Analysis of the laccase gene family and miR397-/miR408-mediated posttranscriptional regulation in *Salvia miltiorrhiza*

Caili Li*, Dongqiao Li*, Hong Zhou, Jiang Li and Shanfa Lu

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

* These authors contributed equally to this work.

**ABSTRACT**

*Salvia miltiorrhiza* is one of the most commonly used traditional Chinese medicine materials. It contains important bioactive phenolic compounds, such as salvianolic acids, flavonoids and anthocyanins. Elucidation of phenolic compound biosynthesis and its regulatory mechanism is of great significance for *S. miltiorrhiza* quality improvement. Laccases (LACs) are multicopper-containing enzymes potentially involved in the polymerization of phenolic compounds. So far, little has been known about LAC genes in *S. miltiorrhiza*. Through systematic investigation of the whole genome sequence and transcriptomes of *S. miltiorrhiza*, we identified 65 full-length *SmLAC* genes (*SmLAC1*–*SmLAC65*). Phylogenetic analysis showed that 62 of the identified *SmLACs* clustered with LACs from *Arabidopsis* and *Populus trichocarpa* in seven clades (C1–C7), whereas the other three fell into one *S. miltiorrhiza*-specific clade (C8). All of the deduced *SmLAC* proteins contain four conserved signature sequences and three typical Cu-oxidase domains, and gene structures of most *LACs* from *S. miltiorrhiza*, *Arabidopsis* and *P. trichocarpa* were highly conserved, however *SmLACs* encoding C8 proteins showed distinct intron-exon structures. It suggests the conservation and diversity of plant *LACs* in gene structures. The majority of *SmLACs* exhibited tissue-specific expression patterns, indicates manifold functions of *SmLACs* played in *S. miltiorrhiza*. Analysis of high-throughput small RNA sequences and degradome data and experimental validation using the 5′ RACE method showed that 23 *SmLACs* were targets of Smi-miR397. Among them, three were also targeted by Smi-miR408. It suggests the significance of miR397 and miR408 in posttranscriptional regulation of *SmLAC* genes. Our results provide a foundation for further demonstrating the functions of *SmLACs* in the production of bioactive phenolic compounds in *S. miltiorrhiza*.

**INTRODUCTION**

Laccases (LACs, benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases widely existing in plants, fungi, bacteria and insects. Laccases proteins have three

---

**How to cite this article** Li C, Li D, Zhou H, Li J, Lu S. 2019. Analysis of the laccase gene family and miR397-/miR408-mediated posttranscriptional regulation in *Salvia miltiorrhiza*. *PeerJ* 7:e7605 DOI 10.7717/peerj.7605
catalytic sites, named T1, T2 and T3, respectively. When a substrate is oxidized at T1, an electron is released and transferred to T2/T3 trinuclear copper cluster (TNC) via cys-his pathway, consequently the free hydrogens are combined with molecule oxygens (O$_2$) and reduced to water molecules (H$_2$O). The resulting oxidized products, which are usually free-radical species, may be involved in subsequent radical–radical coupling (Mayer & Staples, 2002; Giardina et al., 2010) or cross-linking with other substances, including extracellular molecules (Mattinen et al., 2005; Giardina et al., 2010).

So far, genes encoding LACs have been identified from various plant species, such as Arabidopsis (McCaig, Meagher & Dean, 2005; Turlapati et al., 2011), rice (Liu et al., 2017), tobacco (Richardson & McDougall, 1997), ryegrass (Gavnholt, Larsen & Rasmussen, 2002), cotton (Balasubramanian et al., 2016), poplar (LaFayette, Eriksson & Dean, 1999; Lu et al., 2013), sycamore maple (Sterjiades, Dean & Eriksson, 1992) and pear (Cheng et al., 2019). LAC genes present as a multi-gene family in plants. For instance, Arabidopsis has 17 annotated LACs (McCaig, Meagher & Dean, 2005; Turlapati et al., 2011), rice has 30 (Liu et al., 2017) and P. trichocarpa has 49 (Lu et al., 2013). Phylogenetic analysis of LACs from Arabidopsis, rice and/or P. trichocarpa showed that plant LACs could be divided into six clades (C1–C6) (McCaig, Meagher & Dean, 2005; Turlapati et al., 2011). Arabidopsis AtLACs and P. trichocarpa PtLACs distributed in all of the six clades, whereas rice OsLACs distributed in five clades (McCaig, Meagher & Dean, 2005; Turlapati et al., 2011; Liu et al., 2017; Lu et al., 2013).

It has been shown that plant LACs are involved in lignin (Berthet et al., 2011; Brown et al., 2005) and flavonoid biosynthesis (Pourcel et al., 2005). Laccases could polymerize lignin monomers in vitro (Freudenberg, 1959). The expression of some LAC genes was correlated with lignin deposition in xylem (Sterjiades, Dean & Eriksson, 1992; Dean & Eriksson, 1994; Bao et al., 1993; Schuetz et al., 2014). In Arabidopsis, eight out of 17 AtLACs were highly expressed in the inflorescence stems, where lignin was deposited. Double mutation of two stem-expressed AtLACs, lac4 and lac17, resulted in significant decrease of lignin content (Berthet et al., 2011), and in a lac11lac4lac17 triple-mutant, root lignin content was extremely low (Zhao et al., 2013). This suggests that some plant LACs are involved in lignin polymerization. In addition to lignin, LACs also participate in flavonoid biosynthesis. For instance, AtLAC15, which is also known as TRANSPARENT TESTA10 (TT10), may function as an oxidase in the oxidative polymerization of flavonoids. Mutation of AtLAC15 resulted in a delay in developmentally determined browning of the seed coat and an accumulation of more epicatechin monomers and soluble proanthocyanidins than wild-type seeds (Pourcel et al., 2005).

MicroRNAs (miRNAs) are a large family of 21–24 nt endogenous small non-coding ribonucleic acid (RNAs) playing important regulatory roles in many bioprocesses (Brodersen & Voinnet, 2009; Filipowicz, Bhatacharyya & Sonenberg, 2008; Vaucheret, 2006). Various studies have shown that miRNAs, including miR397, miR408, miR528 and miR857, tightly regulate the expression of plant LAC genes at the posttranscriptional level. In woody Citrus, miR397a plays a pivotal role in the tolerance to B-toxicity by targeting LAC17 and LAC4 that are responsible for secondary cell-wall synthesis (Huang et al., 2016). Analysis of Arabidopsis transgenics plants overexpressing Ath-miR397 showed that
Ath-miR397b could regulate lignin content and seed number through modulation of AtLAC4 (Wang et al., 2014). Overexpression of Osa-miR397 in rice significantly downregulated its target, OsLAC, and improved rice yield by increasing grain size and promoting panicle branching (Zhang et al., 2013). In the woody plant P. trichocarpa, Ptr-miR397a acted as a negative regulator of a subset of LAC genes involved in lignin polymerization during wood formation (Lu et al., 2013). Overexpression of Ptr-miR397a in P. trichocarpa severely reduced LAC gene expression, total LAC activity and lignin content (Lu et al., 2013). Overexpression of PbrmiR397a and simultaneous silencing of three LAC genes reduced the lignin content and stone cell number in pear fruit (Xue et al., 2019).

Salvia miltiorrhiza Bunge, known as danshen in Chinese, is one of the most important materials of traditional Chinese medicines (TCMs). It is also an emerging model system for medicinal plant biology (Song et al., 2013). The whole genome of S. miltiorrhiza has recently been sequenced (Zhang et al., 2015; Xu et al., 2016). Phenolic compounds, such as salvianolic acids, flavonoids and anthocyanins, are important bioactive substances in S. miltiorrhiza (Deng et al., 2018; Deng & Lu, 2017; Hou et al., 2013). Elucidating the biosynthetic mechanism of these substances is of great significance for S. miltiorrhiza quality improvement. However, no efforts have been taken to identify and characterize the LAC genes in S. miltiorrhiza, although the importance of LACs has been shown in a variety of plants. In this study, using bioinformatics approaches, we identified 65 SmLAC genes from the S. miltiorrhiza genome. Gene structures, sequence characteristics and gene expression patterns were analyzed. Computational and experimental analysis showed that Smi-miR397 and Smi-miR408 were involved in posttranscriptional regulation of SmLACs. The results provide useful information for further elucidating SmLAC functions.

MATERIALS AND METHODS

Plant materials

Two-year-old plants of S. miltiorrhiza Bunge (line 99-3) were used as a source of plant tissues. The plants were grown in a field nursery under natural conditions at the Institute of Medicinal Plant Development, Beijing, China. Flowers, leaves, stems and roots were harvested in June and then immediately frozen in liquid nitrogen for nucleic acid isolation.

Gene prediction

Protein sequences of 17 A. thaliana LACs described by McCaig, Meagher & Dean (2005) were downloaded from the Arabidopsis Information Resource (TAIR, http://www.Arabidopsis.org/). Amino acid sequences of 49 P. trichocarpa LACs (PtLACs) were downloaded from the P. trichocarpa genome assembly v3.0 (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ptrichocarpa_er) (Lu et al., 2013). BLAST analysis of AtLACs and PtLACs against the genome database for S. miltiorrhiza line 99-3 (Xu et al., 2016) was carried out using BLASTp with value ≤10^{-10}. Gene models were predicted from retrieved genomic DNA sequences through alignment with LAC genes from other plants and RNA-Seq data of S. miltiorrhiza transcriptome (http://www.ncbi.nlm.nih.gov) using the BLASTx and BLASTn program, respectively. The predicted gene models
were checked for accuracy by comparison with another genome database for *S. miltiorrhiza* (http://www.ndctcm.org/shujukujieshao/2015-04-23/27.html) (Zhang et al., 2015). All of the identified laccase sequences were analyzed for conserved domain by blast against the Pfam database (http://pfam.janelia.org). The obtained open reading frame (ORF) sequences for *SmLAC1–SmLAC65* are available in Data S1.

**RNA extraction, reverse transcription and cDNA cloning**

Total RNA was extracted from *S. miltiorrhiza* tissues using the plant total RNA isolation kit (Aidlab, Beijing, China). Ribonucleic acid quality was examined by gel electrophoresis and NanoDrop 2000C spectro-photometer (Thermo Scientific, Waltham, MA, USA). For 5′ and 3′ RACE, 10µg total RNA was converted into cDNA using SMARTer® RACE 5′/3′Kit (TaKaRa Bio, Otsu, Japan). For polymerase chain reaction (PCR) amplification of coding region, 5µg total RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in 20 µl volume. Rapid amplification of 5′ and 3′ cDNAs ends was carried out using the SMART™ RACE cDNA amplification kit (TaKaRa Bio, Otsu, Japan). The primers used for PCR amplification are listed in Tables S1 and S2. Nesting PCR amplification was performed under the following program of touchdown PCR: 95 °C for 5 min, 5 cycles of amplification at 95 °C for 30 s and 72 °C for 3 min, 5 cycles of amplification at 95 °C for 30 s, 70 °C for 30 s and 72 °C for 1 min, 27 cycles of amplification at 95 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Nested PCR amplification was carried out under the following conditions: 95 °C for 5 min, 25 cycles of amplification at 95 °C for 30 s, TM (Based on specific primers) for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Polymerase chain reaction products were gel-purified and cloned into pTOPO-T vector (Aidlab Biotechnologies, Beijing, China), for sequencing by Sanger method (GENEWIZ, Beijing, China). Full-length coding sequences were amplified by PCR using gene-specific forward and reverse primer pairs (Table S3) under the following reaction conditions: initial denaturation at 95 °C for 5 min, 25 cycles of amplification at 95 °C for 30 s, TM (Based on specific primers) for 45 s and 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. Polymerase chain reaction products were purified using the DNA Gel extraction kit (Aidlab, Beijing, China). The purified fragments were cloned into pMD19-T vector (TaKaRa, Otsu, Japan), and then sequenced by Sanger method (GENEWIZ, Beijing, China).

**Bioinformatic analysis and phylogenetic tree construction**

The theoretical isoelectric point (pI) and molecular weight (Mw) of SmLAC proteins were analyzed using the Compute pI/Mw tool on the ExPASy server (http://web.expasy.org/compute_pi/) (Bjellqvist et al., 1994). The conserved domains were identified for SmLACs using the MEME server v4.11.4 (http://meme-suite.org/tools/meme) (Bailey et al., 2009) with the minimum and maximum weight of 3 and 100 amino acid residues, respectively. Protein sequences with three Cu-oxidase domains in the LAC domain were recognized as members of the LAC family. Sequence logos were created on the WebLogo server (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004). The putative signal sequences
and potential glycosylation sites of the proteins were predicted using TargetP and NetNGlyc server v1.1 (http://www.cbs.dtu.dk/) (Emanuelsson et al., 2000), respectively. The default cut-off values were adopted with plant as the organism. Phylogenetic tree was constructed by the neighbor-joining (NJ) method with 1,000 bootstrap replicates using MEGA 7.0 (Kumar, Stecher & Tamura, 2016).

Intron/exon structure was determined through the comparison of the coding sequence of each SmLAC gene with its genomic sequence using the Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/index.php) (Hu et al., 2015). We found the 1,500 bp sequence upstream from each SmLAC initiation codon and used the online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for the prediction of cis-elements. SmLAC proteins were further subject to GO annotations analysis according to the Gene Ontology (GO, geneontology.org).

Quantitative real-time reverse transcription-PCR

Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis of SmLACs in flowers, leaves, stems and roots of 2-year-old S. miltiorrhiza plants were performed using gene-specific primers listed in Table S4. Polymerase chain reaction was performed in a 20 µl volume with 100 ng diluted cDNA, 10 µM forward primer, 10 µM reverse primer and 10 µl 2 × SYBR Premix Ex Taq II (TaKaRa Bio, Otsu, Japan). Quantitative real-time reverse transcription-PCR was carried out with a CFX96 real-time PCR machine (BIO-RAD, American). SmUBQ10 was used as a reference gene (Li et al., 2015). The expression of Smi-miR397.1, Smi-miR397.2 and Smi-miR408 was analyzed using Mir-X miRNA qRT-PCR SYBR Kit (TaKaRa, Otsu, Japan). The primers were listed in Table S4. Polymerase chain reaction cycling began with template denaturation and hot start Taq activation at 95 °C for 30 s, then 40 cycles of 95 °C for 15 s, and 60 °C for 30 s and 72 °C for 30 s extension step. All of the qRT-PCRs were repeated for three biological and three technical replicates. The $2^{-\Delta\Delta Ct}$ method was used for evaluating gene expression levels. Three biological replicates were standardized as described previously (Willems, Leyns & Vandesompele, 2008). All data were subjected to variance (ANOVA) analysis and the results were presented as the mean ± SD. P < 0.05 was considered as statistically significant.

Identification of SmLACs with perfect or near-perfect complementary sequences to miRNAs

Small RNAs sequences from roots (SRX686651), stems (SRX686652), leaves (SRX686653) and flowers (SRX686654) of S. miltiorrhiza were downloaded from SRA database (Xu et al., 2014). The complementarity of SmLACs to small RNAs was searched using psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Dai & Zhao, 2011) with default parameters. The maximum expectation value was 3.0, length for complementarity scoring was 19 bp and the target accessibility-allowed maximum energy to unpair the target site of 25.0 was applied. Flanking length around target site for target accessibility analysis (UPE calculation) was 17 bp upstream and 13 bp downstream and range of central mismatch causing translational inhibition was set 10–11 nucleotides. The predicted small RNAs were
aligned with *S. miltiorrhiza* 99-3 genome using Bowtie (**Langmead et al., 2009**). Secondary structures were predicted on the mfold web server (**Zuker, 2003**).

**Degradome and experimental verification of miRNA-directed cleavage of SmLACs**

Degradome data were aligned to SmLACs using SOAP2.0 software (**Li et al., 2009**) (**http://soap.genomics.org.cn/**) to define the coverage rate. Alignments with scores not exceeding four and no mismatches at the cleavage site (between the 10th and 11th nucleotides) were considered to be products of miRNA cleavage. Experimental validation of miRNA targets was carried out using the modified RNA ligase-mediated rapid amplification of 5′ cDNAs method (5′RLM RACE) as described (**Li et al., 2017**).

Gene-specific primers (GSP and NGSP) were designed by Premier 5.0 software (**Tables S5 and S6**). Two rounds of PCR were performed. Initial PCR reactions were done with the 5′RACE outer primer and the complementary GSP. The nested PCR was performed with the 5′RACE inner primer and the NGSP. Nested PCR products were analyzed on a 1.5% agarose gel. Positive PCR products were cloned into pMD-19T vector (TaKaRa, Otsu, Japan) and sequenced.

**RESULTS**

**Identification of 65 SmLAC genes in *S. miltiorrhiza***

Blast analysis of 17 AtLACs from *Arabidopsis* and 49 PtLACs from *P. trichocarpa* against the current assembly of the whole *S. miltiorrhiza* genome sequence (**Xu et al., 2016**) identified 85 genomic loci putatively encoding LACs. Genomic DNA sequences surrounding the 85 loci were retrieved and computationally predicted for gene models by alignment with RNA-seq data of *S. miltiorrhiza* (**http://www.ncbi.nlm.nih.gov/sra**) and LACs identified in other species (**www.ncbi.nlm.nih.gov/blast/**). Errors from genome sequencing and assembly were corrected after sequence alignment. Since plant LACs consist of three conserved Cu-oxidase domains with signature sequences (**Diamantidis et al., 2000**), the gene models predicted were analyzed for conserved domains by blast analysis against the Pfam database. The results showed that 49 gene models encoded full-length LACs, 16 were laccase gene fragments, whereas the other 20 coded for L-ascorbate oxidase proteins, which were excluded from further analysis. We then obtained full-length coding sequences (CDSs) of the 16 partial SmLACs using experimental approaches, including 5′ RACE, 3′ RACE and PCR amplification of coding regions. Finally, a total of 65 SmLACs were identified (**Data S1**). They were named SmLAC1–SmLAC65, respectively (**Table 1**). Sequence feature analysis showed that the theoretical pI of deduced *S. miltiorrhiza* LAC proteins widely ranged from 5.23 (SmLAC21) to 9.73 (SmLAC43). The molecular weights of the SmLAC proteins range from 54.8 kDa (SmLAC7) to 70.7 kDa (SmLAC63) and the length varied between 504 (SmLAC7) to 630 (SmLAC63) amino acids.

Generally, most of SmLACs consist of 500–600 amino acids and were probably secreted proteins with a few of them predicted to be located in mitochondria and nuclear. Additionally, variable N- or O-glycosylation sites and phosphorylation sites were predicted...
Table 1  Protein sequences of SmLACs.

| Name       | AA Len | pI   | Mw (Da)  | Predicted subcellular location | N-Glyc | O-Glyc |
|------------|--------|------|---------|--------------------------------|--------|--------|
| SmLAC1     | 566    | 8.54 | 62214.94| Secretory                      | 11     | 13     |
| SmLAC2     | 564    | 8.27 | 62644.60| Secretory                      | 5      | 10     |
| SmLAC3     | 578    | 7.64 | 63022.57| Secretory                      | 10     | 13     |
| SmLAC4     | 559    | 8.87 | 61097.94| Secretory                      | 11     | 14     |
| SmLAC5     | 553    | 7.08 | 62556.68| Mitochondrion                  | 5      | 10     |
| SmLAC6     | 573    | 9.21 | 63120.82| Secretory                      | 12     | 8      |
| SmLAC7     | 504    | 5.96 | 54842.84| Secretory                      | 9      | 21     |
| SmLAC8     | 568    | 8.84 | 62949.54| Secretory                      | 7      | 6      |
| SmLAC9     | 612    | 5.93 | 68171.10| Secretory                      | 17     | 12     |
| SmLAC10    | 570    | 5.99 | 62138.75| Secretory                      | 12     | 12     |
| SmLAC11    | 542    | 9.05 | 59612.02| Secretory                      | 11     | 7      |
| SmLAC12    | 565    | 6.62 | 63234.93| Secretory                      | 11     | 12     |
| SmLAC13    | 575    | 8.02 | 65087.05| Secretory                      | 2      | 10     |
| SmLAC14    | 548    | 8.62 | 62462.27| Extracellular                  | 5      | 6      |
| SmLAC15    | 568    | 9.16 | 62486.70| Secretory                      | 17     | 9      |
| SmLAC16    | 577    | 9.17 | 63496.65| Secretory                      | 15     | 11     |
| SmLAC17    | 565    | 8.40 | 62481.15| Secretory                      | 9      | 8      |
| SmLAC18    | 555    | 9.30 | 61366.90| Secretory                      | 11     | 13     |
| SmLAC19    | 529    | 8.72 | 59939.00| Secretory                      | 7      | 8      |
| SmLAC20    | 573    | 6.79 | 64637.23| Secretory                      | 4      | 8      |
| SmLAC21    | 540    | 5.23 | 60626.67| Secretory                      | 5      | 7      |
| SmLAC22    | 566    | 8.02 | 64165.84| Secretory                      | 4      | 9      |
| SmLAC23    | 513    | 8.57 | 56859.20| Secretory                      | 9      | 12     |
| SmLAC24    | 550    | 9.09 | 60671.31| Secretory                      | 8      | 13     |
| SmLAC25    | 561    | 6.11 | 63020.84| Secretory                      | 10     | 7      |
| SmLAC26    | 568    | 8.71 | 62686.13| Secretory                      | 8      | 15     |
| SmLAC27    | 506    | 8.89 | 56727.77| Secretory                      | 8      | 13     |
| SmLAC28    | 562    | 9.41 | 62070.94| Secretory                      | 11     | 10     |
| SmLAC29    | 553    | 7.67 | 62113.75| Secretory                      | 14     | 8      |
| SmLAC30    | 532    | 6.74 | 60027.81| Secretory                      | 5      | 12     |
| SmLAC31    | 556    | 8.44 | 62176.34| Secretory                      | 10     | 8      |
| SmLAC32    | 570    | 9.19 | 63318.38| Secretory                      | 8      | 16     |
| SmLAC33    | 565    | 7.29 | 63504.00| Secretory                      | 11     | 8      |
| SmLAC34    | 566    | 9.29 | 60713.98| Secretory                      | 8      | 26     |
| SmLAC35    | 550    | 6.93 | 62153.83| Secretory                      | 5      | 9      |
| SmLAC36    | 557    | 7.08 | 63050.93| Secretory                      | 5      | 11     |
| SmLAC37    | 562    | 8.84 | 62954.32| Secretory                      | 6      | 6      |
| SmLAC38    | 573    | 8.54 | 63309.99| Secretory                      | 8      | 8      |
| SmLAC39    | 564    | 9.45 | 62407.43| Secretory                      | 7      | 14     |
| SmLAC40    | 528    | 9.21 | 57151.17| Secretory                      | 7      | 19     |
| SmLAC41    | 588    | 8.91 | 64864.89| Nuclear                        | 9      | 15     |

(Continued)
Phylogenetic analysis of LAC proteins

In order to examine the phylogenetic relationships of SmLACs and LAC proteins from *Arabidopsis* and *P. trichocarpa*, a neighbor-joining (NJ) phylogenetic tree was constructed using MEGA7.0 based on 131 full-length protein sequences of SmLACs, AtLACs and PtLACs (Fig. 1). The constructed tree was highly similar to the trees constructed for AtLACs or for AtLACs and PtLACs (*McCaig, Meagher & Dean, 2005; Turlapati et al., 2011; Lu et al., 2013*). Based on the tree constructed in this study and those from previous investigations (*McCaig, Meagher & Dean, 2005; Turlapati et al., 2011; Lu et al., 2013*), we divided the 131 LACs into eight clades (C1–C8), of which seven clades (C1–C7) included proteins from *S. miltiorrhiza, Arabidopsis* and *P. trichocarpa*, whereas C8 were specific to *S. miltiorrhiza* (Fig. 1).
Additionally, 42 pairs of SmLACs seem to be paralogous proteins (Table S7). To check the type of selection that acted on genes in the SmLAC family, the ratio between non-synonymous (Ka) and synonymous substitutions (Ks) was estimated using DnaSP v.5 (Table S7). The results revealed that 13 gene pairs were under strong purifying selection as the Ka/Ks ratio was lower than 1.0, while 18 gene pairs were approximately under positive selection with a Ka/Ks ratio larger than 1.0. These results showed that different SmLACs had undergone different evolutionary forces.

Figure 1 Phylogenetic analysis of SmLAC, AtLAC and PtLAC proteins. The phylogenetic tree of LAC proteins from *S. miltiorrhiza* (red), *Arabidopsis* (blue) and *P. trichocarpa* (green) was constructed using the neighbor-joining method implemented in MEGA7.0 with 1,000 bootstrap replicates.

DOI: 10.7717/peerj.7605/fig-1
Conserved domain analysis showed that all of the 65 identified SmLACs contain three highly conserved Cu-oxidase domains, including Cu-oxidase_3, Cu-oxidase and Cu-oxidase_2 (Fig. 2). Analysis of sequence logos, which reflected the frequency of residue
at each position, showed that the distribution of SmLAC residues at the three conserved domains was similar to other plant LACs (McCaig, Meagher & Dean, 2005; Turlapati et al., 2011). This suggests high degree of conservation among plant laccase proteins. In the conserved domains, there are four deeply conserved signature sequences, which were labeled L1 to L4, respectively (Figs. 2A and 2C). It has been shown that these signature sequences are unique for LACs, and ten histidines, one cysteine and one leucine or methionine in the signature sequences are responsible for binding four Cu atoms, including one type-1 (T1) Cu, one type-2 (T2) Cu and two type-3 (T3) Cu (Figs. 2A and 2C) (Turlapati et al., 2011). L1 contains two conserved histidines, one of which binds T2 Cu, whereas the other one binds T3 Cu (Fig. 2A). L2 also includes two conserved histidines, both of which bind T3 Cu (Fig. 2A). L3 contains three histidines, which bind T1, T2 and T3 Cu, respectively (Fig. 2C). L4 includes five conserved Cu-binding residues, three of which bind T1 Cu, whereas the other two bind T3 Cu (Fig. 2C). Further examination of the signature sequences reveals two equivalent motifs, including HWHG in L1 and HLHG in L3 (Figs. 2A and 2C). In addition, there are other three conserved motifs (PGP, WxDG and TQCP) in the Cu-oxidase_3 domain and one conserved PGQT motif in the Cu-oxidase domain (Figs. 2A and 2B).

Gene structure analysis showed that the exon number in the coding regions of 65 S. miltiorrhiza LAC genes varied between 4 and 10 with the majority (78%) to be 5 or 6 (Fig. 3; Fig. S1). It is consistent with the exon number in AtLACs and PtLACs, most of which have 5 or 6 exon in the coding regions (Fig. 3; Fig. S1). This suggests the similarity of S. miltiorrhiza, P. trichocarpa and A. thaliana LACs in gene structures. Although the exon number of SmLACs is comparable with AtLACs and PtLACs, several SmLACs contain 4 and 10 exons, which represent the greatest difference among the exon numbers of SmLACs, PtLACs and AtLACs. Investigating the structures of genes in each clade showed that C8 genes had intron-exon structures differing from genes in other clades (Fig. 3). This indicates that the ancestors of C8 SmLACs genes could be different from other SmLACs.

**Diverse cis regulatory elements in SmLAC promoters**

To obtain further insights into the possible expression regulation mode of SmLACs, we analyzed the cis-acting elements of the 1,500 bp regulatory sequence upstream of the 65 SmLAC coding sequences (CDS) using the PlantCARE database (Table S8). The TATA-box elements were found in all SmLAC promoter regions. The other cis-elements were found that were responsive to hormones or inducers such as methyl jasmonate (MeJA), ethylene, abscisic acid and salicylic acid. Fifty-four members contain abscisic acid (ABA)-responsive elements (ABREs), 41 members have ethylene-responsive elements (EREs), 50 members contain the methyl jasmonate (MeJA)-responsive element (CGTCA motif) and 55 members contain the salicylic acid (SA)-responsive element (TCA element). This analysis revealed a large number of motifs responding to different inductions, suggesting a complex regulation of SmLAC genes expression. Besides, we identified some biotic and abiotic stress-related cis-acting elements in the upstream regulatory sequences of the SmLACs, such as the TC-rich repeat element (related to defence), and low-temperature...
stress-related (LTR). These results suggest that members of the SmLAC family might play roles in the responses to a variety of abiotic and biotic stresses.

**Expression patterns of SmLAC genes in different tissues**

Since gene expression patterns provide clues for deciphering gene functions, we analyzed the level of SmLAC transcripts in roots, stems, leaves and flowers of two-year-old, field nursery-grown *S. miltiorrhiza* plants using quantitative real-time RT-PCR. Significant differential expression was observed for 65 of identified SmLAC genes (Fig. 4). Among them, 14 (SmLAC1, SmLAC2, SmLAC3, SmLAC4, SmLAC8, SmLAC9, SmLAC16, SmLAC28, SmLAC29, SmLAC36, SmLAC37, SmLAC38, SmLAC54 and SmLAC65) were mainly expressed in flowers, 14 in stems (SmLAC6, SmLAC7, SmLAC12, SmLAC13, SmLAC15, SmLAC30, SmLAC31, SmLAC42, SmLAC43 SmLAC45, SmLAC48, SmLAC49,
SmLAC52 and SmLAC58), 16 in roots (SmLAC5, SmLAC10, SmLAC11, SmLAC14, SmLAC18, SmLAC19, SmLAC21, SmLAC25, SmLAC33, SmLAC35, SmLAC55, SmLAC57, SmLAC59, SmLAC61, SmLAC63 and SmLAC64), three (SmLAC44, SmLAC53 and SmLAC56) in leaves and 14 (SmLAC17, SmLAC20, SmLAC22, SmLAC24, SmLAC26, SmLAC32, SmLAC34, SmLAC39, SmLAC40, SmLAC46, SmLAC47, SmLAC51, SmLAC60 and SmLAC62) in at least two tissues (Fig. 4). The other four, including SmLAC23, SmLAC27, SmLAC41 and SmLAC50, were highly expressed in all of the tissues analyzed. The results indicate that SmLACs play manifold functions in *S. miltiorrhiza*.

The gene expression of SmLACs is highly variable. In fact, folding changes range from units to tens of thousands. Therefore, we verified the amplification efficiency of several PCR primer pairs in low expressed (SmLAC26 and SmLAC41) and highly expressed
(SmLAC28 and SmLAC34) SmLACs. Polymerase chain reaction efficiency for some primer pairs are listed in Table S9 and gels with expression results of SmLAC26, SmLAC28, SmLAC34 and SmLAC41 are shown in Fig. S2.

**MiRNA-mediated posttranscriptional regulation of SmLAC expression**

MiRNAs are a class of small non-coding RNAs with 20–24 nucleotides in length. They play vital roles in plant growth, development and stress responses mainly through targeting gene transcripts for cleavage. In *Arabidopsis*, AtLACs are posttranscriptionally regulated by miR397, miR408 and miR857 (*Abdel-Ghany & Pilon, 2008*). In order to examine whether SmLACs are regulated by miRNAs, we searched *S. miltiorrhiza* small RNAs (*Xu et al., 2014*) potentially targeting SmLACs for cleavage using psRNATarget with default parameters (*Dai & Zhao, 2011*). Using penalty score cutoff threshold 3 for mismatched patterns in the miRNA/target duplexes as the criterion, we identified a total of 34 small RNAs. We mapped the retrieved small RNA sequences to the assembly of *S. miltiorrhiza* genome (*Xu et al., 2016*). Genomic sequences surrounding the matched regions were then analyzed for secondary structures using mfold (*Zuker, 2003*). Finally, two stem-loops structures satisfying the criteria of miRNA precursors (*Meyers et al., 2008*) were identified. They are Smi-MiR397 (Fig. 5A) and Smi-MiR408 (Fig. 5B). Smi-MiR397 generated two overlapped mature miRNAs, termed Smi-miR397.1 and Smi-miR397.2, respectively. Smi-MiR408 generated one mature miRNA, termed Smi-miR408.

Computational prediction showed that 23 of the 65 identified SmLACs had near-perfect complementary region to Smi-miR397.1, Smi-miR397.2 and Smi-miR408 (Fig. 5C). Among them, 11 SmLACs are targets of Smi-miR397.1, and 9 are common targets of Smi-miR397.1 and Smi-miR397.2. Two, including SmLAC3 and SmLAC18 are common targets of Smi-miR397.1, Smi-miR397.2 and Smi-miR408, and SmLAC28 is common targets of Smi-miR397.1 and Smi-miR408. The target site of Smi-miR408 locates at the nonconserved 5‘ terminal region, whereas the site of Smi-miR397 locates in the region encoding the conserved Cu-oxidase domain.

Using the poly(A) adaptor RT-PCR method, we analyzed the expression of Smi-miR397.1, Smi-miR397.2 and Smi-miR408 in roots, stems, leaves and flowers of 2-year-old, field-grown *S. miltiorrhiza* Bunge (line 993). Smi-miR397 exhibited higher expression level in leaves compared with the level in roots, stems and flowers (Fig. 5E). Smi-miR408 showed the highest expression in stems. Its expression in roots and flowers is similar (Fig. 5F).

Plant miRNA-mediated cleavage of transcripts usually locates at a site corresponding to the tenth miRNA nucleotide from the 5‘ end. To validate the cleavage of Smi-miR397 and Smi-miR408 on SmLACs, we analyzed high-throughput *S. miltiorrhiza* degradome data (*Xu et al., 2014*). The results confirmed that the 23 SmLACs were targets of Smi-miR397 and Smi-miR408 (Fig. 6). Furthermore, we performed the modified 5‘ RNA ligase-mediated RACE (5‘RLM RACE) for mapping of cleavage sites. The experiments were carried out on mRNA isolated from roots, stems, leaves and flowers of *S. miltiorrhiza*. The 5‘RLM RACE products revealed that six SmLACs, including SmLAC3, SmLAC18, SmLAC23, SmLAC50, SmLAC55 and SmLAC60, were indeed cleaved by Smi-miR397.
and/or Smi-miR408 in vivo (Fig. 5D). Further investigating the cleavage sites of Smi-miR397 showed that SmLAC3 and SmLAC50 were cleaved by both Smi-miR397.1 and Smi-miR397.2.

Figure 5 SmLACs targeted by Smi-miR397 and Smi-miR408. (A) Hairpin structure of Smi-miR397. Mature miRNA sequences of Smi-miR397.1 and Smi-miR397.2 are indicated by red and red line, respectively. (B) Hairpin structure of Smi-miR408. Mature miRNA sequence of Smi-miR408 is indicated by red. (C) Smi-miR397- and Smi-miR408-targeted SmLACs. (D) Experimental validation of Smi-miR397- and Smi-miR408-mediated cleavage of SmLACs. Cleavage sites were determined by the modified 5′RNA ligase-mediated RACE method. SmLAC sequence of each complementary site from 5′ to 3′ and Smi-miR397/408 sequences from 3′ to 5′ are shown. Smi-miR397/408 complementary sites with the nucleotide positions of SmLAC coding region are indicated. Vertical arrows indicate the 5′ termini of miRNA-guided cleavage products, as identified by 5′-RACE, with the frequency of clones shown. (E) and (F) Expression patterns of Smi-miR397.1, Smi-miR397.2 and Smi-miR408 in roots (Rt), stems (St), leaves (Le) and flowers (Fl) of S. miltiorrhiza, respectively. Expression level of Smi-miR7972 in leaves was arbitrarily set to 1 and the levels of Smi-miR397.1, Smi-miR397.2 and Smi-miR408 were given relative to this. Error bars were indicated by the standard deviations of three biological replicates. Full-size DOI: 10.7717/peerj.7605/fig-5
The 23 miRNA-targeted SmLACs distribute in five clades of the phylogenetic tree (Fig. 1). SmLACs targeted by Smi-miR397 distribute in C1, C2, C3, C4 and C5, and those targeted by Smi-miR408 belong to C5. No miRNA targets were found in C6, C7 and C8. It is consistent with the fact that LACs in a clade show relatively high sequence similarity and usually play redundant functions.

**Putative functions of SmLACs**

All the identified proteins were subjected to GO annotations to understand the biological functions. The GO database contains three ontologies: biological process, cellular component and molecular function. Thirty-five SmLAC proteins were categorized into the
three ontologies (Fig. S3; Table S10). Among the 35 SmLACs, 3 SmLACs (SmLAC63, SmLAC64 and SmLAC65), 13 SmLACs and 34 SmLACs were annotated in the cellular component, molecular function and biological process categories, respectively (Table S10). Further analysis of GO annotations showed that SmLAC genes involved in the biosynthesis of secondary metabolites, such as lignin biosynthetic process, phenylpropanoid metabolic process, plant-type secondary cell-wall biogenesis and proanthocyanidin biosynthetic process (Fig. S3; Table S10).

Since functional redundancy of plant LACs, it is difficult to accurately uncover the role of single SmLAC in plants. However, clues can be acquired from phylogenetic analysis and expression profiling. AtLAC2 that belong to C2 was widely expressed in roots, stems, leaves and flowers with the level in stems relatively high (Turlapati et al., 2011). It was down-regulated under heat, drought, genotoxic (bleomycin and mitomycin), brassinolide, IAA, salicylic acid and zeatin treatments (Turlapati et al., 2011). Knockout mutant of AtLAC2 exhibited reduced root elongation under PEG-induced dehydration conditions (Cai et al., 2006). Although the function of AtLAC2 has not been fully elucidated, current evidence indicates that it is involved in the development of stems and roots and in plant response to various stresses.

Laccases are associated with monolignols or flavonoid metabolites in plants (Table S11). The Arabidopsis AtLAC member of C1 and C2, AtLAC17, AtLAC4, AtLAC10 and AtLAC11 were widely expressed in roots, stems, leaves and flowers with the highest expression level in stems (Turlapati et al., 2011). Double mutation of AtLAC4 and AtLAC17 resulted in significant reduction of lignin content (Berthet et al., 2011). Triple mutation of AtLAC4, AtLAC11 and AtLAC17 resulted in extremely low lignin content in roots (Zhao et al., 2013). Consistently, in Ptr-miR397a-overexpressed P. trichocarpa tran Gençler, the reduction of lignin content in stem xylem was accompanied by significantly decreased expression of a subset of PtLAC genes, the majority of which, such as PtLAC1, PtLAC2, PtLAC14, PtLAC15, PtLAC20, PtLAC23, PtLAC24, PtLAC41, and PtLAC49, are members of C1 and C2 (Lu et al., 2013). This suggests that C1 and C2 LAC members are probably involved in lignin biosynthesis. A total of 12 SmLACs are included in C1 and C2. Five of them, including SmLAC6, SmLAC12, SmLAC15, SmLAC31 and SmLAC43 were predominantly expressed in stems (Fig. 4), and all of them are targets of Smi-miR397 (Fig. 5C). This indicates the putative roles of these five SmLACs in oxidative lignin polymerization in S. miltiorrhiza and suggests the conserved regulatory mechanism of miR397-LAC module in oxidative polymerization in S. miltiorrhiza and Arabidopsis.

It has been shown that AtLAC15 is involved in oxidative polymerization of flavonoids (Pourcel et al., 2005). The seeds of lac15-1 and lac15-2 mutants showed yellow or pale color, resembling a transparent testa phenotype (Cai et al., 2006). SmLAC8 showed very close phylogenetic relationship with AtLAC15 (Fig. 1) and were predominantly expressed in flowers (Fig. 4). This indicates the role of SmLAC8 in the polymerization of flavonoids. In addition, SmLACs in C8 could be involved in the biosynthesis of lineage-specific compounds, such as salvianolic acids, since no Arabidopsis and P. trichocarpa homologs were found for these SmLACs (Fig. 1). Further functional characterization of SmLACs
through genetic transformation and enzyme activity analysis will definitely shed lights on the mechanisms of SmLAC-mediated polymerization of phenolic compounds.

**DISCUSSION**

Laccases widely exist in various plant species, such as *Arabidopsis thaliana* (McCag, Meagher & Dean, 2005; Turlapati et al., 2011), rice (Liu et al., 2017), tobacco (Richardson & McDougall, 1997), ryegrass (Gavnholt, Larsen & Rasmussen, 2002), cotton (Balasubramanian et al., 2016), poplar (LaFayette, Eriksson & Dean, 1999; Lu et al., 2013), sycamore maple (Sterjiades, Dean & Eriksson, 1992) and pear (Cheng et al., 2019). Here, we identified and characterized 65 LAC genes in *S. miltiorrhiza*. They all contain the typical characteristics of three conserved Cu-oxidase domains and SmLACs are mostly secretory proteins. Integrative analysis of gene structures, sequence features, conserved domains and phylogenetic relationships showed that SmLAC genes could be divided into eight clades. Previous results showed that 17 AtLACs and 49 PtLACs were clustered into six clades (McCag, Meagher & Dean, 2005; Turlapati et al., 2011; Lu et al., 2013). Comparison of our results with previous studies showed that, among the seven clades with SmLAC, AtLAC and PtLAC proteins, six clades (C1–C6) were consistent, whereas C7 was an extra. C7 consists of three proteins, including AtLAC6, PtLAC33 and SmLAC17. AtLAC6 and PtLAC33 belonged to C6 in the phylogenetic trees constructed previously (Lu et al., 2013), although it showed relative far relationships with other members in the clade. Adding SmLACs to the tree resulted in separation of AtLAC6 and PtLAC33 from C6. Similar results were observed in a tree constructed for AtLACs and *Citrus sinensis* CsLACs (Xu et al., 2019). This suggests that the relationships between C6 and C7 members are indeed relatively far. C8 was specific to *S. miltiorrhiza*, indicated that SmLACs in C8 could be involved in the biosynthesis of lineage-specific compounds.

It has been reported that these three *Arabidopsis* laccase genes were up-regulated by stress. AtLAC4, AtLAC10 and AtLAC11 that belong to C1 and AtLAC2 and AtLAC17 that belong to C2, are highly expressed in stems (Fig. 1) (Turlapati et al., 2011). Among them, AtLAC2, AtLAC4 and AtLAC17 are targets of Ath-miR397 (Abdel-Ghany & Pilon, 2008). AtLAC2 was widely expressed in roots, stems, leaves and flowers with the level in stems relatively high (Turlapati et al., 2011). It was down-regulated under heat, drought, genotoxic (bleomycin and mitomycin), brassinolide, IAA, salicylic acid and zeatin treatments (Turlapati et al., 2011). Knockout mutant of AtLAC2 exhibited reduced root elongation under PEG-induced dehydration conditions (Cai et al., 2006). Although the function of AtLAC2 has not been fully elucidated, current evidence indicates that it is involved in the development of stems and roots and in plant response to various stresses.

MiRNAs have been demonstrated to play important roles in negatively regulating the expression of laccase genes by degradation of target mRNAs in several plants, such as miR408, miR397 and miR857 in *Arabidopsis* regulating seven laccase genes, ptr-miR397a in *P. trichocarpa* regulating seventeen laccase genes, and Os-miR397 in *Oryza sativa* regulating OsLAC gene (Abdel-Ghany & Pilon, 2008; Lu et al., 2013; Zhang et al., 2013). Our results suggested that 23 SmLACs were predicted to be Sm-miR397 targets. All these SmLACs were clustered within C1, C2, C3, C4 and C5 in the phylogenetic tree.
This distribution indicated the role of Smi-miR397 in controlling cell-wall lignin biosynthesis and stress responses.

CONCLUSIONS
A total of 65 SmLAC genes were identified in S. miltiorrhiza. Genes and the deduced proteins were characterized through a comprehensive approach, including phylogenetic tree construction, laccase domain characterization, gene structure analysis and gene expression profiling. The majority of clades contained members from S. miltiorrhiza, A. thaliana and P. trichocarpa, which indicated that the function of most LACs was conserved among plant species. However, C8 was specific to S. miltiorrhiza. This indicates that the members of the clade play unique functions in S. miltiorrhiza. The Cu_oxidase domains are highly conserved in plant LACs. Gene structures of most LACs from S. miltiorrhiza, Arabidopsis and P. trichocarpa were highly conserved. However, SmLACs encoding C8 proteins were distinct. Although the precise functions of most plant LACs remain unknown, phylogenetic and expression analyses showed that LACs could play conserved and divergent roles in polymerization of phenolic compounds. Posttranscriptional analysis revealed that Smi-miR397 and Smi-miR408 were involved in regulating a subset of S. miltiorrhiza LAC genes.

ACKNOWLEDGEMENTS
We appreciate Prof. Xian’en Li at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College for providing S. miltiorrhiza plant.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This work was supported by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS) (2016-I2M-3-016), the Natural Science Foundation of China (Grant Nos. 31370327 and 81603225), the Beijing Natural Science Foundation (Grant No. 5152021) and PUMC Youth Fund and the Fundamental Research Funds for the Central Universities (Grant No. 3332016072). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
CAMS Innovation Fund for Medical Sciences (CIFMS): 2016-I2M-3-016.
Natural Science Foundation of China: 31370327 and 81603225.
Beijing Natural Science Foundation: 5152021.
PUMC Youth Fund and the Fundamental Research Funds for the Central Universities: 3332016072.

Competing Interests
The authors declare that they have no competing interests.
Author Contributions

- Caili Li conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables.
- Dongqiao Li performed the experiments, analyzed the data, contributed reagents/materials/analysis tools.
- Hong Zhou analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables.
- Jiang Li analyzed the data, contributed reagents/materials/analysis tools.
- Shanfa Lu conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7605#supplemental-information.

REFERENCES

Abdel-Ghany SE, Pilon M. 2008. MicroRNA-mediated systemic downregulation of copper protein expression in response to low copper availability in Arabidopsis. Journal of Biological Chemistry 283(23):15932–15945 DOI 10.1074/jbc.M801406200.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37(Web Server):W202–W208 DOI 10.1093/nar/gkp335.

Balasubramanian VK, Rai KM, Thu SW, Hii MM, Mendu V. 2016. Genome-wide identification of multifunctional laccase gene family in cotton (Gossypium spp.); expression and biochemical analysis during fiber development. Scientific Reports 6(1):34309 DOI 10.1038/srep34309.

Bao W, O’malley DM, Whetten R, Sederoff RR. 1993. A laccase associated with lignification in loblolly pine xylem. Science 260(5108):672–674 DOI 10.1126/science.260.5108.672.

Berthet S, Demont-Caulet N, Pollet B, Bidzinski P, Cézard L, Le Bris P, Borrega N, Hervé J, Blondet E, Balzergue S, Lapierre C, Jouanin L. 2011. Disruption of LACCASE4 and 17 results in tissue-specific alterations to lignification of Arabidopsis thaliana stems. Plant Cell 23(3):1124–1137 DOI 10.1105/tpc.110.082792.

Bjellqvist B, Basse B, Olsen E, Celis JE. 1994. Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 15(1):529–539 DOI 10.1002/elps.1150150171.

Brodersen P, Voinnet O. 2009. Revisiting the principles of microRNA target recognition and mode of action. Nature Reviews Molecular Cell Biology 10(2):141–148 DOI 10.1038/nrm2619.

Brown DM, Zeef LAH, Ellis J, Goodacre R, Turner SR. 2005. Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. Plant Cell 17(8):2281–2295 DOI 10.1105/tpc.105.031542.
Cai X, Davis EJ, Ballif J, Liang M, Bushman E, Haroldsen V, Torabinejad J, Wu Y. 2006. Mutant identification and characterization of the laccase gene family in Arabidopsis. Journal of Experimental Botany 57(11):2563–2569 DOI 10.1093/jxb/erl022.

Cheng X, Li G, Ma C, Abdullah M, Zhang J, Zhao H, Jin Q, Cai Y, Lin Y. 2019. Comprehensive genome-wide analysis of the pear (Pyrus bretschneideri) laccase gene (PbLAC) family and functional identification of PbLAC1 involved in lignin biosynthesis. PLOS ONE 14(2):e0210892 DOI 10.1371/journal.pone.0210892.

Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo generator. Genome Research 14(6):1188–1190 DOI 10.1101/gr.849004.

Dai X, Zhao PX. 2011. psRNATarget: a plant small RNA target analysis server. Nucleic Acids Research 39(Suppl 2):W155–W159 DOI 10.1093/nar/gkr319.

Dean JFD, Eriksson K-EL. 1994. Laccase and the deposition of lignin in vascular plants. Holzforschung 48(s1):21–33 DOI 10.1515/hfsg.1994.48.s1.21.

Deng Y, Li C, Li H, Lu S. 2018. Identification and characterization of flavonoid biosynthetic enzyme genes in Salvia miltiorrhiza (Lamiaceae). Molecules 23(6):1467 DOI 10.3390/molecules23061467.

Deng Y, Lu S. 2017. Biosynthesis and regulation of phenylpropanoids in plants. Critical Reviews in Plant Sciences 36(4):257–290 DOI 10.1080/07352689.2017.1402852.

Diamantidis G, Effosse A, Potier P, Bally R. 2000. Puriﬁcation and characterization of the ﬁrst bacterial laccase in the rhizospheric bacterium Azospirillum lipoferum. Soil Biology and Biochemistry 32(7):919–927 DOI 10.1016/S0038-0717(99)00221-7.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. Journal of Molecular Biology 300(4):1005–1016 DOI 10.1006/jmbi.2000.3903.

Filipowicz W, Bhattacharyya SN, Sonenberg N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews Genetics 9(2):102–114 DOI 10.1038/nrg2290.

Freudenberg K. 1959. Biosynthesis and constitution of lignin. Nature 183(4669):1152–1155 DOI 10.1038/1831152a0.

Gavnholt B, Larsen K, Rasmussen SK. 2002. Isolation and characterisation of laccase cDNAs from meristematic and stem tissues of ryegrass (Lolium perenne). Plant Science 162(6):873–885 DOI 10.1016/S0168-9452(02)00035-3.

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G. 2010. Laccases: a never-ending story. Cellular and Molecular Life Sciences 67(3):369–385 DOI 10.1007/s00018-009-0169-1.

Hou X, Shao F, Ma Y, Lu S. 2013. The phenylalanine ammonia-lyase gene family in Salvia miltiorrhiza: genome-wide characterization, molecular cloning and expression analysis. Molecular Biology Reports 40(7):4301–4310 DOI 10.1007/s11033-013-2517-3.

Hu B, Jin J, Guo A-Y, Zhang H, Luo J, Gao G. 2015. GS2D 2.0: an upgraded gene feature visualization server. Bioinformatics 31(8):1296–1297 DOI 10.1093/bioinformatics/btu817.

Huang J-H, Qi Y-P, Wen S-X, Guo P, Chen X-M, Chen L-S. 2016. Illumina microRNA profiles reveal the involvement of miR397a in Citrus adaptation to long-term boron toxicity via modulating secondary cell-wall biosynthesis. Scientific Reports 6(1):22900 DOI 10.1038/srep22900.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7):1870–1874 DOI 10.1093/molbev/msw054.
LaFayette PR, Eriksson KEL, Dean JF. 1999. Characterization and heterologous expression of laccase cDNAs from xylem tissues of yellow-poplar (Liriodendron tulipifera). Plant Molecular Biology 40(1):23–35 DOI 10.1023/A:1026437406859.

Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10(3):R25 DOI 10.1186/gb-2009-10-3-r25.

Li C, Li D, Li J, Shao F, Lu S. 2017. Characterization of the polyphenol oxidase gene family reveals a novel microRNA involved in posttranscriptional regulation of PPOs in Salvia miltiorrhiza. Scientific Reports 7(1):44622 DOI 10.1038/srep44622.

Li C, Li D, Shao F, Lu S. 2015. Molecular cloning and expression analysis of WRKY, transcription factor genes in Salvia miltiorrhiza. BMC Genomics 16(1):200 DOI 10.1186/s12864-015-1411-x.

Li R, Yu C, Li Y, Lam T, Yiu S, Kristiansen K, Wang J. 2009. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25(15):1966–1972 DOI 10.1109/bioinformatics/btp336.

Liu Q, Luo L, Wang X, Shen Z, Zheng L. 2017. Comprehensive analysis of rice laccase gene (OsLAC) family and ectopic expression of OsLAC10 enhances tolerance to copper stress in Arabidopsis. International Journal of Molecular Sciences 18(2):209 DOI 10.3390/ijms18020209.

Lu S, Li Q, Wei H, Chang M-J, Tunlaya-Anukit S, Kim H, Liu J, Song J, Sun Y-H, Yuan L, Yeh T-F, Peszlen I, Ralph J, Sederoff RR, Chiang VL. 2013. Ptr-miR397a is a negative regulator of laccase genes affecting lignin content in Populus trichocarpa. Proceedings of the National Academy of Sciences of the United States of America 110(26):10848–10853 DOI 10.1073/pnas.1308936110.

Mattinen M-L, Kruus K, Buchert J, Nielsen JH, Andersen HJ, Steffensen CL. 2005. Laccase-catalyzed polymerization of tyrosine-containing peptides. FEBS Journal 272(14):3640–3650 DOI 10.1111/j.1742-4658.2005.04786.x.

Mayer AM, Staples RC. 2002. Laccase: new functions for an old enzyme. Phytochemistry 60(6):551–565 DOI 10.1016/S0031-9422(02)00171-1.

McCaig BC, Meagher RB, Dean JF. 2005. Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in Arabidopsis thaliana. Planta 221(5):619–636 DOI 10.1007/s00425-004-1472-6.

Meyers BC, Axtell MJ, Bartel B, Bartel DP, Baulcombe D, Bowman JL, Cao X, Carrington JC, Chen X, Green PJ, Griffiths-Jones S, Jacobsen SE, Mallory AC, Martienssen RA, Poethig RS, Qi Y, Vaucheret H, Voinnet O, Watanabe Y, Weigel D, Zhu J-K. 2008. Criteria for annotation of plant microRNAs. Plant Cell 20(12):3186–3190 DOI 10.1105/tpc.108.064311.

Pourcel L, Routaboul J-M, Kerhoas I, Caboche M, Lepiniec Lïc, Debeaujon I. 2005. TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in Arabidopsis seed coat. Plant Cell 17(11):2966–2980 DOI 10.1105/tpc.105.035154.

Richardson A, McDougall GJ. 1997. A laccase-type polyphenol oxidase from lignifying xylem of tobacco. Phytochemistry 44(2):229–235 DOI 10.1016/S0031-9422(96)00489-X.

Schuetz M, Benske A, Smith RA, Watanabe Y, Tobimatsu Y, Ralph J, Demura T, Ellis B, Samuels AL. 2014. Laccases direct lignification in the discrete secondary cell wall domains of protoxylem. Plant Physiology 166(2):798–807 DOI 10.1104/pp.114.245597.

Song JY, Luo HM, Li CM, Sun C, Xu J, Chen SL. 2013. Salvia miltiorrhiza as medicinal model plant. Yao XueXueBao 48:1099–1106.

Sterjiades R, Dean JFD, Eriksson K-EL. 1992. Laccase from sycamore maple (Acer pseudoplatanus) polymerizes monolignols. Plant Physiology 99(3):1162–1168 DOI 10.1104/pp.99.3.1162.
Turlapati PV, Kim KW, Davin LB, Lewis NG. 2011. The laccase multigene family in Arabidopsis thaliana: towards addressing the mystery of their gene function(s). Planta 233(3):439–470 DOI 10.1007/s00425-010-1298-3.

Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes & Development 20(7):759–771 DOI 10.1101/gad.1410506.

Wang C-Y, Zhang S, Yu Y, Luo Y-C, Liu Q, Ju C, Zhang Y-C, Qu L-H, Lucas WJ, Wang X, Chen Y-Q. 2014. MiR397b regulates both lignin content and seed number in Arabidopsis via modulating a laccase involved in lignin biosynthesis. Plant Biotechnology Journal 12(8):1132–1142 DOI 10.1111/pbi.12222.

Willems E, Leyns L, Vandesompele J. 2008. Standardization of real-time PCR gene expression data from independent biological replicates. Analytical Biochemistry 379(1):127–129 DOI 10.1016/j.ab.2008.04.036.

Xu X, Jiang Q, Ma X, Ying Q, Shen B, Qian Y, Song H, Wang H. 2014. Deep sequencing identifies tissue-specific microRNAs and their target genes involving in the biosynthesis of tanshinones in salvia miltiorrhiza. PLOS ONE 9(11):e111679 DOI 10.1371/journal.pone.0111679.

Xu H, Song J, Luo H, Zhang Y, Li Q, Zhu Y, Xu J, Li Y, Song C, Wang B, Sun W, Shen G, Zhang X, Qian J, Ji A, Xu Z, Luo X, He L, Li C, Sun C, Yan H, Cui G, Li X, Li X’en, Wei J, Liu J, Wang Y, Hayward A, Nelson D, Ning Z, Peters R J, Qi X, Chen S. 2016. Analysis of the genome sequence of the medicinal plant Salvia miltiorrhiza. Molecular Plant 9(6):949–952 DOI 10.1016/j.molp.2016.03.010.

Xu X, Zhou Y, Wang B, Ding L, Wang Y, Luo L, Zhang Y, Kong W. 2019. Genome-wide identification and characterization of laccase gene family in Citrus sinensis. Gene 689:114–123 DOI 10.1016/j.gene.2018.12.015.

Xue C, Yao J-L, Qin M-F, Zhang M-Y, Allan AC, Wang D-F, Wu J. 2019. PbrmiR397a regulates lignification during stone cell development in pear fruit. Plant Biotechnology Journal 17(1):103–117 DOI 10.1111/pbi.12950.

Zhang G, Tian Y, Zhang J, Shu L, Yang S, Wang W, Sheng J, Dong Y, Chen W. 2015. Hybrid de novo genome assembly of the Chinese herbal plant danshen (Salvia miltiorrhiza Bunge). GigaScience 4(1):62 DOI 10.1186/s13742-015-0104-3.

Zhang Y-C, Yu Y, Wang C-Y, Li Z-Y, Liu Q, Xu J, Liao J-Y, Wang X-J, Qu L-H, Chen F, Xin P, Yan C, Chu J, Li H-Q, Chen Y-Q. 2013. Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. Nature Biotechnology 31(9):848–852 DOI 10.1038/nbt.2646.

Zhao Q, Nakashima J, Chen F, Yin Y, Fu C, Yun J, Shao H, Wang X, Wang Z-Y, Dixon RA. 2013. LACCASE is necessary and nonredundant with Peroxidase for lignin polymerization during vascular development in Arabidopsis. Plant Cell 25(10):3976–3987 DOI 10.1105/tpc.113.117770.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Research 31(13):3406–3415 DOI 10.1093/nar/gkg595.