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Phylogeny of *Leptographium Qinlingensis* Cytochrome P450 Genes and Their Expression When Grown on Different Media or Treated With Terpenoids

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Abstract

Leptographium qinlingensis is a fungal associate of the Chinese white pine beetle (*Dendroctonus armandi*) and a pathogen of the Chinese white pine (*Pinus armandi*) that must overcome the terpenoid oleoresin defences of host trees. We identified and phylogenetically analysed the cytochrome P450 (CYP) genes in the transcriptome of *L. qinlingensis*. Through analyses of the growth rates on different nutritional media and inhibition by terpenoids, the expression profiles of six CYPs in the mycelium of *L. qinlingensis* grown on different media or treated with terpenoids were determined. The CYP evolution predicted that most of the CYPs occurred in a putative common ancestor shared between *L. qinlingensis* and *G. clavigera*. This fungus is symbiotic with *D. armandi* and has more similarity with *G. clavigera*, which can retrieve nutrition from pine wood and utilize monoterpenes as the sole carbon source. Some CYP genes might be involved in the metabolism of fatty acids and detoxification of terpenes and phenolics, as observed in other blue-stained fungi, which also indicates the pathogenic properties of *L. qinlingensis* in Chinese white pine.

Introduction

Pathogens overcome the effects of terpenoids produced by conifers by active detoxification mechanisms. The ascomycete *Leptographium qinlingensis* is an active participant in the large-scale death of *Pinus armandi*, and it is associated with the Chinese white pine beetle (*Dendroctonus armandi*) (Chen and Tang, 2007; Chen et al., 2010). Although trees have accumulated defence chemicals, the associated fungi can kill host trees during summertime attacks (Boone et al., 2011; Clark et al., 2012). Beetles and fungi have evolved efficient systems for overcoming the toxicity of host defence chemicals (Hofstetter et al. 2005; Kopper et al. 2005), and fungal associates could also increase the concentrations of monoterpenes and sesquiterpenes in the phloem and xylem of host trees (Lee et al. 2006; Pham et al. 2014).

The constitutive and induced defensive system of *P. armandi* consists of a multitude of monoterpenes, sesquiterpenes and diterpenes (Chen et al. 2006) and can stop or delay beetles from entering trees (Erbilgin et al. 2003). Moreover, monoterpenes present in the resin, including pinene, limonene and carene, can injure or kill beetles and inhibit fungal growth (Reid and Purcell 2011; Dai et al. 2015a, 2015b). Previous studies have shown that symbiotic fungi destroy bleeding cells, block resin canals in host trees and kill epithelial cells, thereby resulting in disorders of the nutrient and water metabolism of the host (Chen and Tang 2002). The nitrogen concentrations in phloem infected with beetle-associated fungi were increased compared to those in uninfested phloem (Ayres et al. 2000). Associated fungi have been shown to provide nutritional support to bark beetles (Ayres et al. 2000; Bentz and Six 2006), metabolize terpenoids (Lah et al. 2013; Cheng et al. 2016), and produce pheromone compounds that affect beetle behaviour (Six 2012).

Fungal cytochrome P450 enzymes are important in specialized fungal metabolism, such as detoxifying host chemical defence compounds. CYP monooxygenases of symbiont yeast of *Dendroctonus rhizophagus* could be involved in the metabolism of terpenoids (Hernández-Martínez et al. 2016). Members of the CYP53 family can modify antifungal chemicals, such as benzoic acid and similar phenolic compounds (Faber et al. 2001; Matsuzaki and Wariishi 2005; Podobnik et al. 2008). CYP65A family genes of *Fusarium* species could hydroxylate an intermediate in the biosynthetic pathway of the sesquiterpenoid mycotoxin trichothecene (Kimura et al. 2007). CYPs are also involved in the de novo synthesis of secondary metabolites. In *Grosmannia clavigera*, which is a pathogen of pines associated with *Dendroctonus ponderosae*, CYP65BJ1 is located in a secondary metabolite biosynthetic gene cluster and highly upregulated after treatment with monoterpenes that may produce aromatic

polyketides (Lah et al. 2013). In pathogenic fungi, these compounds (e.g., aflatoxin, fumonsin, trichothecene, gliotoxin) are often toxic to the host species and represent important virulence factors (Proctor et al. 2003; Yu et al. 2004; Yu and Keller 2005; Balibar and Walsh 2006; Kimura et al. 2007). Moreover, three phytotoxins (6-methoxymethyleugenin, maculosin and cerevisterol) of *P. armandi* seedlings are synthesized by *L. qinlingensis* (Li et al. 2012).

Ophiostoma piceae is a wood-staining fungus that grows on a mixture of monoterpenes and diterpenes, although compared with *G. clavigera*, it cannot utilize monoterpenes as a carbon source (DiGuistini et al. 2011; Haridas et al. 2013). The wood of trees, logs and lumber has a high carbon/nitrogen ratio (Zabel and Morrell 1992). Compared with *O. piceae*, which grows more efficiently in drier pine wood, *G. clavigera* colonizes healthy or stressed living pine trees and can manage the high concentrations of defence chemicals produced by its pine host. Thus, *O. picea*e has slower growth rates than *G. clavigera* on rich media and wood (Wang et al. 2013; Haridas et al. 2013). *O. piceae* and *G. clavigera* can grow on a variety of sugars (mannose, maltose and starch, a stored tree nutrient) and can acquire additional sugars by degrading wood hemicelluloses (Zabel and Morrell 1992; Fischer and Holl 1992; Fleet et al. 2001; Schirp et al. 2003). However, triglycerides and fatty acids can occasionally be used as carbon sources, which are ultimately processed through ß-oxidation and glycolysis pathways (Wang et al. 2010).

Treatment with a terpenoid blend or pine phloem extract for associated fungi of bark beetles (*D. ponderosae* and *D. armandi*) always induces specific CYPs (Lah et al. 2013; Dai et al. 2015b). Thus, cytochrome P450 enzymes that are highly induced by terpenes and metabolize or utilize monoterpenes are considered the major mechanisms that enable fungal resistance to monoterpenes (Lah et al. 2013; Wang et al. 2014). The nutrition of culture media could have an effect on the tolerance of *G. clavigera* to terpenes (Kligun et al. 2017).

In the work reported here, we identified and phylogenetically analysed CYPs in the transcriptome of *L. qinlingensis*. Analyses of the growth rates on different nutrition media, inhibition of growth by terpenoids and expression profiles of six CYPs in the mycelium of *L. qinlingensis* grown on different media or treated with terpenoids indicated that CYPs may detoxify pine defence compounds and could be influenced by different nitrogen/carbon sources.

Materials And Methods

Strain and Growth Conditions

Leptographium qinlingensis (NCBI Taxonomy ID: 717526) was deposited at the College of Forestry, Northwest A&F University (Yangling, China).

Leptographium qinlingensis was grown on an MEA medium containing 1% Oxoid Malt Extract Agar and 1.5% Agar Technical (Oxoid Ltd., Basingstoke, Hampshire, UK) and topped with cellophane, and the pH was adjusted to 5 ~ 6.

Fungal growth under different nutrition

We characterized the effect of different nutrients on the growth rate of *L. qinlingensis*. The fungal strain was acclimatized at room temperature for 1 week on 25 mL MEA media following long-term storage at 4°C. According to the treatment for the mountain pine beetle-fungal symbiont *Grosmannia clavigera* (DiGuistini et al. 2011), mycelial plugs were transferred to a new Petri dish containing 25 mL of six different media [wood (W): 10 g/plate Chinese white pine sawdust; 1.5% granulated agar; starch (S); organic nitrogen (ON); inorganic nitrogen (IN); olive oil (OO); Chinese white pine methanol extract (CWPE): complete medium (0.17% YNB, 1.5% granulated agar, 1%

maltose, 0.1% PHP, 0.3% asparagine) with 200 μl of the crude Chinese white pine methanol extract (Dai et al., 2015).

All plates were incubated at 28°C in the dark, and growth (in cm) was measured every 4 days in four directions and averaged until the strain brought the fungus to the edge of the plate. For the six different nutrition media, the growth rates were obtained by calculating the area of the colony. To assess whether different parameters affect the growth rate, we performed curve fitting with a logistic equation [$Y = A/(1 + B \cdot e^{-kt})$, where Y is the size of the colony (cm^2) and t is the culture time] using SPSS software (IBM SPSS Statistics, Chicago, IL, USA).

Inhibition of Terpenoids

Monoterpenes (+)-limonene (95%), (+)-3-carene (90%), (±)-α-pinene (98%), (-)-β-pinene (99%), and turpentine were selected as fungistats for MIC screening and mixed at a ratio of 5:3:1:1. A 1% malt extract microdilution susceptibility assay was performed according to the Clinical and Laboratory Standards Institute M38-A2 protocol to evaluate the initial MIC. The final terpenoid concentration ranged from 10%~0.0465% (v/v) for all terpenoids. An equal volume of 1×10^5 spores was mixed with the 1% malt extract microdilution susceptibility assay. The MIC of terpenoids was defined as the lowest concentration of the drug that produced no visible growth following 72 h of incubation at 27°C. The MIC determination was repeated five times.

To determine the magnitude of the synergy, the MICs for the monoterpene mixture can be compared with the MICs for (+)-limonene, (+)-3-carene, (+)-α-pinene and (-)-β-pinene alone. The synergy index (SI) was determined using the equation $SI = QA/Qa + QB/Qb$ according to the method for antibacterial or fungicide mixtures (Zwart Voorspuij and Nass 1957; Kull et al. 1961).

Identification of *Leptographium qinlingensis* P450s

Total RNA was isolated from mycelia grown on MEA medium for 7 days according to the protocol supplied with the E.Z.N.A.™ Fungal RNA Kit (Omega Bio-Tek, Norcross, GA, USA), and its integrity was assessed on 1% agarose gels and quantified by spectrophotometry with a NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). The purity was estimated by the A260/A280 equation (μg/mL = A260 × dilution factor × 40).

Samples were shipped on dry ice to Annoroad Gene Technology Co., Ltd. (Beijing, China) for paired-end sequencing. During the QC steps, an Agilent 2100 Bioanalyser and ABI StepOnePlus Real-Time PCR System were used for quantification and qualification of the sample library. Finally, the library was sequenced using an Illumina HiSeq™ 2000 system. Raw data were processed with Perl scripts to ensure the quality of the data used in further analyses. For paired-end sequencing data, both reads were filtered out if any reads of the paired-end reads were adaptor-polluted.

The reads were assembled using Trinity (Grabherr et al. 2011), and unigene sequences were identified as candidate coding regions with TransDecoder to find an open reading frame (ORF).

Trinotate was used to perform the functional annotation of unigenes and ORFs. The functional annotation included homology searches of known sequence data (BLAST), protein domain identification (PFAM), protein signal peptide and transmembrane domain prediction (SignalP), and comparison to current annotation databases, namely, the UniProt (Universal Protein), eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous

Groups) and GO (Gene Ontology) pathway databases. Protein function information could be predicted from the annotation of the most similar proteins in those databases.

To identify all of the unique P450 transcripts in the hybrid assembly, we assessed these unigenes and translated ORFs against the BLASTx, BLASTp, PFAM, and eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) databases (e-value < 0.00001) to identify potential P450 sequences. The remaining unigenes were identified as potential P450 genes in *L. qinlingensis* (Table S1).

We downloaded the P450 protein sequences from *Grosmannia clavigera* kw1407 (53), *Neurospora crassa* OR74A (41), *Sporothrix schenckii* 1099-18 (40) and *Ophiostoma piceae* UAMH 11346 (43) for the phylogenetic analysis of potential P450 genes (ORFs with at least 200 codons) (37) in *L. qinlingensis*. To identify the different P450 variants expressed in fungi, a phylogenetic inference analysis of the P450 sequences by the maximum likelihood method was performed with MEGA6 (Tamura et al. 2011). The JTT + F model was supported by the test (-lnL = 998.482), with a gamma parameter value of G = 0.66. To estimate the support of each node, bootstrap values were calculated after 1000 pseudoreplicates.

A pair of primers for 6 annotated P450 sequences was designed to screen the putative P450 genes (Table S2). PCR amplifications were performed in a C1000 thermocycler (Bio-Rad, Hercules, CA, USA). P450 genes were amplified under the indicated conditions in 20 µL reactions containing 1 µl cDNA, 0.25 µM of each primer and 1× EcoTaq PCR SuperMix (TransGen Biotech, Beijing, China). An initial 5 min step at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at Tm and 30 s at 72°C, with a final extension for 10 min at 72°C.

The PCR products were visualized on 1% agarose gels stained with 1× DuRed and compared with a 2K plus DNA marker (TransGen Biotech, Beijing, China). Amplicons were purified, and the reaction product was cloned using the pMD™ 18-T Vector (TaKaRa, Dalian, China). Cloning reactions were transformed into DH5α chemically competent *Escherichia coli* cells, and a total of 5 clones with inserts were sequenced directly by GenScript USA Inc. The sequences were manually edited with DNAMAN to obtain the insert sequences. Blastx searches of partial-length sequences were performed against the NCBI database.

Information on the *L. qinlingensis* CYP65 genes was determined based on corresponding genes from *Magnaporthe oryzae*, *N. crassa*, *Sordaria macrospora*, *Penicillium marneffei* and *Talaromyces stipitatus* from the NCBI, and information on the CYP56BJ gene was determined based on corresponding genes from the genus *Grosmannia* (*G. clavigera*, *G. aureum*, *G. penicillata*) as well as *Leptographium longiclavatum* and *L. terebrantis* (Lah et al. 2013), and these data were used in the phylogenetic analyses.

Real-Time Fluorescent Quantitative PCR

We generated and analysed transcript-level data from two sets of growth conditions. For the first set of conditions, mycelia were generated from a suspension of 5 × 105 spores spread on cellophane on the surface of six different nutrition media as above.

In the second set of conditions, mycelia were generated from spores grown on 1% MEA (0.83% malt extract agar and 0.75% technical agar (BD Difco, Sparks, MD, USA)) covered with cellophane for 5 days. The young germinating mycelia were treated with 4 monoterpenes ((+)-limonene, (+)-3-carene, (±)-α-pinene and (-)-β-pinene) and turpentines at the same MIC screening for 24 h. However, terpenoids were added at three concentrations: 5%, 10% and 20% (v/v) in dimethyl sulfoxide (DMSO) solution. We used mycelia grown on 1% MEA with DMSO as a control.

Total RNA isolation of the fungi was performed as described above. cDNA synthesis was performed using the protocol described in the FastQuant RT Kit (with gDNase) (Tiangen Biotech Co., Beijing, China) using 2 μg total RNA in a 20 μl final reaction volume. The cDNA synthesis program was as follows: 42°C for 15 min and 95°C for 3 min. The cDNA was stored at -20°C.

For six P450 genes and the reference gene EF (Dai et al. 2015b), specific primers were designed using Primer Premier 5.0 (Table S2). To estimate the qPCR efficiency and validate the primers for each gene, a linear regression analysis was performed between the mean values of the quantification cycles (Cq) of different dilutions (1.0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of cDNAs and the initial concentration. These dilutions were made from a cDNA pool, and 2 μl of each dilution was used as a qPCR template. PCR was performed three times for each gene, and its efficiency was estimated with the Eq. $(10^{-1/slope} - 1) \times 100$, where the E value and R^2 are shown in Table S2. Moreover, a melting curve reaction was performed to evaluate their specificity.

The reaction was carried out in a 20 μl volume that included 0.4 μm of each primer, 1 μl cDNA template (100 ng/μl), 8 μl ddH2O, and 10 μl TransStart® Tip Green qPCR SuperMix (TransGen Biotech). All samples were placed in the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A 3-step amplification process was performed: 95°C for 30 s, 40 cycles of 95°C for 5 s, Tm (melting temperature of primers) of each pair of primers (Table S2) for 15 s and 72°C for 20 s. Each treatment contained three technical replicates, and each technical replicate contained three biological replicates.

Statistical Analysis

The relative expression values for all of the genes were determined using the Ct ($\Delta\Delta Ct$) method (Livak and Schmittgen 2008) and analysed with Microsoft Excel 2003 (v.11.0.5612). Outlier values identified by a PCR system were excluded from our analysis. To evaluate significant differences in the expression for each gene, $2^{-\Delta\Delta Ct}$ values transformed at log2 were subjected to a one-way ANOVA to determine whether the gene expression differed among the treatments. The $2^{-\Delta\Delta Ct}$ values and standard error (SE) were transformed at $\log_2$ to generate graphs. All of the statistical analyses were performed with SPSS 18.0 (IBM SPSS Statistics, Chicago, IL, USA) and plotted with SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA)

Results

Fungal growth in different culture media

We compared the growth of *L. qinlingensis* on six culture media with different carbon and nitrogen sources at 28°C (Fig. 1). The growth of *L. qinlingensis* on media with organic nitrogen, inorganic nitrogen and wood was fast, and the growth on media with starch and Chinese white pine methanol extract was slow. However, *L. qinlingensis* showed the lowest growth rate on the medium with olive oil.

The logistic curve fit the growth curve of *L. qinlingensis* on the six culture media ($R^2 > 0.97$). According to the logistic curve fitting of the growth curve, the growth inflexion day of *L. qinlingensis* growth on organic nitrogen, inorganic nitrogen and wood media occurred after approximately 8 d and the growth inflexion day on starch medium or complete medium with Chinese white pine methanol extract occurred after approximately 12 d (Table 1). However, the growth inflexion day occurred after over 20 d for the medium with olive oil as the only carbon source (Table 1).

Table 1
Growth curve of *L. qinlingensis* in different culture medium after Logistic curve fitting

Media	A	B	k	R^2	Inflexion day
S	64.375	74.981	0.36	0.985	11.99
ON	63.369	549.432	0.831	1.00	7.59
IN	64.309	359.495	0.742	0.998	7.93
OO	69.626	3444.738	0.403	0.979	20.21
CWPE	63.454	117.289	0.406	0.988	11.74
W	64.051	1388.080	0.897	0.999	8.06

Logistic equation: $Y = A/(1 + B \cdot e^{-kt})$, Y means size of the colony (cm^2), t means culture time, A is maximum size of the colony, B is parameter, and k is maximum of relative growth rate.

MIC of the terpenoids alone and the terpenoid mixture

The monoterpenes showed different degrees of *L. qinlingensis* reproduction inhibition. Limonene, β-pinene and 3-carene were more effective at inhibiting spore germination than α-pinene and turpentine. The MIC of (+)-α-pinene was 2.5×10^{-2} μl/100 μl, and that of the other monoterpenes ((+)-limonene, (+)-3-carene, (-)-β-pinene) was 6.25×10^{-3} μl/100 μl (Table 2). Turpentine mainly consists of α-pinene and had the same MIC as (+)-α-pinene (Table S3). However, the mixture of four monoterpenes had a much lower MIC at 1.56×10^{-3} μl/100 μl compared with the individual components. Furthermore, the value of the synergy index (SI) was 0.23 < 1 (Table 2), indicating a synergistic effect among monoterpenes when they were mixed as a fungicide.

Table 2
Minimum inhibitory concentration (MIC) of monoterpenes alone and mixture and corresponding synergy index (SI) against *L. qinlingensis*

MIC(μl/100μl) of Monoterpenes alone			
(+)-Limonene	(+)-3-Carene	(-)-β-Pinene	(+)-α-Pinene
Q_A	Q_B	Q_C	Q_D
6.25×10^{-3}	6.25×10^{-3}	6.25×10^{-3}	2.5×10^{-2}
MIC(μl/100μl) of Mixture			
1.56×10^{-3}			
Q_a/50%	Q_b/30%	Q_c/10%	Q_d/10%
7.8×10^{-4}	4.68×10^{-4}	1.56×10^{-4}	1.56×10^{-4}
SI*	0.23		

*SI = QA/Qa + QB/Qb, where QA and QB are the concentrations of A and B acting alone, which produced an end point (i.e. MIC of four monoterpens, respectively), Qa and Qb are the concentrations of A and B in the mixture, which produced an end point. Value of SI < 1 indicates a synergistic effect, while SI > 1 means an antagonist effect (Zwart Voorspuij and Nass 1957; Kull et al. 1961).

Transcriptome assembly and annotation

Among the predicted ORFs, 17,040 corresponded to our acceptance criteria (see Methods), and 10,735 of these ORFs were at least 200 amino acids long. Within the annotated transcriptome of *L. qinlingensis*, we identified genes and gene families for secondary metabolite processing and cytochrome P450. We also identified homologous *O. piceae*, *G. clavigera* and *N. crassa* proteins based on reciprocal best BLAST hits. Some of the major gene families for secondary metabolite processing in *L. qinlingensis* are shown in Table 3.

Table 3
Major gene families in *L. qinlingensis* (Lq) and in three other ascomycetes

Gene family	Lq	Op*	Gc*	Nc*
MFS transporters	9	289	227	161
ABC transporters	74	34	40	36
ATPases	98	308	349	356
NAD binding proteins	39	258	254	211
FAD binding proteins	71	130	146	122
Cytochrome P450s[#]	56	45	54	43
Methyltransferases	86	112	159	126
Transcription factors	111	115	133	106
Glycosyl transferases	35	63	64	76

***O. piceae* (Op); *G. clavigera* (Gc); *Neurospora crassa* (Nc) (Haridas et al. 2013)

[#] The *L. qinlingensis* potential P450 genes according to the functional annotations were listed in Supplementary material Table S1.

CYPome of L. qinlingensis

We identified 56 cytochrome P450 (CYP) genes in the 17,040 ORFs of the *L. qinlingensis* transcriptome (Table S1). Thirty-nine CYP genes with ORFs at least 200 amino acids long were used for phylogenetic analyses with the CYPome from *G. clavigera, O. piceae*, *S. schenckii* and *N. crassa.* We found more examples of recognizable orthologues of P450s for *G. clavigera* in our comparison than in the other fungal species (Fig. 2). According to the nomenclature of 54 CYP genes of *G. clavigera*, the CYP genes of *L. qinlingensis* represent 18 different CYP families.

Six CYP genes were amplified and sequenced for accurate sequence information using primers designed according to transcriptome annotation. The sequences were submitted to the Cytochrome P450 Nomenclature Committee (Nelson 2009) as CYP61A1, CYP582C, CYP537D6, CYP65BJ4, CYP578E and CYP52Z4. The specific sequences of CYPs were submitted to GenBank under accession numbers MT178256-MT178261. The amino acid sequence had the highest identity with *G. clavigera* except for CYP52Z4, which was between partial-length sequences with respect to the matched GenBank sequences (Table 4). CYP52Z4 had high identity with the n-alkane-inducible cytochrome p450 protein of *Pochonia chlamydosporia* (Table 4).

Table 4
Putative amino acid identity of P450 genes isolated from *L. qinlingensis* with P450 sequences from other species

Gene name /Accession No.	BLAST matches in GenBank			Identity (%) *
	Species	P450 name	Accession No.	
CYP61A1 /MT178256	*Grosmannia clavigera* kw1407	CYP61A1	EFX05849.1	95.09
	Ophiostoma piceae UAMH 11346	cytochrome p450 61	EPE03416.1	84.58
	Sporothrix brasiliensis 5110	C-22 sterol desaturase	KIH88936.1	82.71
CYP582C /MT178257	*Grosmannia clavigera* kw1407	CYP582C1	EFX03427.1	88.67
	Coniochaeta ligniaria NRRL 30616	cytochrome p450 monooxygenase	OIW25416.1	65.02
	Coniochaeta sp. 2T2.1	cytochrome P450	KAB5518869.1	62.38
CYP537D6 /MT178258	*Grosmannia clavigera* kw1407	CYP537D3	EFX05326.1	87.70
	Ophiostoma piceae UAMH 11346	cytochrome p450	EPE03590.1	71.17
	Sporothrix insectorum RCEF 264	benzoate 4-monooxygenase cytochrome p450	OAA63392.1	65.99
CYP65BJ4 /MT178259	*Grosmannia clavigera* kw1407	CYP65BJ1	EFX04804.1	93.21
	Talaromyces marneffei ATCC 18224	cytochrome P450 monooxygenase	EEA18658.1	55.49
	Valsa mali var. pyri	Isotrichodermin C-15 hydroxylase	KUI53331.1	54.86
CYP578E /MT178260	*Grosmannia clavigera* kw1407	CYP578E2	EFX06222.1	79.80
	Lophiostoma macrostomum CBS 122681	cytochrome P450	KAF2653719.1	63.64
	Colletotrichum fructicola Nara gc5	cytochrome P450	ELA29077.1	62.50
CYP52Z4 /MT178261	*Pochonia chlamydosporia* 123	n-alkane-inducible cytochrome p450 protein	RZR69190.1	71.03
	Phaeoacremonium minimum UCRPA7	n-alkane-inducible cytochrome p450 protein	EON99497.1	68.44
	Lophiotrema nucula	n-alkane-inducible cytochrome P450	KAF2120036.1	64.26

*Predicted by BLASTp (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1990).

One gene was classified into the CYP65B family, whose members in other fungi were shown to be involved in terpene bioconversions (Kimura et al. 2007). The phylogenetic analysis with a maximum likelihood tree (model: T92 + G + I, -lnL = 5724.207, G = 1.69, I = 0.19) suggested that CYP65BJ4 of *L. qinlingensis* was conserved within the CYP65BJ1 subclade of the *Grosmannia* genus (Fig. 3).

RT-qPCR

To determine whether the P450 genes were involved in the utilization of different nutrition (carbon and nitrogen) sources, we analysed six CYP gene expression profiles of *L. qinlingensis* grown on the following culture media: W (wood), S (starch), ON (organic nitrogen), IN (inorganic nitrogen), OO (olive oil) and CWPE (Chinese white pine methanol extract). Statistically significant differences were found among these culture media for six CYPs (Table 5). In mycelia grown on complete medium with Chinese white pine methanol extract (CWPE), six CYPs were significantly overexpressed (Fig. 4). However, the expression of CYPs was significantly downregulated in mycelia grown on inorganic nitrogen medium (Fig. 4). Significant overexpression of CYP582C and CYP52Z4 was found in mycelia grown on the other three kinds of media (wood, starch and olive oil), and the medium with olive oil as the only carbon source significantly downregulated CYP61A1 (Fig. 4).

Table 5
Statistics significant of P450 genes expression from *L. qinlingensis* in different culture medium

Gene	df	F	Sig.
CYP61A1	6	10.190	**< 0.001**
CYP582C	6	42.723	**< 0.001**
CYP537D6	6	5.964	**0.003**
CYP65BJ4	6	12.822	**< 0.001**
CYP578E	6	8.532	**0.001**
CYP52Z4	6	26.418	**< 0.001**

Values in bold indicate significant difference in different culture medium with one-way ANOVA (α = 0.05). Multiple comparisons among different times with Tukey tests are shown in Fig. 4 with different letters.

To discover these six *L. qinlingensis* CYPs with a possible role in the detoxification of pine defence chemicals, we analysed the expression profiles of CYPs from mycelia grown on MEA medium treated with monoterpenes and turpentine at three different concentrations for 24 h. The transcription levels of most CYPs were significantly changed after exposure to the terpenoids (Table 6). CYP61A1 was only significantly downregulated after treatment with limonene at a 10% concentration (Fig. 5). The transcription level of CYP582C was significantly overexpressed after treatment with 3-carene and β-pinene at a 5% concentration but downregulated after treatment with limonene and turpentine at 20% (Fig. 5). For CYP537D6, significant overexpression was found only after treatment with 10% α-pinene and 5% β-pinene (Fig. 5). The transcription level of CYP65BJ4 was significantly downregulated after treatment with 10% and 20% β-pinene and turpentine. Treatment with 3-carene caused overexpression at 5% and 20% but downregulation at 10% for CYP65BJ4, although the opposite changes in expression were observed after treatment with limonene (Fig. 5). For CYP578E, significant overexpression was found after treatment with all terpenoids at almost all concentrations. Similar to CYP578E, the transcription level of CYP52Z4 was overexpressed

after treatment with α-pinene, 3-carene and β-pinene but downregulated after treatment with limonene and turpentine at 20% (Fig. 5).

Table 6
Statistics significant of P450 genes expression from *L. qinlingensis* in MEA with different terpenoids

Gene	df	(+)-α-Pinene		(-)-β-Pinene		(+)-3-Carene		(+)-Limonene		Turpentine	
		F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.
CYP61A1	3	1.791	0.227	1.950	0.200	3.579	0.066	9.743	**0.005**	1.948	0.201
CYP582C	3	4.124	**0.048**	10.171	**0.004**	12.120	**0.002**	13.095	**0.002**	4.278	**0.044**
CYP537D6	3	4.875	**0.033**	12.123	**0.002**	3.750	0.060	1.058	0.417	1.856	0.215
CYP65BJ4	3	3.646	0.064	18.754	**0.001**	5.409	**0.025**	7.509	**0.010**	5.086	**0.029**
CYP578E	3	4.837	**0.033**	9.964	**0.004**	17.547	**0.001**	9.608	**0.005**	5.074	**0.029**
CYP52Z4	3	5.172	**0.028**	4.677	**0.036**	4.511	**0.039**	7.654	**0.010**	5.754	**0.021**

Values in bold indicate significant difference among different concentrations of the same stimulus with one-way ANOVA (α = 0.05). Multiple comparisons among different times with Tukey tests are shown in Fig. 5 with different letters.

Discussion

We compared the CYPome of *L. qinlingensis* to those of the bark beetle-associated fungi *G. clavigera* and *O. piceae* and other ascomycetes *N. crassa* and *S. schenckii*. With 56 CYPs, the CYPome was small relative to that of the ascomycete *Aspergillus oryzae* (153 CYPs) and basidiomycete *Postia placenta* (250 CYPs) (Nelson 2011; Ide et al. 2012) but similar to that of the bark beetle-associated fungi *G. clavigera* (54 CYPs) and *O. piceae* (45 CYPs) (Lah et al. 2013; Haridas et al. 2013). The CYP evolution predicted that most *L. qinlingensis* CYPs had a putative common ancestor with *G. clavigera*. *G. clavigera* is an associate fungus of *D. ponderosae*, which is similar to *L. qinlingensis* with *D. armandi* (Lee et al. 2006; Li et al. 2012). A few *L. qinlingensis* CYPs were assigned to families whose members have been functionally characterized, such as CYP51F1 or 14 α-demethylase and CYP61A1 or sterol Δ22-desaturase (Kalb et al. 1987; Kelly et al. 1995; Skaggs et al. 1996). Furthermore, CYP53 family genes have been shown to hydroxylate phenolics (Matsuzaki and Wariishi 2005; Podobnik et al. 2008), and the CYP504 family encodes enzymes that hydroxylate phenylacetic acid (Ferrer-Sevillano and Fernández-Cañón 2007). The phylogeny of CYP65BJ4 indicated high homology with CYP65BJ1 in *G. clavigera*, which was the most highly upregulated CYP after treatment with a monoterpene blend (Lah et al. 2013).

Host defence chemicals, including terpenoids and phenolics, are toxic to bark beetle-associated fungi when they colonize pine trees together (Erbilgin et al. 2003; Chen et al. 2006). The associated fungi have to cope with defence chemicals through detoxification or retrieve nutrients form the host by accessing sugars and triglycerides (Lah et al. 2013; Kligun et al. 2017). The growth rates of *L. qinlingensis* on different media show that Chinese white pine sawdust could supply enough carbon and nitrogen sources as complete medium. Compared with *O. piceae*, which grows more efficiently in drier pine wood, *L. qinlingensis* and *G. clavigera* colonize healthy or stressed living pine trees associated with bark beetles and must first cope with high concentrations of defence chemicals produced by their pine host. Thus, *O. piceae* has slower growth rates than *G. clavigera* on rich media and wood (Wang et al. 2013; Haridas et al. 2013). $NaNO_3$ can represent an inorganic nitrogen source, which is similar to the role of

asparagine, which represents an *organic nitrogen* source. Similar to *O. piceae* and *G. clavigera*, which can acquire additional sugars by degrading wood hemicelluloses, *L. qinlingensis* can grow on a variety of sugars (mannose, maltose and starch, a stored tree nutrient) (Zabel and Morrell 1992; Fischer and Holl 1992; Fleet et al. 2001; Schirp et al. 2003).

However, *L. qinlingensis* cannot utilize olive oil as a carbon source well, which is possibly because this medium consists of fatty acids. Fatty acids can be used as a carbon source in *G. clavigera*, although their utilization might require processing via ß-oxidation and glycolysis pathways (Wang et al. 2010). The Chinese white pine methanol extract can reduce *L. qinlingensis* growth on abundant carbon and nitrogen sources, which is similar to lodgepole pine methanol extract, which inhibits *G. clavigera* and *N. crassa* (DiGuistini et al. 2011). Moreover, terpenoids from the host phloem can inhibit spore germination at certain concentrations, and the mixture of monoterpenes has a synergistic effect compared to the monoterpenes alone. However, monoterpenes in *L. qinlingensis* and *G. clavigera* are not only used for detoxification but also represent an energy source when no other carbon source is available (Dai et al. 2015b; Wang et al. 2013; DiGuistini et al. 2011).

CYP monooxygenases of fungi are important in specialized metabolism pathways, such as detoxifying host chemical defence compounds, including terpenoids (Hernández-Martínez et al. 2016). Following treatment with either a complex terpenoid blend or lodgepole pine extract containing phenolics, many CYP genes were induced in *G. clavigera* (Hesse-Orce et al. 2010; DiGuistini et al. 2011). The six CYP genes CYP61A1, CYP582C, CYP537D6, CYP65BJ4, CYP578E and CYP52Z4 in *L. qinlingensis* significantly responded to the Chinese white pine methanol extract. In *G. clavigera*, CYP genes induced by lodgepole pine extract are classified into the same CYP family known to degrade phenylacetic acid or detoxify benzoic acid and other phenolics (Matsuzaki et al. 2008; Podobnik et al. 2008; Mendonça et al. 2009; Davies 2010; Ide et al. 2012; Lah et al. 2013). Host pine sawdust contains a variety of carbon sources, including mannose, triglycerides and fatty acids, and induced oxidoreductase genes (e.g., P450s) that code for putative proteins involved in the modification of aromatic compounds, including phenolics (Haridas et al. 2013). Additionally, *CYP582C* and *CYP52Z4* were significantly overexpressed in mycelia grown on pine sawdust. The upregulation of these two genes in mycelia grown on olive oil was similar to that of CYPs in *G. clavigera* and *O. piceae*, and might be involved in hydroxylate fatty acids (Nakayama et al. 1996; Kitazume et al. 2000, 2002).

Monoterpenes are well-known biocides for microorganisms, including fungi associated with beetle vectors (Raffa and Smalley 1995; Wang et al. 2013). Treatments with terpenoids can rapidly upregulate the expression of genes involved in oxidative processes in *O. piceae* (Haridas et al. 2013). Moreover, a gene cluster with three CYP genes involved in metabolizing terpenes in *G. clavigera* was found after treatment with a terpene blend (DiGuistini et al. 2011; Lah et al. 2013). The expression of CYP genes in *L. qinlingensis* changed the effect of the monoterpene type and concentration. Limonene induced more CYP genes than other monoterpenes, especially CYP61A1, which is named sterol Δ22-desaturase and is involved in ergosterol biosynthesis (Kelly et al. 1995; Skaggs et al. 1996). The bioconversion of limonene is usually initiated by CYPs in several microorganisms (Duetz et al. 2003), and the bacterium *Rhodococcus erythropolis* processes limonene through the fatty acid β-oxidation pathway (Van Der Werf and Boot 2000).

We studied the nutrition utilization of *L. qinlingensis* grown on host tree wood, multiple sugars and fatty acids. The host chemical compound tolerance of *L. qinlingensis* was determined with the MIC test, and the induction of CYP genes by monoterpenes and pine extract was identified. This fungus is symbiotic with *D. armandi* and has considerable similarity with *G. clavigera*, which can retrieve nutrition from pine wood and utilize monoterpenes as a

carbon source (DiGuistini et al. 2011; Lah et al. 2013; Dai et al. 2015b). Some CYP genes might be involved in fatty acid metabolism and detoxify terpenes and phenolics, similar to other blue-stained fungi, which also indicates the pathogenic properties of *L. qinlingensis* in Chinese white pine. However, to fully reveal the ability of *L. qinlingensis* to detoxify host chemical compounds, additional information is required about the associated metabolic enzymes and membrane transporters (Wang et al. 2013; Haridas et al. 2013).

Declarations

Acknowledgment

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Compliance with ethical standards

Conflict of interest

The authors report no conflicts of interest.

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Figures

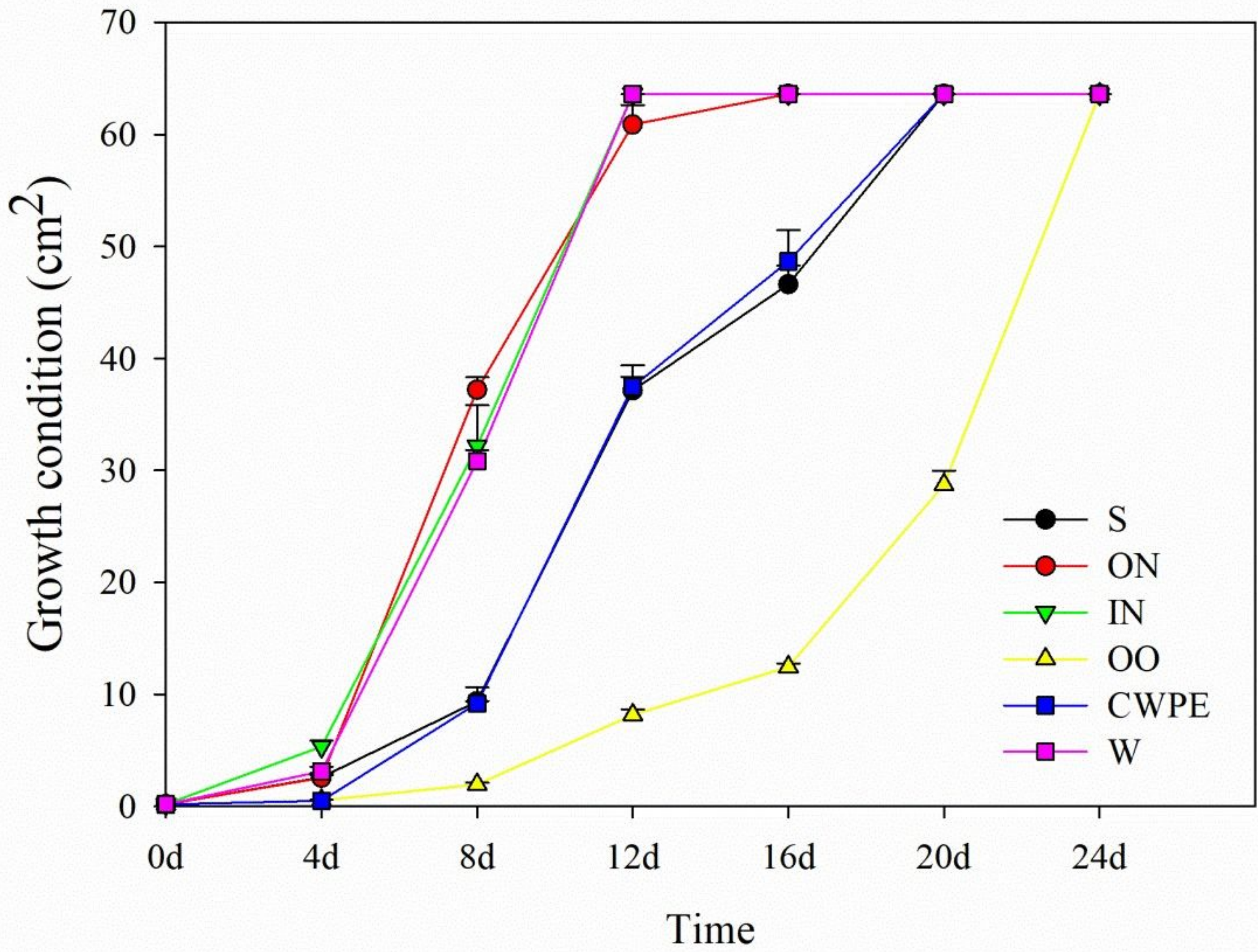

Figure 1

Growth rate for L. qinlingensis in different culture medium at 28°C. W: wood, S: starch, ON: organic nitrogen, IN: inorganic nitrogen, OO: olive oil, CWPE: Chinese white pine methanol extract.

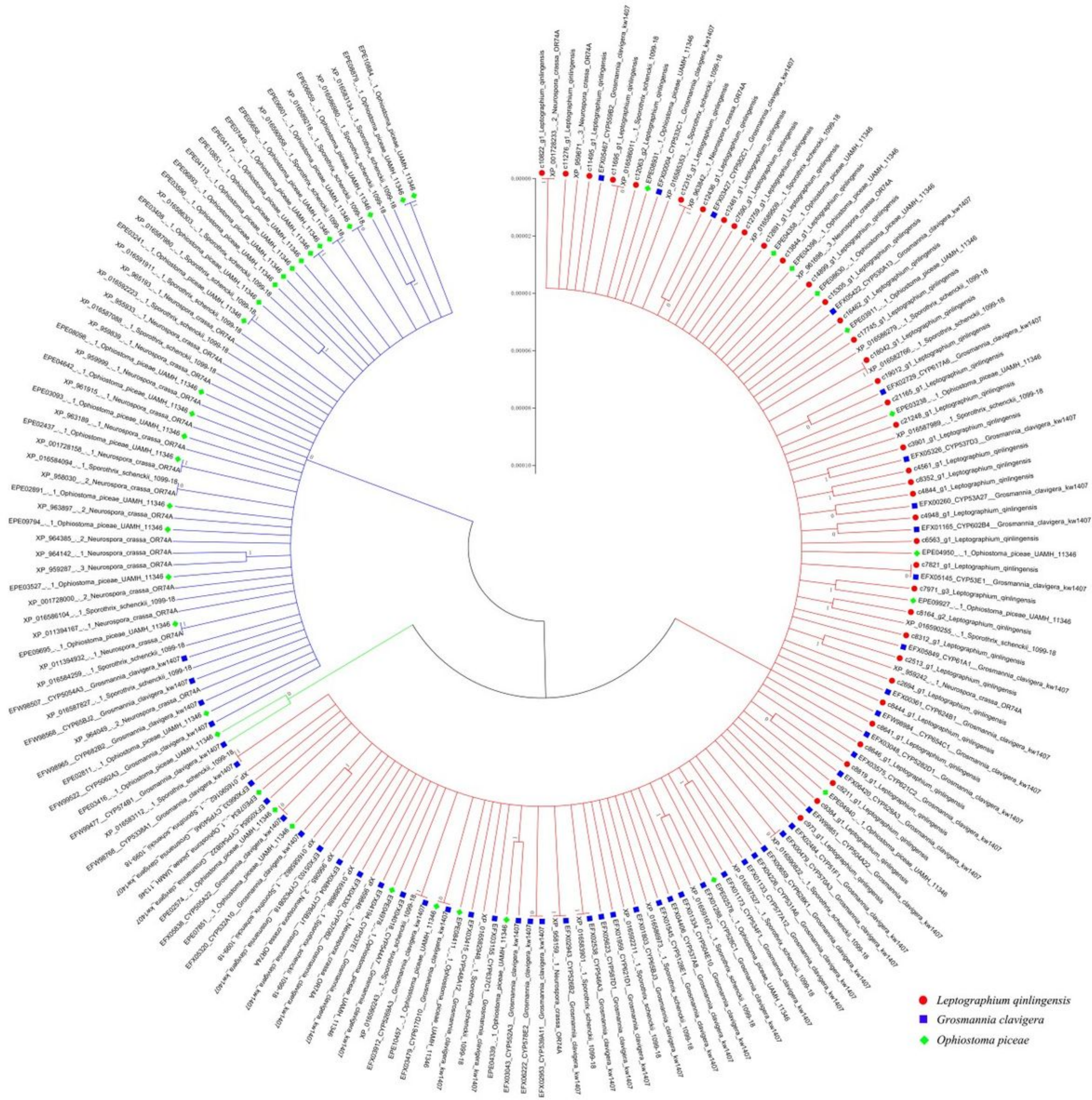

Figure 2

A maximum likelihood phylogeny tree of cytochrome P450s. Circular phylogram of the 39 predicted P450 protein sequences from L. qinlingensis, along with the P450s identified from the genome sequences of the Grosmannia clavigera kw1407, Ophiostoma piceae UAMH 11346, Sporothrix schenckii 1099-18 and Neurospora crassa OR74A. Some branch lengths are longer than might be expected due to their partial sequence length.

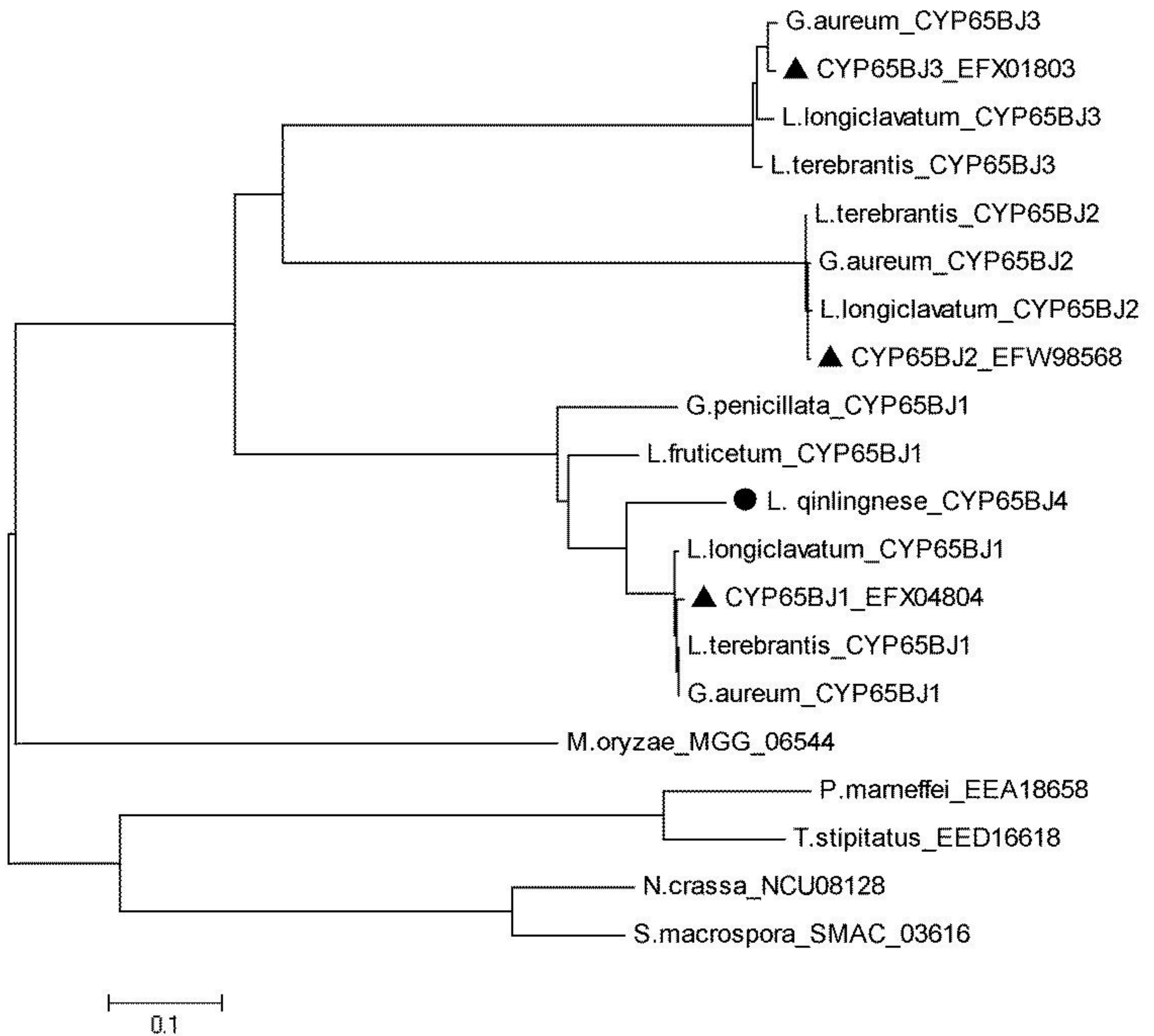

Figure 3

A maximum likelihood phylogeny tree of CYP65BJ4 of L. qinlingensis with other CYP65BJ genes from genus Grosmannia (G. clavigera, G. aureum, G. penicillata, Leptographium longiclavatum and L. terebrantis). Other Sordariomycete species (Magnaporthe oryzae, Neurospora crassa, Sordaria macrospora, Penicillium marneffi, and Talaromyces stipitatus) with CYP65 genes were used as outgroup according to Lah et al., 2013.

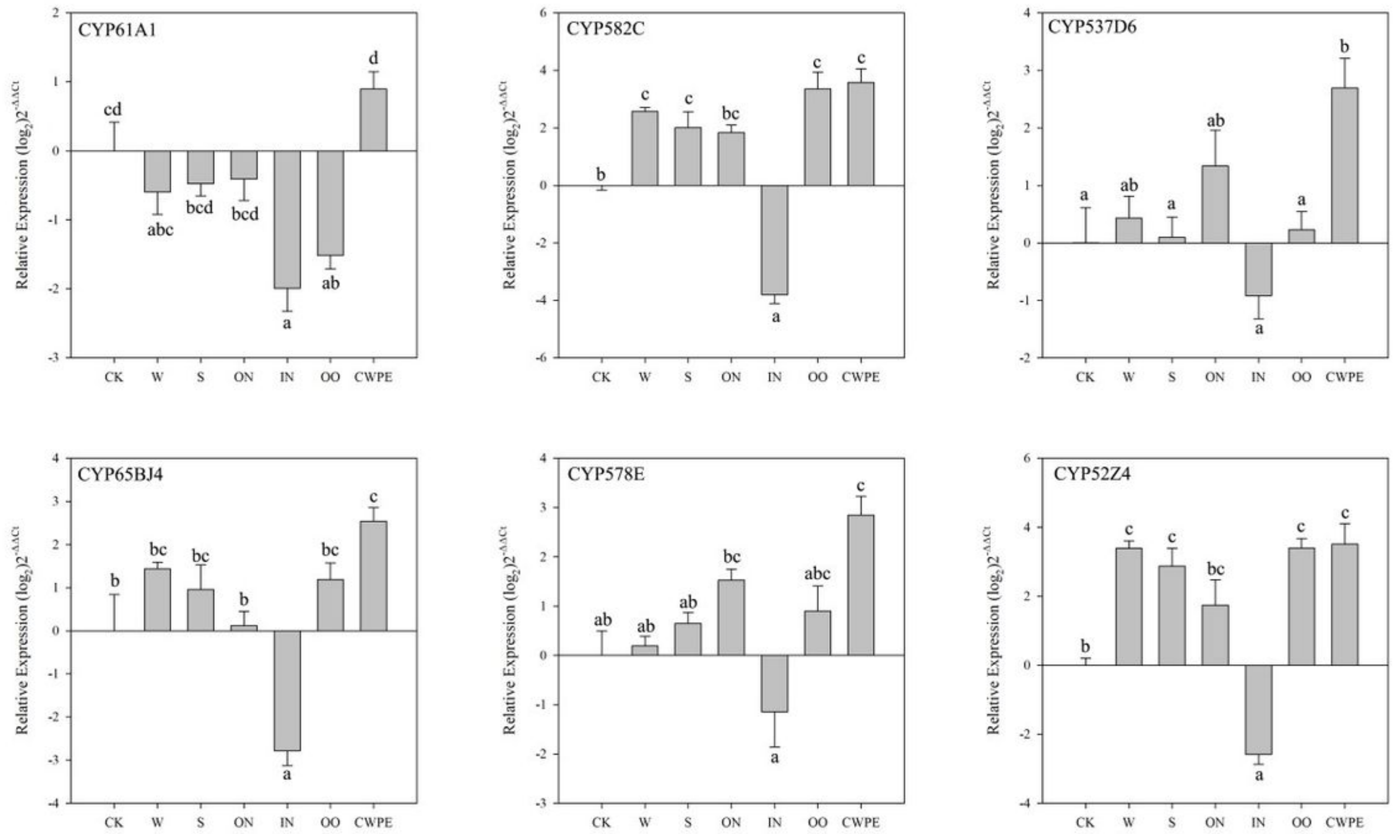

Figure 4

Quantitative expression of the six P450 genes (mean ± SE) in L. qinlingensis grown on different culture medium. W: wood, S: starch, ON: organic nitrogen, IN: inorganic nitrogen, OO: olive oil, CWPE: Chinese white pine methanol extract. CYPs expressions were normalized with respect to EF1. The $2^{-\Delta\Delta Ct}$ and SE values were transformed at log2 for plotting. Different letters indicate significant differences at $P < 0.05$ (Tukey test, no letter means no significant difference among all kind medium)

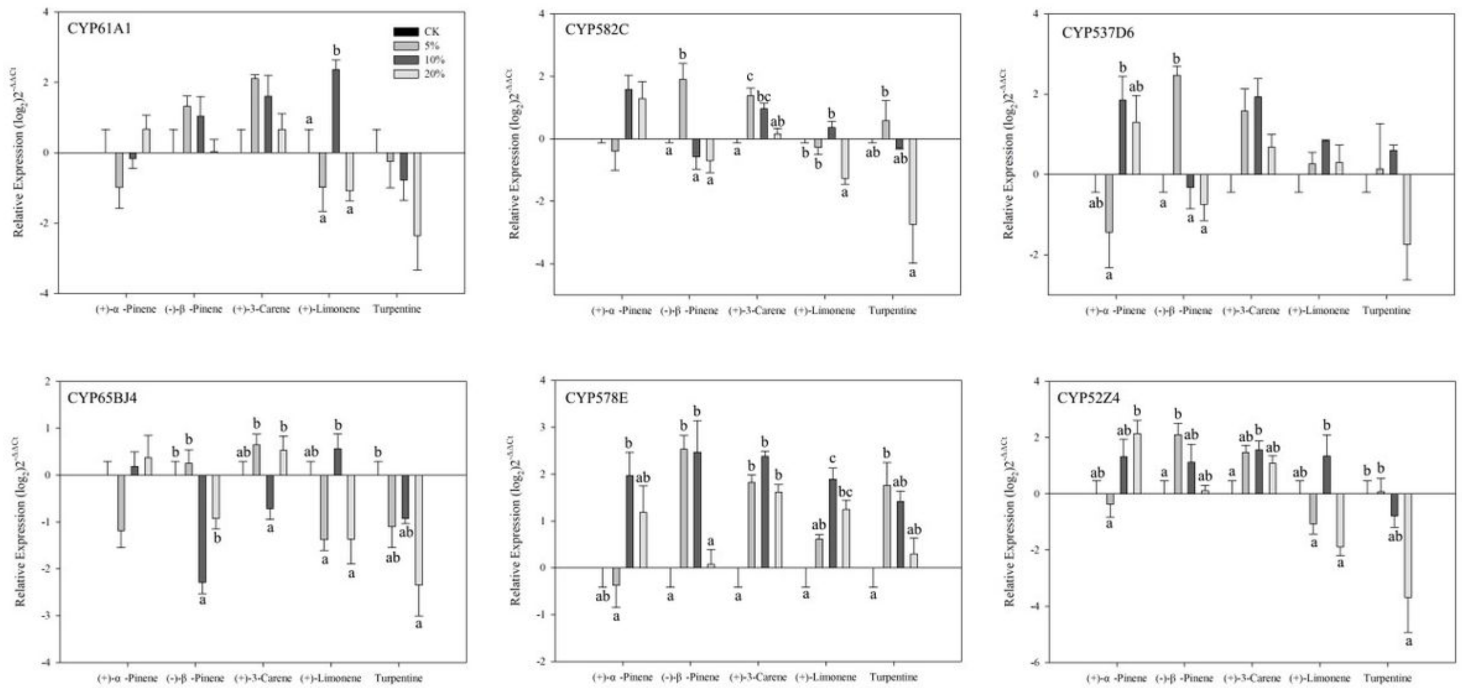

Figure 5

Quantitative expression of the six P450 genes (mean ± SE) in L. qinlingensis following treatment with different terpenoids. CYPs expressions were normalized with respect to EF1. The 2−ΔΔCt and SE values were transformed at log2 for plotting. Different letters indicate significant differences at $P < 0.05$ (Tukey test, no letter means no significant difference) among different concentrations of the same stimulus.

Supplementary Files

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