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Identification of laccase-like multicopper oxidases from the pathogenic fungus *Setosphaeria turcica* and their expression pattern during growth and infection

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Abstract *Setosphaeria turcica* (syn. *Exserohilum turcicum*) is the pathogenic fungus of maize (*Zea mays*) causing northern leaf blight, which is a major maize disease worldwide. Laccase-like multicopper oxidases (LMCOs) are generally found in different fungi and play important physiological roles during growth and pathogenesis of the fungus. Nine LMCOs were found in the *S. turcica* genome using a Hidden Markov Model for three Pfam copper oxidase families. They shared a low homology of 19.79%–48.70% and were classified into five LMCO super families, but had conserved amino acid residues in the Cu-binding sites. Transcription levels of LMCOs were detected by quantitative real-time PCR during different stages of invasion, i.e. in non-germinated conidia, during formation of germ tubes, appressoria and penetration pegs as well as during hyphal growth after penetration. *StLAC6* and *StLAC8* were highly expressed in mycelium and expression of *StLAC2* was significant in non-germinated conidia. During infection, the expression of *StLAC1* and *StLAC8* was high during appressorium formation and the expression of *StLAC6* was high during penetration peg formation. The laccase activity and gene expression of LMCOs cultivated with the laccase inducers $CuSO_4$, ABTS and resveratrol was detected. When treated with Cu^{2+}, the laccase activity significantly increased. Furthermore, the expression of all genes was significantly increased, except that of *StLAC7*. In the presence of the phenolic phytoalexin resveratrol, laccase activity did not increase, but the expression levels of *StLAC2*, *StLAC4* and *StLAC5* were up-regulated. These results suggest that LMCOs in *S. turcica* play different roles during fungal growth and infection processes.

Keywords *Setosphaeria turcica* · *Exserohilum turcicum* · Laccase-like multicopper oxidase · Infection · Development · Expression pattern

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Introduction

Laccases (benzenediol: dioxygenoxidoreductases, EC 1.10.3.2), which are a part of the protein superfamily of multicopper oxidases (MCOs), are polyphenol oxidases with four copper ions per molecule. They catalyse the reduction of O_2 to water with concomitant one-electron oxidation of various aromatic substrates, such as phenols, anilines and benzenethiols. Laccases are widely distributed in higher plants and fungi and were recently found in

several bacteria and insects (Giardina et al. 2010). In higher plants, laccases oxidize phenols to polyphenolic compounds, which spontaneously polymerize. Furthermore, laccases are particularly abundant in white-rot fungi and have been repeatedly linked to lignin degradation (Baltierra-Trejo et al. 2015; Khambhaty et al. 2015).

MCO classification is mainly based on the protein sequence because many of the prime substrates and natural functions of MCOs are unknown. Considering the complexity of their origin, substrates and functions, the term 'laccase-like multicopper oxidase' (LMCO) was established to distinguish them from the term 'laccase', originally identified from the lacquer tree (Reiss et al. 2013; Mathews et al. 2016). LMCOs are induced by different culture conditions and play different physiological roles in the growth and pathogenesis of fungi, such as in pigmentation (Lin et al. 2012; Sapmak et al. 2015), fruiting body formation (Courty et al. 2009), morphogenesis (Sakamoto et al. 2015; Nakade et al. 2011), infection of the host (Kuo et al. 2015), stress defence and xenobiotic compound degradation (Balcázar-López et al. 2016).

Many fungi have more than one LMCO gene (Hoegger et al. 2006; Cázares-García et al. 2013) and the expression pattern of the multi-gene family has been explored in various plant pathogenic fungi (Ruhl et al. 2013; Kilaru et al. 2006). The coprophilous fungus *Coprinopsis cinerea* has 17 different laccase genes (Kilaru et al. 2006). In the important wheat pathogen *Gaeumannomyces tritici*, the transcription of the laccase gene *LAC1* is constitutive, *LAC2* is Cu^{2+}-inducible and transcription of *LAC3* is observed only during infection of wheat (Litvintseva and Henson 2002). Transcriptional and enzyme profiling of 12 laccase genes in the genome of the white-rot wood-decay basidiomycete *Pleurotus ostreatus* was investigated by RNA sequencing. Here, it was found that *Lacc2* and *Lacc10* are the main sources of laccase activity (Castanera et al. 2012).

Setosphaeria turcica (syn. *Exserohilum turcicum*) is the causal agent of northern leaf blight, which is a major maize disease worldwide, resulting in devastating yield losses if efficient control strategies are not implemented (Shen et al. 2013). The fungus invades epidermal cells of maize leaves, circumvent plant defences and colonise host tissues, thereby causing disease. Histopathological studies of *S. turcica* infection showed that crude laccases could accelerate pathogen infection, resulting in bigger lesions on the leaves (Zhan et al. 2011). To date, not much is known about the LMCO genes of *S. turcica* and their function in growth and pathogenesis. The main objective of the present study was to identify the LMCO gene family and explore different functions of LMCOs from *S. turcica* by investigating their expression pattern during growth and simulated infection processes.

Materials and methods

Strains and culture conditions

Isolate 01–23 of *S. turcica* was cultured on home-made potato dextrose agar (PDA, Shen et al. 2013), in potato dextrose broth (PDB, prepared as PDA, but omitting the agar) as well as in Fries medium (Shen et al. 2013; Gu et al. 2014).

Identification of LMCO genes

The whole genome and protein sequences of *S. turcica* isolate Et28A were obtained from the Joint Genome Institute (http://genome.jgi.doe.gov). Predicted proteins from the genome were scanned with HMMER3.0 (Mistry et al. 2013). The proteins contained three Pfam copper oxidase families, Cu-oxidase (PF00394), Cu-oxidase_2 (PF07731) and Cu-oxidase_3 (PF07732) (Messerschmidt and Huber 1990). A Hidden Markov Model (HMM) for *S. turcica* was constructed by using the sequences of these three families. The HMM build from the HMMER Suite was used in the making of the model. All proteins with an E-value $\leq 10^{-3}$ were selected.

Cloning of full-length genes

The mycelium of *S. turcica* (isolate 01–23), grown on PDA, was used to extract genomic DNA using the CTAB method (Shen et al. 2013) and the full-length genes of LMCOs were amplified (primers shown in Table S1). To identify putative LMCO genes in *S. turcica*, the PCR products of these full-length genes, with genomic DNA as template, were sequenced and compared with public sequences in NCBI.

Bioinformatic analysis of LMCOs

The sequences of LMCOs in *S. turcica* were analysed with the following programmes: SMART (http://smart.embl-heidelberg.de/) to identify and analyse the features of protein motifs; Prot Param (http://web.expasy.

org/cgi-bin/protparam/) to predict the protein molecular weight and *p*I; PROSITE (http://prosite.expasy.org/) to predict any N-glycosylation sites; SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) to predict the presence and location of signal peptide cleavage sites; ClustalX 2 (Larkin et al. 2007) to align the amino acid sequences and Mega 5.1(Tamura et al. 2011) to construct an NJ phylogenetic tree of the amino acid sequences of the LMCOs. TRANSFAC (http://www.gene-regulation.com) was also used to predict the *cis*-acting elements of transcription factors. The Laccase and Multicopper Oxidase Engineering Database (LccED, https://lcced.biocatnet.de/) was used to classify the proteins (Sirim et al. 2011).

Sample collection, RNA extraction and cDNA preparation

Mycelium of *S. turcica* (isolate 01–23), was collected from PDB medium after being cultured for 7 days at 25 °C (Fig. 1a). Non-germinated conidia were harvested and washed with distilled water from PDA plates after the isolate was cultured for 15 days (Fig. 1b). To simulate the infection processes, a volume of 25 μL conidial suspension with 10^4 conidia/mL was incubated on the surface of a 20 μm thick cellophane membrane (Solarbio Life Science Co. Ltd., PEK, China) on water agar at 25 °C (Shen et al. 2013; Cao et al. 2011). Light microscopy was used to determine the developmental stages and fungal developmental stages during the infection processes of *S. turcica* are shown in Fig. 1. In order to measure gene expression levels during infection, samples were collected during formation of germ tubes (Fig. 1c), appressoria (Fig. 1d), penetration pegs (Fig. 1e) and hyphal growth after penetrating the cellophane membrane (Fig. 1f) (Cao et al. 2011). When more than 80% of the conidia developed to the appropriate developmental stages, sample droplets were collected, centrifuged at 8228×*g* for 5 min to remove water and preserved in liquid nitrogen until sufficient material for RNA extraction was collected. To measure the transcription levels of LMCO genes after application of inducers of laccase activity, the fungus was grown in Fries medium supplemented with either of the inducers $CuSO_4$ (Sangon Biotech, SHH, China), ABTS (Roche, IN, USA) or resveratrol (Sangon Biotech, SHH, China) at 0.03 g/L as well as without these inducers (control) at 25 °C for 7 days with shaking at 150 rpm. Three independent biological replications were made and within each replication, three samplings took place. RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, MA, USA) and used as the template to make cDNA using the TransScript II One-Step gDNA Removal kit and the cDNA Synthesis SuperMix kit (Transgen Biotech, PEK, China).

Quantitative real-time PCR

The expression levels of the LMCOs multigene family genes were investigated through qPCR. The primers for qPCR amplification, which were designed based on the sequences of non-conserved regions of these homologous genes and the primers for *β-tubulin*, which was used as the reference gene, are shown in Table S1. qPCRs were performed using a CFX Connect™ Thermal Cycler and Optics Module (Bio-Red, CA, USA). All qPCR experiments were run with cDNA synthesized from three biological replications and each sample had three technical replications. Quantification of the relative expression was performed using the $2^{-\Delta\Delta CT}$ method.

Assay of laccase activity by the oxidation of ABTS

The laccase activity was assayed in cultures of *S. turcica* grown in Fries medium supplemented with either of the inducers $CuSO_4$, ABTS or resveratrol at 0.03 g/L as well as without these inducers (control) at 25 °C for 7 days with continuous shaking at 150 rpm. The mycelium samples were filtered and ground in liquid nitrogen to extract crude laccases in PBS buffer (Sangon Biotech, SHH, China). The protein concentrations of samples were measured using the Bradford Protein Assay Kit (Sangon Biotech, SHH, China). Absorbance changes were monitored during oxidation of 2 mM ABTS at 420 nm ($\varepsilon_{420} = 36\ mM^{-1} \cdot cm^{-1}$) at room temperature using a spectrophotometer U*V*/VIS 2802PC (Unico, NJ, USA). One activity unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min (Ma et al. 2017). The laccase activity is presented as specific activity (units per mg protein). Three independent biological replications were made and each sample had three technical replications.

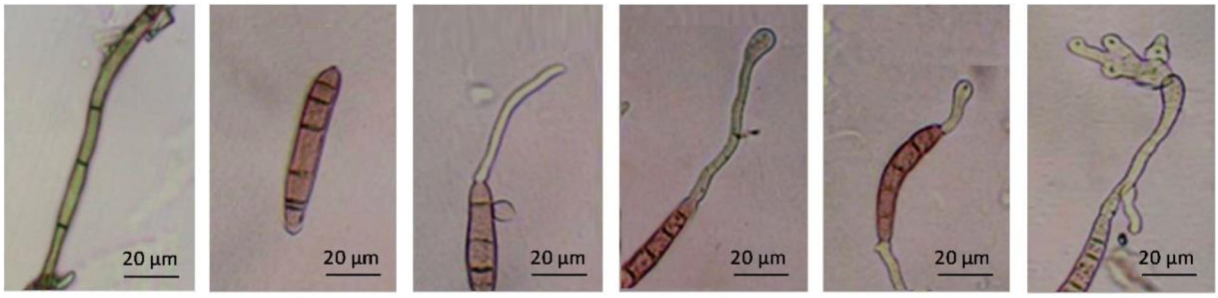

Fig. 1 Developmental stages of conidia germinated on a cellophane membrane for simulating the infection processes of *S. turcica*. Samples for qPCR were collected when more than 80% of conidia developed to the appropriate developmental stage. **a**: Hyphae isolated from a liquid culture incubated for 7 days in PDB; **b**: non-germinated conidia collected from a 15-day-old culture on PDA plates; **c**: germ tube formation on cellophane membrane; **d**: appressoria formation on cellophane membrane; **e**: penetration peg formation on cellophane membrane; **f**: hyphal growth after penetration on cellophane membrane

The enzyme activity of each technical replication was measured in triplicate.

Statistical analyses of data

Sample collection was performed from three biological replications, each with three technical replications. Data from each biological replication for gene expression by qPCR and enzyme activity were analysed separately by ANOVA using the Data Processing Station software (Tang and Zhang 2013) and the results from representative replications are shown. Means marked with different letters are significantly different at $P \leq 0.05$.

Results

Identification and analysis of LMCOs in *S. turcica*

Nine putative LMCOs were identified by a Hidden Markov Model using three conserved domains (PF00394, PF07731 and PF07732) of the classical multicopper oxidase in the genome of *S. turcica* (isolate Et28A), which matched the models with an E-value$\leq 10^{-3}$, as shown in Table S2. StLAC1 (EOA82311), StLAC2 (EOA90070), StLAC3 (EOA85295), StLAC4 (EOA85022), StLAC5 (EOA86979), StLAC6 (EOA85322), StLAC7 (EOA88313) and StLAC8 (EOA82285) had all three conserved domains whereas StLAC9 (EOA90498) had only two domains (PF07731 and PF07732). To check for their presence in *S. turcica* isolate 01–23, the respective encoding genes were cloned and the PCR-products of these full-length genes with the genomic DNA as template are shown in Fig. 2. The sequences were completely consistent with the published sequences from *S. turcica* isolate Et28A in NCBI, indicating that the nine complete sequences of the LMCO genes in *S. turcica* isolate 01–23 were identified.

The sequences for the genes are shown in Fig. 3a and the amino acid sequences of the putative LMCOs were analysed (Fig. 3b and Table 1). The LMCOs of

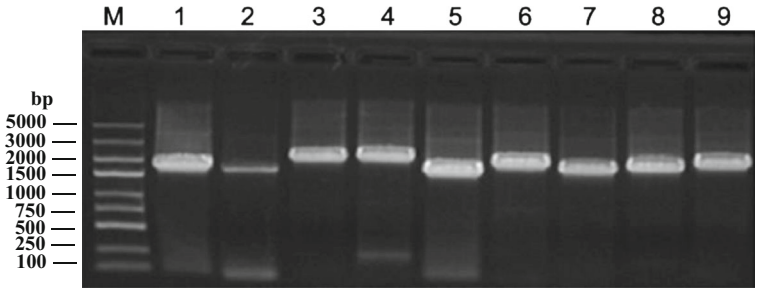

Fig. 2 PCR identification of putative LMCO genes in *S. turcica*. Lanes 1–9: *StLAC1*, *StLAC2*, *StLAC3*, *StLAC4*, *StLAC5*, *StLAC6*, *StLAC7*, *StLAC8* and *StLAC9*; M: DL-5000 DNA marker

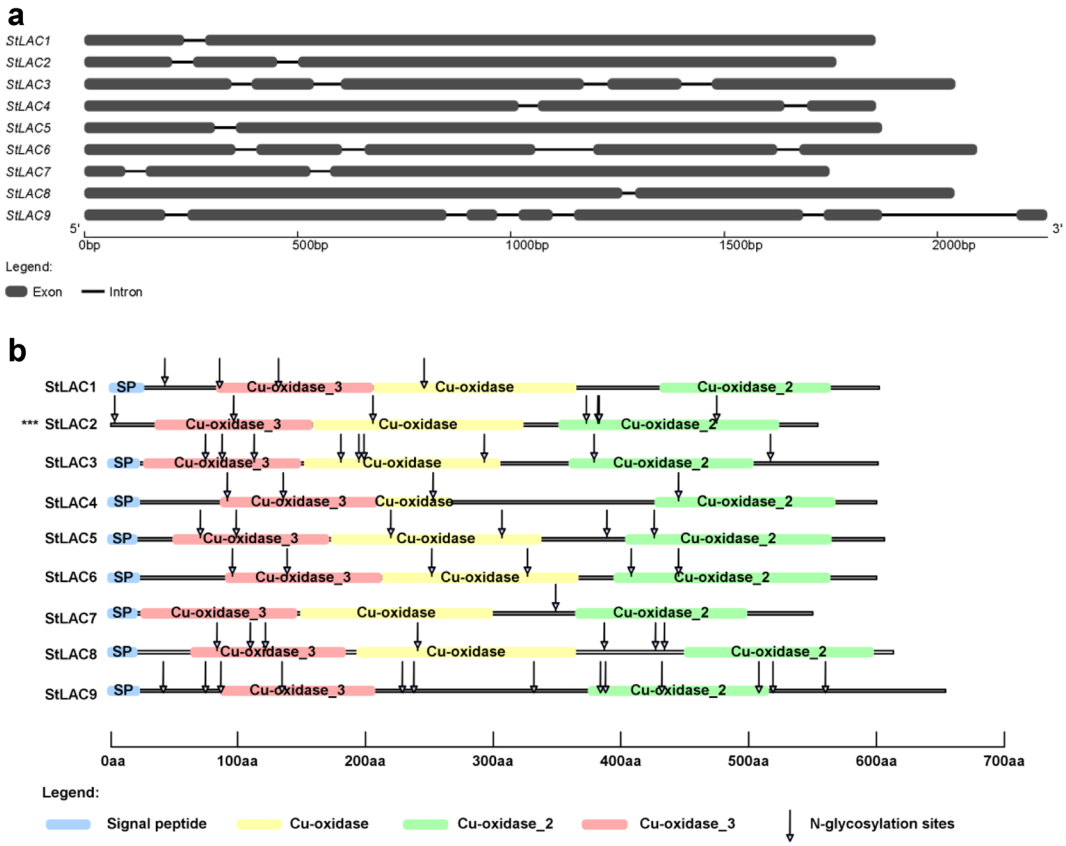

Fig. 3 Gene and protein characteristics of LMCOs in *S. turcica*. **a**: gene structure of LMCOs; **b**: protein characteristics of LMCOs. ***Protein of StLAC2 without signal peptide may not be glycosylated (in vivo) even though they contain potential motifs

S. turcica had 550–613 amino acid residues with the predicted protein having a molecular weight of 60.83–68.10 kDa. The predicted *p*I of these proteins was between 4.98 and 7.16 and all of them had 1–13 predicted potential glycosylation sties. All the LMCOs had a signal peptide of 19–23 amino acid

Table 1 GenInfo identifiers, amino acid length, predicted molecular weight (kDa), estimated *p*I, signal peptide length and the numbers of N-glycosylation sites of LMCOs in *S. turcica*

Gene name	GenInfo identifier	Amino acid length	Molecular weight (kDa)	*p*I	Length of signal peptide	N-glycosylation sites
StLAC1	482805212	601	65.73	5.65	23	5
StLAC2	482813379	554	61.64	5.00	0	7
StLAC3	482808362	601	65.85	4.98	21	9
StLAC4	482808089	600	65.94	7.16	21	4
StLAC5	482810173	606	67.79	6.56	19	6
StLAC6	482808389	600	65.85	5.07	21	6
StLAC7	482811557	550	60.83	6.87	19	1
StLAC8	482805186	613	68.10	5.59	19	7
StLAC9	482813807	564	64.31	5.38	21	13

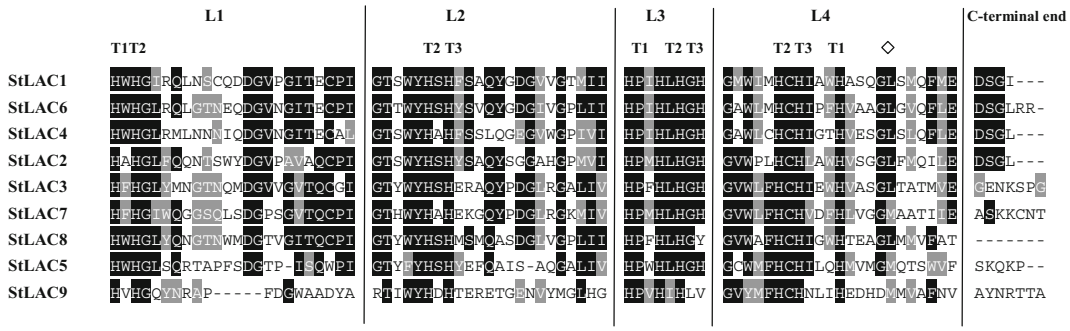

Fig. 4 Alignment of the four types of fungal laccase signature sequences of the copper-binding regions L1-L4 as well as the C-terminal end of StLAC1–9. The 10 conserved histidine residues and one cysteine residue are indicated in a complete set of four copper binding sites. The diamond symbol shows the residue at the axial ligand position of the T1-centre

residues at the N-terminus, except StLAC2 with no signal peptide.

Classification and phylogenetic analysis

The nine LMCOs were aligned and the sequences of the copper-binding regions of four types of fungal laccase signatures (L1-L4) as well as the C-terminal end are shown in Fig. 4. The sequences of L1-L4 in nine LMCOs had a low similarity to each other, but all these fragments typically contained ten histidine residues and one cysteine residue, indicating a functional LMCO with a complete set of four copper binding sites. The copper atom in the substrate binding site is a type 1 copper and defines the redox potential and thus the substrate range of the enzymes. The axial ligand for StLAC1, StLAC2, StLAC3, StLAC4, StLAC6 and StLAC8 is a leucine, which suggests that they are medium or high redox potential laccases. In StLAC5, StLAC7 and StLAC9, the axial ligand is a methionine, which suggests low redox potential laccases. Compared to the others, StLAC1, StLAC2, StLAC4 and StLAC6 had DSGL/I as C-terminal plug, which is conserved in ascomycetes laccases.

Pair-wise alignment was performed of the amino acid sequences of the nine LMCOs in *S. turcica* (Table 2). The proteins were assigned to homologous families to estimate their functions. The identity (%) of the nine LMCOs in *S. turcica* varied from 19.79–48.70%. StLAC1, StLAC4 and StLAC6 shared the highest identity (above 40%). As shown in Fig. 5, phylogenetic comparison of LMCOs in *S. turcica* was performed to other fungal LMCOs with known functions or reported previously, such as those from *Clavariopsis aquatica*, *Gaeumannomyces tritici* and *Podospora pauciseta*. The comparison revealed that the nine proteins belonged to different superfamilies. Based on the phylogenetic analysis and the blast results from the Laccase Engineering

Table 2 Identity (%) among protein sequences of the members of LMCOs in *S. turcica* by pair-wise alignment

Identity	StLAC1	StLAC6	StLAC4	StLAC2	StLAC3	StLAC7	StLAC8	StLAC5	StLAC9
StLAC1	100.00	48.70	42.39	35.61	30.66	29.08	31.78	24.68	21.69
StLAC6		100.00	43.15	36.99	30.75	28.71	30.75	22.86	22.00
StLAC4			100.00	35.82	28.49	25.65	29.16	24.63	21.89
StLAC2				100.00	29.65	30.67	29.59	22.74	22.62
StLAC3					100.00	37.36	35.63	26.77	22.44
StLAC7						100.00	29.14	22.16	20.80
StLAC8							100.00	25.24	21.28
StLAC5								100.00	19.79
StLAC9									100.00

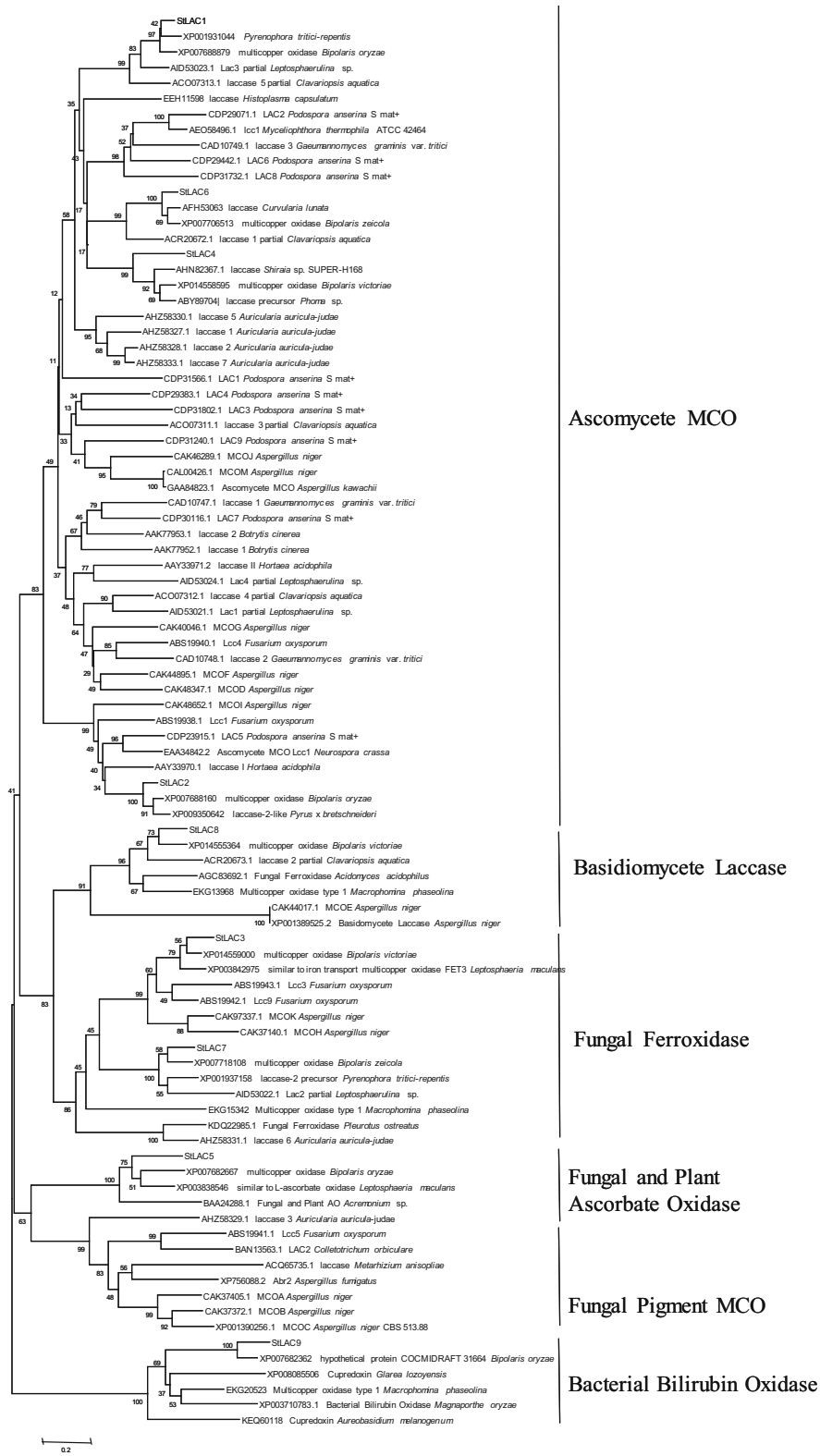

Fig. 5 Phylogenetic analysis of LMCOs in *S. turcica*. GeneBank accession numbers and described names of LMCOs are depicted together with the scientific names of the fungal strains (NCBI). The phylogenetic tree was constructed by the Neighbour-Joining method with the bootstrap values based on 1000 replications. The scale bar indicates a distance equivalent to 0.2 amino acid substitutions per site

Table 3 Number of transcriptional regulatory elements located in the promoter region 2000 bp upstream of the LMCOs genes in *S. turcica*

Gene	*NIT2*	*HSF*	*ADR1*	*MATalpha2*	StuAp	STRE	XRE	MRE	CRE-A
StLAC1	2	3	3		1				3
StLAC6		1	1	1			1		2
StLAC4	1	3							
StLAC2	1	3					3	1	
StLAC3		3	3	2			2	2	3
StLAC7	1	2		3		1	3		1
StLAC8		4	2	1					3
StLAC5	1	6	2						1
StLAC9		6	2	1	1				

The transcription factors include *NIT2* (the major positive-acting nitrogen regulatory gene), *HSF* (heat shock transcription factor), *ADR1* (a positive regulator of peroxisomal protein genes), *MATalpha2* (the determined mating type gene in *S. cerevisiae*) and *StuAp* (a gene regulating developmental complexity). Other responsive elements include STRE (stress responsive element), XRE (xenobiotic-responsive element), MRE (metal- responsive element) and CRE-A (carbon catabolite repression responsive element)

Database (LccED), which was designed to serve as a tool for systematic sequence-based classification and analysis of the diverse MCO family, StLAC1, StLAC2, StLAC4 and StLAC6 were grouped in the first clade and belong to superfamily B, which is known as ascomycete MCOs. However, StLAC2, which has no signal peptide, was in a different terminal branch. StLAC3 and StLAC7 belong to Superfamily E, which is known as fungal ferroxidases. StLAC5, StLAC8 and StLAC9 were in a different clade and belong to Super family F (known as fungal and plant ascorbate oxidases), Super family A (known as basidiomycete laccases) and Super family I (known as bacterial bilirubin oxidases), respectively.

Transcriptional regulatory elements of LMCOs from *S. turcica*

The promoter regions 2000 bp upstream of the LMCOs genes in *S. turcica* were analysed to determine the transcriptional regulatory elements of the fungus and the results are shown in Table 3. Certain *cis*-acting elements of transcription factors were found in different genes including *NIT2* (the major positive-acting nitrogen regulatory gene), in *StLAC1*, *StLAC2*, *StLAC4*, *StLAC5* and *StLAC7;* as well as *HSF* (heat shock transcription factor) in all nine LMCOs of *S. turcica*. *ADR1* (a positive regulator of peroxisomal protein genes) was found in *StLAC1*, *StLAC3*, *StLAC5*, *StLAC6*, *StLAC8* and *StLAC9*, *MATalpha2* (the determined mating type gene in *Saccharomyces cerevisiae*) was found in *StLAC3*, *StLAC6*, *StLAC7*, *StLAC8* and *StLAC9* and finally, *StuAp* (regulates developmental complexity) was found in *StLAC1* and *StLAC9*. The promoter regions also contained STRE (stress response element) in *StLAC7*, XRE (xenobiotic-responsive element) in *StLAC2*, *StLAC3*, *StLAC6* and *StLAC7*, MRE (metal-responsive element) in *StLAC2* and *StLAC3* and finally CRE-A (responsible for carbon catabolite repression) in *StLAC1*, *StLAC3*, *StLAC5*, *StLAC6*, *StLAC7* and *StLAC8*.

The promoter regions of these genes had different transcriptional regulatory elements even in the same superfamily of MCOs (e.g. *StLAC1*, *StLAC2*, *StLAC4* and *StLAC6*). This indicates that the expression may be influenced by different exogenous materials and environments.

LMCO expression pattern during different fungal growth and infection stages

The relative expression of nine LMCOs were analysed by qPCR separately in all samples, but only expression of *StLAC1*, *StLAC2, StLAC4, StLAC5, StLAC6, StLAC7* and *StLAC8* was detected. The expression levels of these seven genes were determined during different developmental stages on cellophane membranes, i.e. in non-germinated conidia, during formation of germ tubes, appressoria and penetration pegs, during hyphal growth after penetration as well as in mycelium cultured in PDB for 7 days (Fig. 6). The expression level in mycelium was set as a control for normalization in

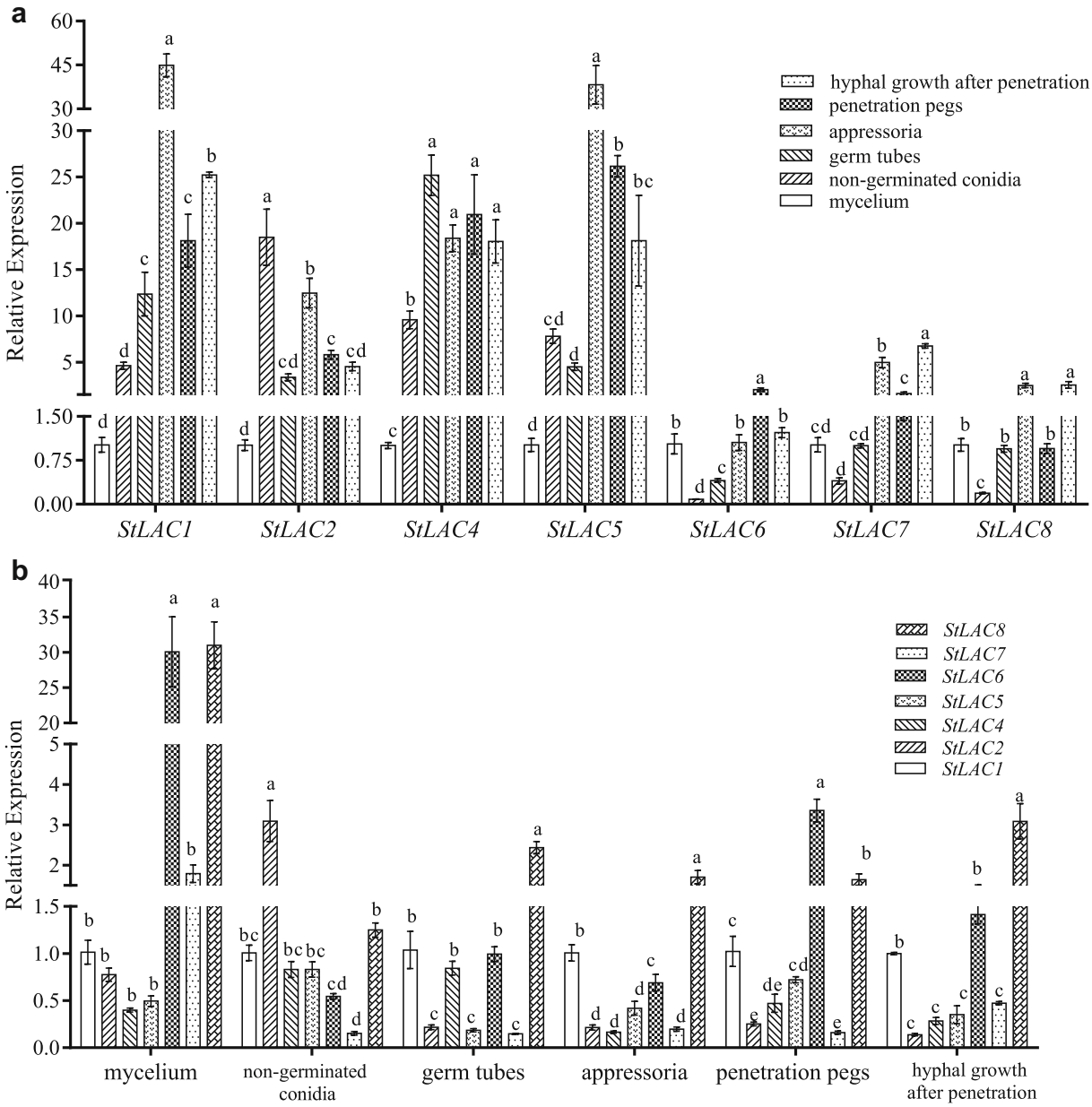

Fig. 6 Expression profiles of LMCO genes at different developmental stages of *S. turcica*. **a**: Expression levels of the LMCO genes during different developmental stages were normalized to the expression level of each gene in mycelium grown in PDB; **b**: Expression level of the LMCO genes during the same stage were normalized to the expression of *StLAC1* measured in the same samples. Columns marked with different letters are significantly different at $P \leq 0.05$. Error bars represent standard error of the mean

Fig. 6a. The *StLAC1* expression level was set as a control for normalization in Fig. 6b.

Single gene expression analysis indicated that the transcription level of *StLAC1* was significantly up-regulated during appressorium formation and it was 44.85 fold higher than that in mycelium. *StLAC2* had the highest expression level in the non-germinated conidia. The *StLAC6* expression level in the non-germinated conidia was the lowest, only 0.09 fold of that in mycelium and it gradually increased significantly during conidial

development of penetration pegs. The transcription levels were significantly higher in hyphal growth after penetration than in mycelium, except for *StLAC6* and *StLAC2*.

Gene expression analysis at single developmental stages indicated that in mycelium, *StLAC6* and *StLAC8* had the significantly highest expression (30.08 and 31.01 fold compared to *StLCA1*, respectively), but no significant difference in expression levels was observed for the other LMCOs compared to *StLCA1* in mycelium. In non-germinated conidia, *StLAC2* was significantly expressed. *StLAC8* was highly expressed when conidia germinated to form germ tubes and appressoria. When penetration pegs were formed, the expression of *StLAC6* was significantly up-regulated. The transcription pattern during hyphal growth after penetration was different from that in mycelium incubated in PDB, as *StLAC8* showed higher expression during hyphal growth after penetration than *StLAC6*, whereas the expression levels of *StLAC6* and *StLAC8* was not significantly different from mycelium incubated in PDB.

LMCO expression pattern after application of potential laccase inducers

The laccase activity was measured after treatment with potential laccase inducers (Fig. 7) and expression of seven genes was analysed (Fig. 8). When treated with resveratrol (a phenolic phytoalexin produced in plants) and ABTS (a typical laccase substrate), the laccase activity did not significantly differ from the control without inducer. Analysis of the expression levels after resveratrol treatment showed that the expression of *StLAC2*, *StLAC4* and *StLAC5* were significantly up-regulated by 9.20, 11.20 and 9.40 fold, respectively, but *StLAC1* decreased significantly by about 0.09 fold. The expression levels of *StLAC6* and *StLAC8*, which were highly expressed in mycelium, were not significantly changed by resveratrol treatment. Treated with 0.03 g/L ABTS, the expression levels of *StLAC5*, *StLAC6* and *StLAC8* were significantly increased by 6.97, 1.68 and 3.23 fold, respectively, compared to the control, whereas changes in the expression of other genes were not significantly altered. When Cu^{2+}, as a structural component of the catalytic centre of MCOs, was added to the culture, the laccase activity increased significantly, while the expression levels of all detected genes increased significantly except for *StLAC7*, the expression of which decreased by 0.31 fold.

Discussion

In the phytopathogenic fungus *S. turcica* (syn. *Exserohilum turcicum*), nine LMCOs were identified with conserved copper-binding sites characteristics and low sequence identity and they were classified into five different super families. The LMCOs have predicted protein masses of 60.83–68.10 kDa and 1–13 predicted potential glycosylation sites, which is consistent with most fungal laccases with molecular masses of 55–85 kDa and carbohydrate content of 10–20% (even up to 25%, Maestre-Reyna et al. 2015). The identity of the amino acid sequences of LMCOs in *S. turcica* was low

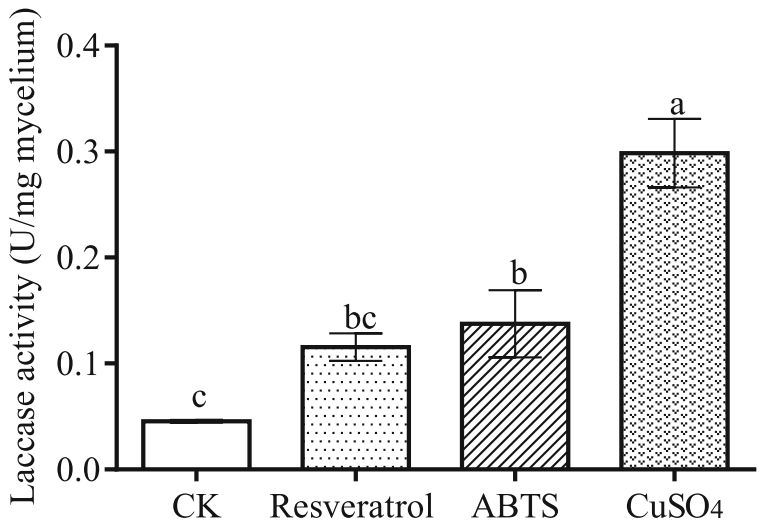

Fig. 7 Intracellular laccase activity after treatment with the potential laccase inducers resveratrol, ABTS, $CuSO_4$, and with none inducer as control (CK). Columns marked with different letters are significantly different at $P \leq 0.05$. Error bars represent standard error of the mean

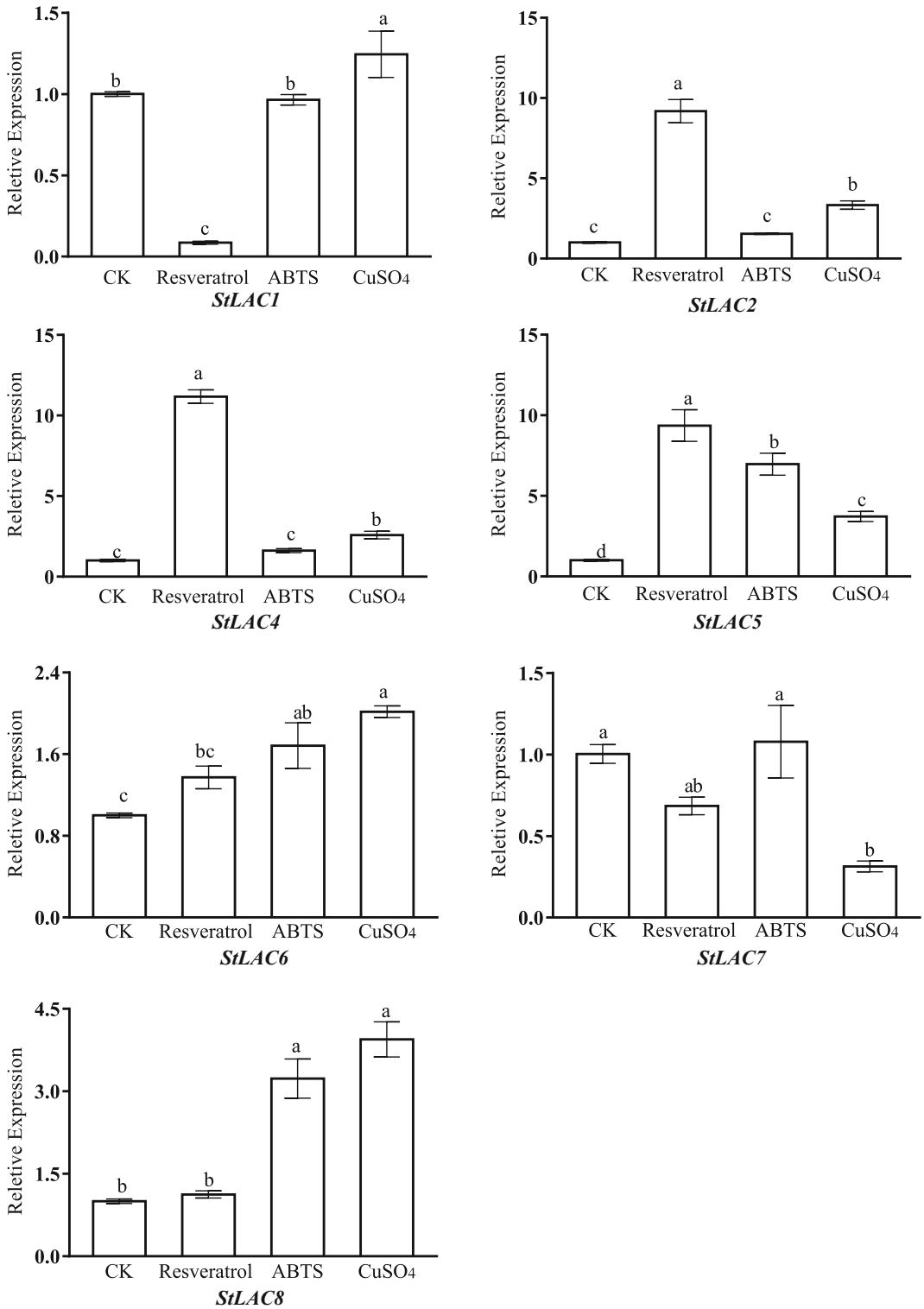

Fig. 8 Expression levels of LMCO genes after treatment with the potential laccase inducers resveratrol, ABTS and $CuSO_4$ as well as with no inducer as control (CK). Columns marked with different letters are significantly different at $P \leq 0.05$. Error bars represent standard error of the mean

(19.79–48.70%), compared to 30–88% among nine laccases in *Trichoderma* spp. (Cázares-García et al. 2013) and 45–89% among six laccases found in the basidiomycete *Pleurotus ostreatus* (Park et al. 2015). StLAC1, StLAC2, StLAC4 and StLAC6 are ascomycete MCOs based on the LccED annotation (Sirim et al. 2011). Compared to other LMCOs, they are laccases sensu stricto and have the DSGL/I plug at the C-terminal end, which is conserved in ascomycete laccases and is known to fold into the oxygen channel and create a robust knot-like structure that imparts high stability and a wider pH profile (Fig. 4, Kumar et al. 2003; Andberg et al. 2009; Kallio et al. 2011). However, it is difficult to distinguish between laccases and other LMCOs from their consensus sequences. Thus, it was shown that LMCOs in other superfamilies also have the catalytic characteristics and functions of laccases (Durand et al. 2013; Xie et al. 2018).

In *S. turcica*, the expression of the nine LMCOs was significantly different between different developmental stages of the fungus. Seven of the nine genes were expressed, except for *StLAC3* and *StLAC9*, with *StLAC6* and *StLAC8* being highly expressed in mycelium cultured in PDB and *StLAC2* highly expressed in non-germinated conidia. The LMCO expression pattern was investigated in different white-rot fungi and the results suggest that they are involved in fungal morphogenesis (Sakamoto et al. 2015). In *Gaeomannomyces tritici*, LAC1, LAC2 and LAC3, which clustered among the same superfamily ascomycete MCOs, had different expression patterns (Litvintseva and Henson 2002). lcc9 and lcc10 from *Laccaria bicolor* represent a basidiomycete laccase and a ferroxidase, respectively. They were mostly expressed in mycelium, whereas basidiomycete laccase lcc7 was highly expressed in fruiting bodies (Courty et al. 2009). It appears that LMCOs clustered in the same super family may be expressed at different development stages, whereas those in different super families are expressed at the same stage.

StLAC2 is an intracellular laccase, which plays an important role in non-germinated conidia. StLAC2 is a laccase sensu stricto, but compared with other LMCOs, StLAC2 is the only one with no signal peptide, indicating that it cannot be secreted. It had a different expression pattern than the other LMCOs, since *StLAC2* was most highly expressed in non-germinated conidia (Fig. 6a). The results are consistent with our previous finding with a gene-knockout of *StLAC2* (Ma et al. 2017). Thus, it was found that △*StLAC2* mutants were unable to produce conidia and the melanin level of mutants was partly decreased. The cell walls of hyphae of △*StLAC2* were thinner than wildtype hyphae and hydrophobicity was reduced, suggesting another function of *StLAC2* in mycelium than in conidia.

LMCOs of *S. turcica* are involved in different infection stages, illustrating the diversity of their functions, including synthesis of melanin and lignin degradation. Infection is initiated when conidia germinate. The conidia form germ tubes attached to the host cell wall and they swell at the tip, forming appressoria with melanin in the wall to increase turgor pressure to perforate the host cell wall (Cao et al. 2011). Laccases are involved in 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis in the pathogenic fungi *Aspergillus fumigatus* (Upadhyay et al. 2013) and *Talaromyces* (*Penicillium*) *marneffei* (Sapmak et al. 2015). Thus, knocking out LMCO genes lead to a colour change. In *S. turcica*, the highest expression of *StLAC1* in appressoria indicated the contribution to synthesis of melanin. On the other hand, conidia can produce germ tubes and secrete cell wall-degrading enzymes to facilitate host penetration, including laccases, which can be involved in biodegradation of lignin (Dashtban et al. 2010; Bugg et al. 2011). Our previous study showed that when crude laccases from *S. turcica* were treated to the maize leaves, the plant cell wall was degraded, suggesting that laccases could play a role in lignin degradation (Zhan et al. 2011). Considering the expression of LMCOs during the infection processes, only expression of *StLAC6* increased continuously during formation of germ tubes and penetration pegs and it was mainly expressed during penetration peg formation (Fig. 6). Sequence analysis showed that StLAC6 is secretable and has a relatively high redox potential (Xu et al. 1998, Fig. 4). StLAC6 clustered with LAC6 (CDP29442.1) and LAC8 (CDP31732.1) in *Podospora pauciseta* (Fig. 5), and are known to play roles in lignocellulose degradation and detoxification of phenolic substrates (Xie et al. 2014). The expression of *StLAC8* was constitutively high during the infection processes and it clustered with the basidiomycete laccases with a high redox potential, which might even have lignolytic activity. So *StLAC6* and *StLAC8* are speculated to be involved in the infection by contributing the degradation of lignin.

The complex expression of the LMCOs in *S. turcica* may be due to the transcriptional regulatory elements in the promoter region of the genes. There are two possible explanations. One is that the activation of LMCOs is a response to different nutrient conditions during infection. Appressorial metabolism is dominated by fatty acid β-oxidation, the glyoxylate cycle and the export of acetyl-CoA from the peroxisomes and is different from the glucose metabolism in mycelium (Fernandez and Wilson 2014). In the promoter regions of the nine genes, there are transcriptional regulatory elements: the major positive-acting nitrogen regulatory gene *NIT2* (Fu and Marzluf 1990), CRE-A is responsible for carbon catabolite repression (Kim et al. 2005) and *ADR1* is a positive regulator of peroxisomal protein genes (Simon et al. 1991). These transcriptional regulatory elements could be responsible for the different expression levels during the different developmental stages of *S. turcica*. Significantly increased expression levels of LMCOs in *S. turcica* during appressorium formation were investigated and the highest transcription level of *StLAC1* during appressorium formation was 44.85 fold higher than that in mycelium. In the promoter region of *StLAC1*, there are two copies of the transcriptional regulatory element *NIT2*, 3 of *ADR1* and 3 of CRE-A and these could respond to the different levels of nutrients and peroxisomes in appressoria. Another explanation for increased gene expression could be that LMCO activation responds to the metabolites from plant defence such as reactive oxygen species, phytoalexins and phenolic and aromatic compounds related to lignin derivatives, which are recognized to be inducers of laccase activity (Piscitelli et al. 2011). For example, there is a xenobiotic-responsive element (XRE) in *StLAC2*, *StLAC3*, *StLAC6* and *StLAC7* and heat shock transcription factor elements (*HSF*) in all of nine LMCOs (Kües and Rühl 2011; Hashikawa et al. 2007). *StLAC2* has 3 XRE so the expression was increasing in the presence of resveratrol. Resveratrol is a xenobiotic phytoalexin and can strongly stimulate the expression of the laccase gene *Bclcc2* in *Botrytis cinerea* (Schouten et al. 2002; Choquer et al. 2007). Accordingly, the highest expression level of *StLAC6* was detected during the penetration peg formation when lignin of the cell wall is degraded and release phenolic compounds. Furthermore, it was found that expression levels of *StLAC6* increased in the presence of ABTS, which is a synthetic mediator of lignin catalysis by laccases (Munk et al. 2015). Because of the coexistence of the different transcriptional regulatory elements, it is necessary to study further the molecular mechanisms of regulation.

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Compliance with ethical standards This work does not contain any study with animals and/or humans.

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