV-----
<i>LacD. T. hirsuta</i>	MSWSPTAVTILGLC--GAAMAAIGPVTDLIDVKNKEIAPDGLLRDTVLGGT-----
<i>LacP. ostreatus</i>	MFGARILATLTALHLLHGTAAIGPTGMYIVNEVDSPDGFARSAAVARSVPATDPTF
<i>LacC. T. hirsuta</i>	MGLQHFATISIALSLSLGRAVYGAIGPVTTLTANADISPDGYTRATVLANGV-----
<i>Lac3. T. versicolor</i>	MGRVSSLCALTAVIHSF--GRVSAAGPVTDLTISNADVSPDGYTRAAVLANGV-----
<i>Lac4. T. versicolor</i>	MGRVSSLCALTAVIHSF--GRVSAAGPVTDLTISNADVSPDGYTRAAVLANGV-----
<i>Lcc2304. T. versicolor</i>	-----AIGPVTNLITISNADVSPDGYTRAAVLANGV-----
<i>LacA. T. hirsuta</i>	MSRFQSLTITISLV--AVAHAAVGFVADLTITDAASPDGFSRQAVVNGV-----
<i>LacB. T. hirsuta</i>	MPRVFPLGIIVALVLA--VSVLGAIGPKGNLIVNNAIAPDGFNRDAIVVNGV-----
	:
	Cu-I Domain
<i>T. hirsuta</i> Bm-2	----FPGPLITGNTGDNFQINVIDNLNATMLKSTTVHWHGFFQKGTNWADGPAFVNQCP
<i>LacD. T. hirsuta</i>	----FPGPLVQGGKGDHFKINVDKLTNETMLTSTTIHWHGLFQHTTNWADGPAFVTCQP
<i>LacP. ostreatus</i>	ATVSIPIGVLVQNGKDNFQINVNQLSDTMTLKTSTIHHWHGFFQAGSNWADGPAFVTCQP
<i>LacC. T. hirsuta</i>	----YPGPLITGNTGDNFQITVEDQLTNSMTLKTSTIHHWHGFFQEGTNWADGPAFVNQCP
<i>Lac3. T. versicolor</i>	----FPGPLITGKNGDN--QINVDNLNSETMLKSTSTIHHWHGFFQKGTNWADGPAFVNQCP
<i>Lac4. T. versicolor</i>	----FPGPLITGKNGDNFQINVDNLNSETMLKSTSTIHHWHGFFQKGTNWADGPAFVNQCP
<i>Lcc2304. T. versicolor</i>	----FPGPLITGKNGDNFQINVDNLNSETMLKSTSTIHHWHGFFQKGTNWADGPAFVNQCP
<i>LacA. T. hirsuta</i>	----TPGFLVAGNIGDRFQINVIDNLNTHMTLKTSTIHHWHGFFQHTTNWADGPAFVNQCP
<i>LacB. T. hirsuta</i>	----FPGPLITGKNGDNFQINVIDNLNTHMTLKTSTIHHWHGFFQHTTNWADGPAFVNQCP
	:
	Cu-II Domain
<i>T. hirsuta</i> Bm-2	IATGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>LacD. T. hirsuta</i>	IIAGNDFLYNFQVQDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>LacP. ostreatus</i>	VASGDSFLYNFNVQDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>LacC. T. hirsuta</i>	IIASGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>Lac3. T. versicolor</i>	IATGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>Lac4. T. versicolor</i>	IATGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>Lcc2304. T. versicolor</i>	IATGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>LacA. T. hirsuta</i>	IIISPGHSLYDFQVQDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
<i>LacB. T. hirsuta</i>	IIASGNSFLYDFHVPDQAGTFWYHSHLSTQYCDGLRGPMVYVDFNDPHADLYDVDDDESTVI
	:*:
<i>T. hirsuta</i> Bm-2	TLSDWHTAAKL---GPAFPVGSSTLNLGRFSGGNSDTLAVINVEGKKAFAFVLSL
<i>LacD. T. hirsuta</i>	TLADWYHTAAPIEANGPGIPT--SDSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>LacP. ostreatus</i>	TLEDWYHIVAPQ---NAAIPT--PDSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>LacC. T. hirsuta</i>	TLSDWYHTAAAL---GAAPFAGADSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>Lac3. T. versicolor</i>	TLSDWYHTAASL---GAAPFVGSSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>Lac4. T. versicolor</i>	TLSDWYHTAASL---GAAPFVGSSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>Lcc2304. T. versicolor</i>	TLSDWYHTAASL---GAAPFVGSSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>LacA. T. hirsuta</i>	TLADWYHTAAKL---GPRFLGADATLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
<i>LacB. T. hirsuta</i>	TLADWYHTAAKL---GPKFPLGAQSTLNLGRFSGGNSDTLAVINVEGKRYRFRLLNI
	*:
<i>T. hirsuta</i> Bm-2	SCDPNYVFSIDGHNMIIIEVDVNEHPLLVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>LacD. T. hirsuta</i>	ACDPNYNFTIDGHNMIIIEADGENTQPLKVDKIELVAQRYSFVLEAQNQVNNYIRALP
<i>LacP. ostreatus</i>	SCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>LacC. T. hirsuta</i>	SCDPNHTFSIDGHNMIIIEVDVNEHPLLVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>Lac3. T. versicolor</i>	SCYPNYVFSIDGHNMIIIEADVNEHPLTVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>Lac4. T. versicolor</i>	SCDPNYVFSIDGHNMIIIEADVNEHPLTVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>Lcc2304. T. versicolor</i>	SCDPNYVFSIDGHNMIIIEADVNEHPLTVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>LacA. T. hirsuta</i>	SCDPNHTFSIDGHNMIIIEVDVNSQPLEVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
<i>LacB. T. hirsuta</i>	SCDPNHTFSIDGHNMIIIEVDVNSQPLEVDSIQIFAGQRYSFVLTAQTDIDNYFIRALP
	*:
<i>T. hirsuta</i> Bm-2	---NAGTVSFGGVSAILRYSGAAEIDPTTFFVTKTNPLEANLVPLEDAAPGDFVAG
<i>LacD. T. hirsuta</i>	DRQNLNATGFANGINSAILRYKGAIEAPNTQSPVAFLETDLHPLTNPAAPGHPYK
<i>LacP. ostreatus</i>	---NLGSGTGDGINSAILRYAGATEDDPTTSSSTST--PLETNLVPLENPGAPGPTVPG
<i>LacC. T. hirsuta</i>	---NTGTTDFADGINSAILRYSGAEIEPTNTQTTSVAPLVETDVLPLDSAPAGGAEVAG
<i>Lac3. T. versicolor</i>	---SAGTTSFDGINSAILRYSGASEVDPTTETTSVPLEDEANLVPLDSAPAGGDNIG
<i>Lac4. T. versicolor</i>	---SAGTTSFDGINSAILRYSGASEVDPTTETTSVPLEDEANLVPLDSAPAGGDNIG
<i>Lcc2304. T. versicolor</i>	---SAGTTSFDGINSAILRYSGASEVDPTTETTSVPLEDEANLVPLDSAPAGGDNIG
<i>LacA. T. hirsuta</i>	---NFGNVGFDGINSAILRYDGAPAVEPTNTQTTSVPLEDEANLVPLDSAPAGGSSSG
<i>LacB. T. hirsuta</i>	---NFGTTFGAGGVSAILRYKGAFAPEPTTQSTTSVPLEVETNLHPLASMRVPLGVPG
	*:
<i>T. hirsuta</i> Bm-2	GVDYALNLDIFSFGNSNF--FINDATFVPPTVFLVLLQILSGASSAADLLPSGSLFSLPSNS
<i>LacD. T. hirsuta</i>	GVDYALNLDINFTKGEF--LINNVSKPPTVFLVLLQILNGTLDADHLMPHGSVYSLPFS
<i>LacP. ostreatus</i>	GADININLMAFDFTTFTLTINGVPFIPPTAFVLLQILSGASSAADLLPSGSIYALEPNK
<i>LacC. T. hirsuta</i>	GVDYALNLAISFGNSAF--FINDATYTSPTVFLVLLQILSGAQTADLLPSGSIYALEPNK
<i>Lac3. T. versicolor</i>	GVDYALNLDINFTKGEF--FINDVSVSPTVFLVLLQILSGTSAADLLPSGSLFALPSS
<i>Lac4. T. versicolor</i>	GVDYALNLDINFTKGEF--FINDVSVSPTVFLVLLQILSGTSAADLLPSGSLFALPSS
<i>Lcc2304. T. versicolor</i>	GVDYALNLDINFTKGEF--FINDVSVSPTVFLVLLQILSGTSAADLLPSGSLFALPSS
<i>LacA. T. hirsuta</i>	GVDKAINLMAFNFGNSNF--FINGASFVPPTVFLVLLQILSGAQTADLLPSGSIYALEPNK
<i>LacB. T. hirsuta</i>	GVDKAINLMAFNFGNSNF--FINNATFTPTVFLVLLQILSGAQTADLLPPGSIYALEPNK
	*:
	Cu-III Domain
<i>T. hirsuta</i> Bm-2	SIELSFPIATNAPGAPHPFHLLHGHFTSVVRSAGGSDANYVNPAAADVSTGT--AGDNV
<i>LacD. T. hirsuta</i>	TIELSIIPGAA--PGHPFHLLHGHFTSVVRSAGSDHYNKPMRDTVSGT--SPTDNLV
<i>LacP. ostreatus</i>	VVEISMPALAV--GHPFHLLHGHFTSVVRSAGSTTYNFTPARRDVNTGT--GANDNV
<i>LacC. T. hirsuta</i>	TVELSFPIATNAGAPHPFHLLHGHFTSVVRSAGSVYNNPVRDVTSTGT--AGDNV
<i>Lac3. T. versicolor</i>	TIELSFPIATNAPGAPHPFHLLHGHFTSVVRSAGSDTYNFTPARRDVNTGT--AGDNV
<i>Lac4. T. versicolor</i>	TIELSFPIATNAPGAPHPFHLLHGHFTSVVRSAGSDTYNFTPARRDVNTGT--AGDNV
<i>Lcc2304. T. versicolor</i>	TIELSFPIATNAPGAPHPFHLLHGHFTSVVRSAGSDTYNFTPARRDVNTGT--AGDNV
<i>LacA. T. hirsuta</i>	SIELSFPIATNAPGAPHPFHLLHGHFTSVVRSAGSTTYNFTPARRDVNTGT--AGDNV
<i>LacB. T. hirsuta</i>	SIELTLPATSL-APGHPFHLLHGHFTSVVRSAGSTTYNFTPARRDVNTGT--AGDNV
	*:
	Cu-IV Domain
<i>T. hirsuta</i> Bm-2	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>LacD. T. hirsuta</i>	TVRFVTDNPGPWFLLCHIDWHLEAGLAIVFABDTKTDFVNPVPPQDWRDLCPAYDKAMNI
<i>LacP. ostreatus</i>	TIRFTVTDNPGPWFLLCHIDWHLEAGLAIVFABDTKTDFVNPVPPQDWRDLCPAYDKAMNI
<i>LacC. T. hirsuta</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>Lac3. T. versicolor</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>Lac4. T. versicolor</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>Lcc2304. T. versicolor</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>LacA. T. hirsuta</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
<i>LacB. T. hirsuta</i>	TIRFTTDPGPWFLLCHIDFHLEAGFAIVFSEDTSEVSVTDPSAASNLCPYTNALDSA
	*:

Fig. 4 Phylogenetic relationship of laccase genes of *Trametes hirsuta* Bm-2 with other laccase genes. The MEGA 7.0 program and the Likelihood method with 1000 bootstrap were used to construct the tree from cDNA sequences obtained from the database within the NCBI. The *lac-B* and *lac-T* genes of *T. hirsuta* Bm-2 are shown with circle symbols

that further studies are still required in these and other non-coding regions to elucidate the molecular mechanisms that control translation.

The results of this study suggest that the presence of regulatory motifs in the 5'UTR of the mRNA expressed in the inducing medium could favor the production of these enzymes at the translational level. However, it is important to consider that the regulation of the 5'UTR region not only involves the length or number of regulatory structures, but also depends on the combined effects of multiple factors that influence protein translation.

Conclusions

In this study, *lac-B* and *lac-T* genes of the strain Bm-2 of *T. hirsuta* were isolated. Both genes exhibited the same characteristics of laccase type, such as the copper-binding sites, glycosylation sites and signal peptide. The structural difference in the 5'UTR region of both genes could influence the transcriptional and/or translational processes of the genes. The perspectives of these studies include the approach of strategies for the use of modified 5'UTRs to increase the yield of recombinant proteins.

Fig. 5 Predicted secondary structure of 5'UTR regions in *lac-B*

Fig. 6 Predicted secondary structure of 5'UTR regions in *lac-T*

Table 3 Regulatory elements within the 5'UTR regions in laccase genes from *T. hirsuta* Bm-2

Motifs	<i>lac-B</i>	<i>lac-T</i>
G quadruplex	1	2
uORF	1	2
IRES	1	1
Hairpin	6	12

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding publication of this paper.

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