

New Phytologist Supporting Information

Article title: **Localised laccase activity modulates distributions of lignin polymers in gymnosperm compression wood**

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The following Supporting Information is available for this article:

Fig. S1 Additional wood sections labeled with DMAC-SA.

Fig. S2 Additional wood sections labeled with NBD-CA.

Fig. S3 Additional wood sections labeled sequentially with NBD-CA and DMAC-PA.

Fig. S4 Additional wood sections labeled with DMAC-SA.

Fig. S5 Multiple alignment of laccase proteins.

Fig. S6 Immunoblotting with anti-CoLac1 and anti-CoLac3 antibodies.

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Table S1 Primers used in this study.

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Methods S1 Additional experimental procedures.

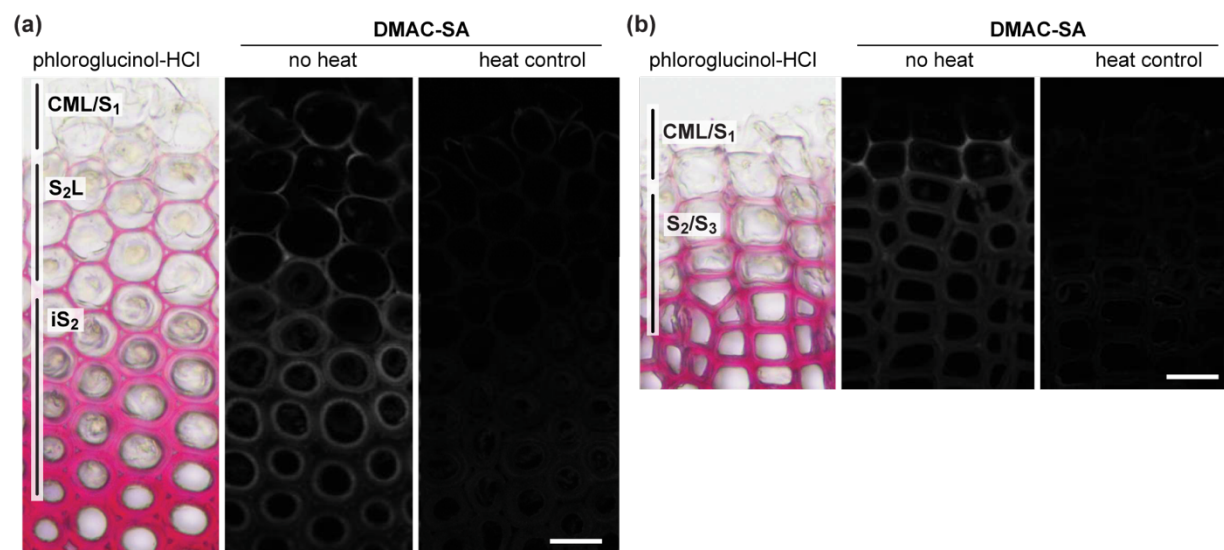


Fig. S1 Laccase-dependent incorporation of DMAC-SA into differentiating compression and normal wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood transverse sections were labeled with DMAC-SA in the presence of catalase, and then visualised as DMAC fluorescence with a confocal microscope. Sections imaged by phloroglucinol-HCl lignin stain, and background control sections labeled after heat treatment (85°C, 1 h) are also shown. Regions where the incorporation of fluorescence-tagged monolignols was detected in different cell wall compartments are indicated on the sections stained with phloroglucinol-HCl. Scale bars denote 20 μ m.

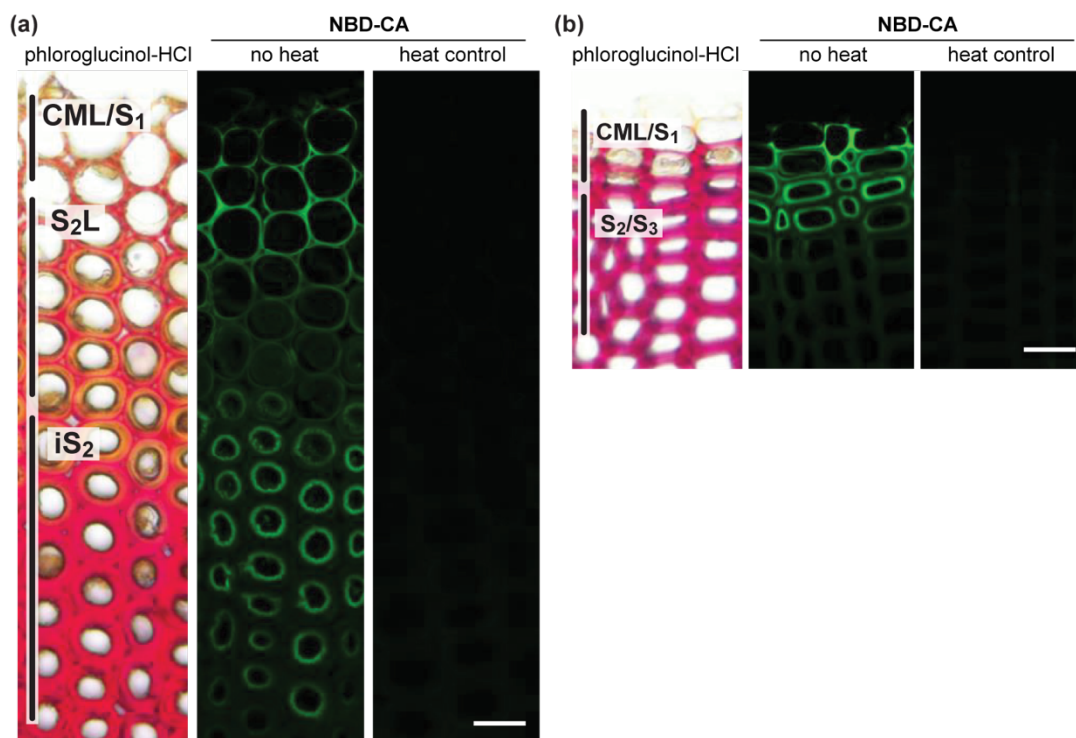


Fig. S2 Laccase-dependent incorporation of NBD-CA into differentiating compression wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood transverse sections were labeled with NBD-CA in the presence of catalase, and then visualised as NBD fluorescence with a confocal microscope. Sections imaged by phloroglucinol-HCl lignin stain, and background control sections labeled after heat treatment (85°C, 1 h) are also shown. Scale bars denote 20 µm.

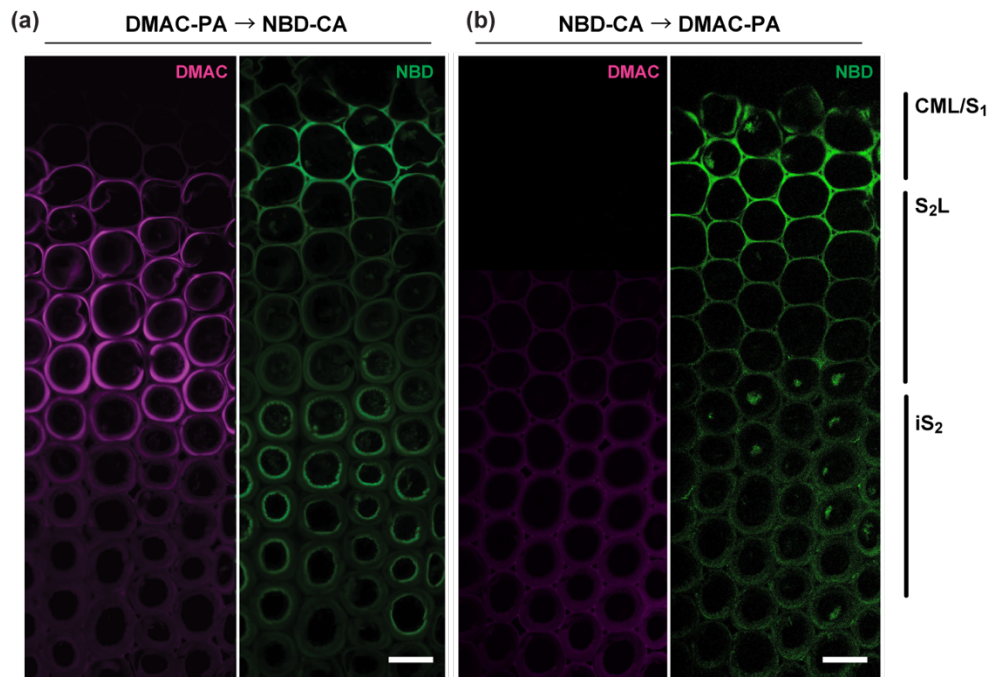


Fig. S3 Laccase-dependent incorporations of DMAC-PA and NBD-CA into differentiating compression wood tracheids of *C. obtusa* seedlings. Sections were incubated sequentially with DMAC-PA and then NBD-CA (DMAC-PA → NBD-CA; a) or in reverse order (NBD-CA → DMAC-PA; b), and then visualised as DNAC (magenta) and NBD (green) fluorescence. The preferential incorporation of DMAC-PA into S₂L was observed in the sections labeled with DMAC-PA → NBD-CA but not with those labeled with NBD-CA → DMAC-PA. Scale bars denote 20 μ m.

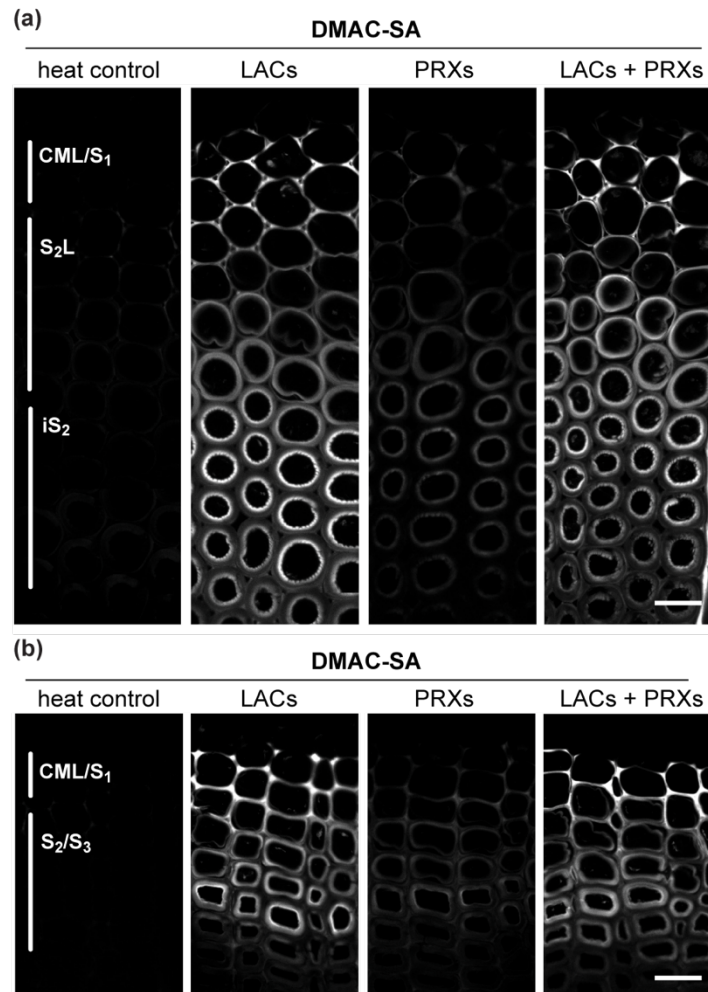


Fig. S4 Laccase- and peroxidase-dependent incorporations of DMAC-SA into differentiating compression and normal wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood sections were labelled with DMAC-SA in the presence of catalase for detection of laccase/O₂ (LACs), glucose and glucose oxidase for peroxidase/H₂O₂ (PRXs), and with H₂O₂ for both laccase/O₂ and peroxidase/H₂O₂ (LACs + PRXs) oxidation activities. Regions where the incorporation of fluorescence-tagged monolignols was detected are indicated on the heat control sections. Scale bars denote 20 μm.

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CoLac1 1 MILAMQTFYGRVLVPLLALWFTVQFVSAKHTVITRRYKFNVOQNTNVTRLCTKALLTVNG
CoLac2 1 -----MLCFVMIV----ILSVTVEAKTRHYNFITRLKKNVTRRLCHTKKPIVTVNG
CoLac3 1 ---MARSVDVKISHKLLIEILGLLILAQGVAAVTRTSEFNVRLLKNVTRLCKTKPLITVNG
AtLac4 1 -----MGSHMWVFLFLVSFFSVFPAPSESMVRHYKFNVMKKNVTRLCSKPTVTVNG
AtLac11 1 -----MKMGFLFLFCYLLAFITGYSFPVDAAVKKVQFDVQVKNISRIICNAKPIVTVNG
AtLac17 1 ---MALQ-----LLLAVFSCVLLLPQAFGITRHYVLEIKMQNVTRLCITKSLVSVNG
ZmLac3 1 -----SRNTHVDEIVITETKVTRLCHBKETILAVNG

CoLac1 61 QYGGPIIVAREGDRVVVNVVNNVQNNVSIHWHGTRQLRS GWADGPAYITQCP IOTGQSYV
CoLac2 45 KYPGPTIYAREGDTVLVNVVNHVKYNNVSIHWHGTRQLRS GWADGPAYITQCP IOTRQSFV
CoLac3 59 KSPGPKIYAREGDNVVVKVNHVKDNVSIHWHGTRQLRS GWADGPAYITQCP IOTGMYV
AtLac4 53 RYPGPTIYAREDDTLIKVNVNHVKYNNVSIHWHGTRQLRS GWADGPAYITQCP IOTGQVYT
AtLac11 52 MFPGPTIYAREGDRVLIINVTNHVQYNNVSIHWHGTRQLRS GWADGPAYITQCP IOTGQSYL
AtLac17 51 QFPGPKLIAREGDVLIKVVNVQVNNVSIHWHGTRQLRS GWADGPAYITQCP IOTGQSYV
ZmLac3 30 QFPGPTIYARKDDVVIVNVVNVQYKNNVSIHWHGTRQLRS GWADGPAYITQCP IOTGQANFT

CoLac1 121 YKFIIKRORGLTWLWHAHISWLRASLHGPIIIVPKKNASVYPPRPHQEVPIVFGEWGNADT
CoLac2 105 YNFTITGQRTLLWHAHISWLRATLHGPIIVILPKKHVPYPPKPKHKEVTLVLGEWNNADT
CoLac3 119 YNFTITGQRTLLWHAHISWIRASVYGAFTIIVPKLHVYPPKPKHKEATILGEEWNTDT
AtLac4 112 YNFTITGQRTLLWHAHISWLRATVYGAIVILPKRGPVYPPKPKDNKVIILGEEWNSDT
AtLac11 113 YNFTITGQRTLLWHAHISWLRATVYGAIVILPAPGKPYPPKPKSNIIILGEEWNKDV
AtLac17 111 YNFTITGQRTLLWHAHISWLRSTVYGPLIILPKRGPVYPPKPKHKEVPMIFGEWNNADT
ZmLac3 90 YKIIIFTEEGTLWHAHSEFDRAIVHGAIVIHPRKRGTVYPPKPKHKEPMIILGEEWNNADV

CoLac1 181 ETVINQALQSGAQNVSDAYTMMNGLPGLMYNCSANDTFRLLKVIIPGKTYLLRLVNAALNSD
CoLac2 165 EAVINQAMKTCGAPNASNAHTINGKPEPLFNCSTKDTTHALAVEPDKTYLLRLVNAALNDQ
CoLac3 179 EKVISQALQGGGPNVSDCYSLNGHPGLYNCSANDYTLKVIIPGKTYLLRLVNAALNDE
AtLac4 173 ENIINQALKSGLAPNVSDSHMINGHPGVRNCP SQG-YKLSVENGKTYLLRLVNAALNEE
AtLac11 172 ETAVNQAQNLGAPPFMSDAHTINGKPEPLFPCSEKHTFVIEAEAGKTYLLRLVNAALNDE
AtLac17 171 EAIIRQATQGGGPNVSDAYTINCLPGLYNCSAKDTFRLLKVIIPGKTYLLRLVNAALNDE
ZmLac3 150 EQLLESQRTEGGDVNHS DANTINGQPGDFAPCKEDTFKMSVEHGHKTYLLRLVINAGLTNE

CoLac1 241 LFFGTANHTLTVVEADAVYVKPFOTNIILITPGOTTNVLFTAMSQ---PPNATFLILLAGP
CoLac2 225 VFFSIGHNHLKVVVEADAVYTKPFETKATLISP GOTTNVLDDTSKK---TGRYFMTVRA
CoLac3 239 MFFSITANHSVTVVEADAVYTKPFETDIFLITPGOTTNVLHTHPNTSLHHHKNFATAARF
AtLac4 232 LFFKTAGHIFTVVEADAVYVKPFOTDITLAPGOTTNVLTTASKS----AGKYLVTASP
AtLac11 232 LFFGTAGHMTVVEADAVYTKPFETKATL LGGOTTNVLVKTDRS----PNRYFMAASP
AtLac17 231 LFFSITANHTVTVVEADAVYVKPFETETLITAPGOTTNVLTKTSS----YPSAFFMTARP
ZmLac3 210 MFFAVAGHRLTVVGTDRYLRPFTVDYILISP GOTTNMLLEANCATDGSANSRNVYMAARP

CoLac1 298 FATGTAA-FDNSTASAGVLEVVANG-----TPVNTSSLPMMRITLPAVNDTCFRSOFSSQK
CoLac2 280 FMDAIP-VDNLTSAAGILQVIGTS-----NTSNPIMVPIPKINDTAFYTNSSSS
CoLac3 299 YATGGQT-FDNTTIAAGILVYSSKKK-----HFNFTHFKNSTLEKLPFNDTSFATNYTLK
AtLac4 287 FMDAIP-VDNVTATATVHYSGLT-----SSPTIITLPPFONATSANNVTFI
AtLac11 287 FMDAPVS-VDNKTVTAILQYKGV-----NTVLPITLKLPLPNDTSFALDYNKGK
AtLac17 288 YVTGGQT-FDNSTVAGILEVEPPKQTKGAHSRTSIKNLQLFKEIILPALNDTNEATKESNK
ZmLac3 270 FFDNTAVNVDDKNTAIVEMTDAP-----PSASAGPPDSIDLPAMDIDIAAATAATTAQ

CoLac1 351 MRSIIGNPFPNAPASAFQKVDKQFLFTVGLGLNFCFQ--GQTCQGN--TKFRASINNISFI
CoLac2 328 LKSLNSALYPAK-VPOVDNRNLFAMGLALDKCST---CVNG---SRASASINNVSVFV
CoLac3 352 LKSLANAQYPA-VPKTVDKRFFTVGLGONPCPK--GFKCGAPNN--TKFTASINNVSVFV
AtLac4 335 LRSLSKKYPAL-VPTTIDHHLFFTVGLGLNACPT---CKRANG--SRVVASINNVTFI
AtLac11 335 LKSLNTPNFPAL-VPLKVDRLFTYIGLGINACPT---CVNG---TNLAASINNVTFI
AtLac17 347 LRSLSKNFPAN-VPLNVDKRFFTVGLGTNPNHKNQTCQGTNTMFAASISNISFT
ZmLac3 322 LRSLVTKHEPID-VPEMVEDEHMLVTVISVNTIPCEP--NKTCAFGN--NRLAASLNNVSVFM

CoLac1 408 QPTTALLOAYYFNOSNGVYNTTFPDNPPFFNYTG-TFPN-NTRTLNDRVELLPFNTTV
CoLac2 379 MPTIALLQAVVNNIS-GVFTKDFPDNPPFFNYTG-TFPK-NLFTSKGTRATSLTYNSTV
CoLac3 408 MPTVALLQSHYSGMKKGVYKTNFNDNPPFFNYTG-TFPN-NTSPMNGTRAKVLKFNSTV
AtLac4 388 MPKTALLPAHYFNIS-GVFTTDFPKNPPHVFNYSG-GSVT-NMATEGTRLYKLPYNATV
AtLac11 386 MPKTALLKAHYSNIS-GVFTTDFDRPPKAFNYTG-VPLTANLGTSTGTRLSRVKFNSTI
AtLac17 406 MPTKALLOSISYSGSHGVYSPKFPWSPVFPNYTG-TFPN-NTMVSNGTNLMVLPNTSV
ZmLac3 378 NPTIDILDAYVDSIS-GVYEPDFENKPFVFNFTAPNPPQDLWFTKRGTKVNVVEVGTIL

CoLac1 466 QLVLDQTSIVTFESHPLHLHGFFNFIVGQGMGNVNASDPANFNLVDPERNTVGVPSGG
CoLac2 436 QLVLDQTSILTVDNHPVHLHGFFNFIVGQGFNGVNSKDDPANFNLVDPAERNVGVPTGG
CoLac3 466 QLVLDQTSIAGIESHPLHLHGFFNFIVGQGFNGVNTKDKSPKFNLVDPVGVNNGG
AtLac4 445 QLVLDQTVIAPENHPVHLHGFFNFIVGQGLGNFMSTKDKPNFNLVDPVERNTHGVPSGG
AtLac11 444 ELVLDQTNLTVESHPLHLGYNFFVVGQGVNFDPKDPAFNLVDPERNTVGVPTGG
AtLac17 464 ELVLDQTSILGAESHPLHLHGFFNFVVGQGFNGVDPNDPRNFNLVDPVGVNNGG
ZmLac3 437 EVVFDQATILGAESHPLHLHGFFNFVVGQGFNGVDPNDPRNFNLVDPVGVNNGG

CoLac1 526 WAALRFLADNPGVFMHCHLEPHTSWGLKMAVVLNNGGRSOSLPLPPKDLPPC
CoLac2 496 WTAIRFRADNPGVFMHCHLEVHTTWGLKMAFVVENGG-AEQSVLPPPPDLPPC
CoLac3 526 WVAIRFRADNPGVFMHCHLEVHTSWGLKMAVIVKNGKG-PLQSLPPPPADLPPC
AtLac4 505 WVVI RFRADNPGVFMHCHLEVHTTWGLKMAFVVENGG-PNQSILPPPKDLPPC
AtLac11 504 WAAIRFRADNPGVFMHCHLEVHTTWGLKMAFVVENGGT-PELSVLPKPPKDLPPC
AtLac17 524 WAAIRFRADNPGVFMHCHLEVHTTWGLRMAVVLVDGDK-PDQKLLPPPADLPPC
ZmLac3 497 WAAIRFRADNPGVFMHCHFDHRHTVWGMVTVIVKNGKG-PDAQMMRPPPNMPPC

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Fig. S5 Multiple alignment of laccase proteins. Amino acid sequences of the three *C. obtusa* laccases tested in this study (CoLac1, CoLac2 and CoLac3) were aligned with Arabidopsis and maize laccases. The copper-binding domain motifs (pink line), substrate-binding loops (blue box), and the residues that may interact with monoglignol substrates (orange stars) (Xie *et al.*, 2020) are highlighted.

Reference
Xie, T., Liu, Z. and Wang, G. 2020. Structural basis for monoglignol oxidation by a maize laccase. *Nat. Plants*, 6: 231-237.

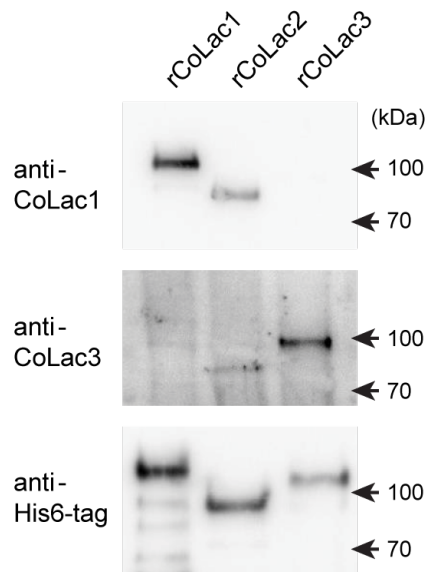


Fig. S6 Selectivity of anti-CoLac1 and anti-CoLac3 antibodies examined by immunoblotting against recombinant laccase proteins. Immunoblotting was performed with anti-CoLac1, anti-CoLac3 and anti-His6-tag antibodies against recombinant CoLac1, CoLac2 and CoLac3 proteins (rCoLac1, rCoLac2 and rCoLac3, respectively). Although there is no detectable cross-binding of anti-CoLac1 and anti-CoLac3 against rCoLac3 and rCoLac1, respectively, a weak binding of anti-CoLac1 against rCoLac2 was detected. Given the negligible *CoLac2* transcript detected in the tested *C. obtusa* wood tissues (Fig. 6b), however, it should not have affected our observations regarding the immunolocalisations of CoLac1 and CoLac3 as described in the main text (Fig. 6c).

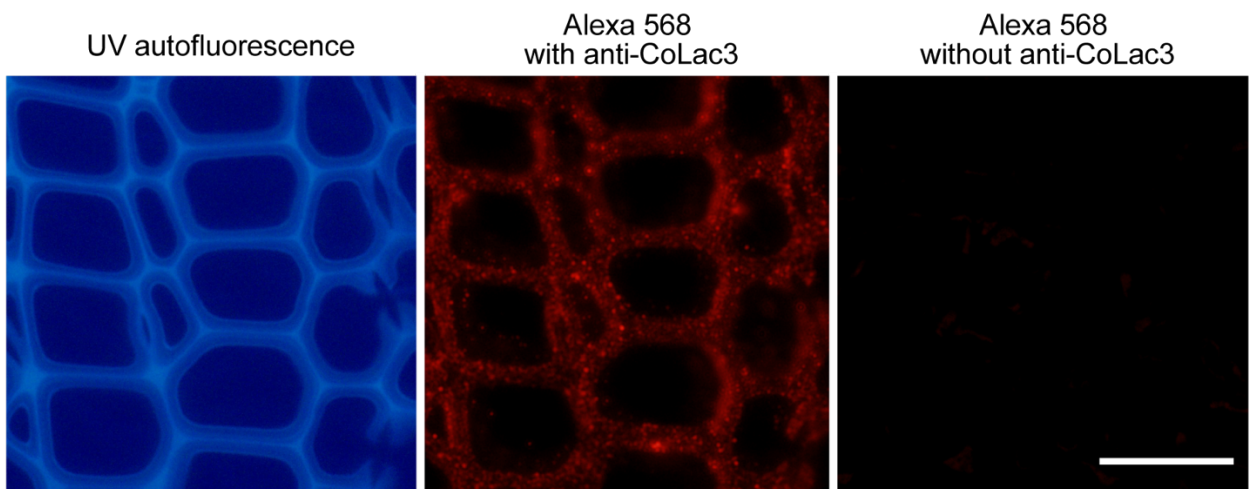


Fig. S7 Immunolocalisation of CoLac3 in cell walls of differentiating normal wood of *C. obtusa* seedlings. Sections were visualised by autofluorescence from deposited lignin (left) and Alexa 568 secondary antibody fluorescence from CoLac3 epitope (center). Control sections visualised without antibody treatment are also shown (right). Scale bars denote 20 μm .

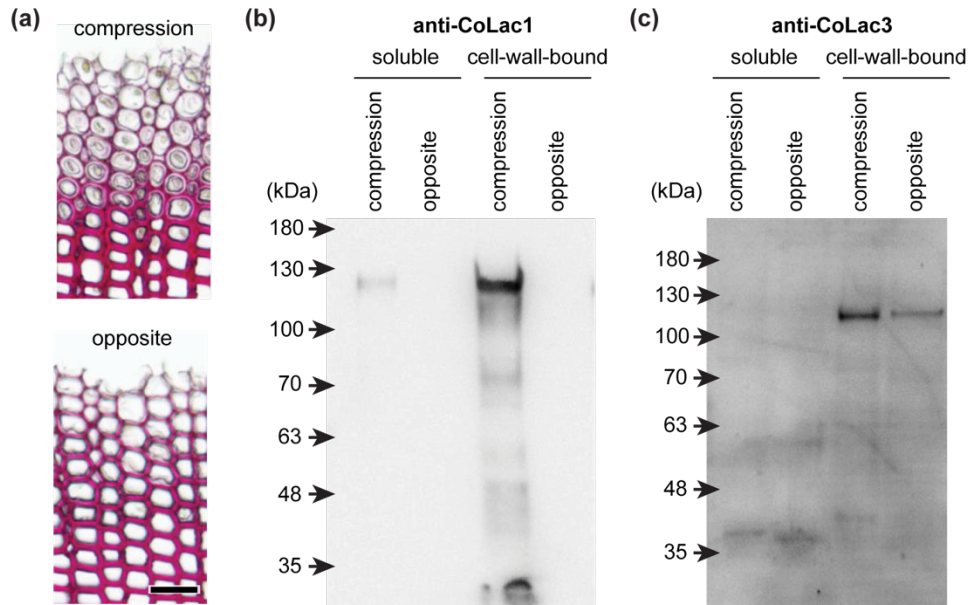


Fig. S8 Immunodetection of CoLac1 and CoLac3 in crude enzyme preparations prepared from differentiating compression and normal-wood-forming opposite tissues of a bent mature *C. obtusa* tree. Soluble and cell-wall-bound protein fractions were extracted from differentiating compression wood tissues and normal-wood-forming opposite wood tissues as a control. Transverse sections of the compression wood and opposite wood control tissues were stained by phloroglucinol-HCl and shown in (a) (scale bar denotes 50 μ m). CoLac1 was majorly detected in cell-wall-bound protein fraction (and also slightly in soluble protein fraction) from compression wood, but not in fractions from opposite wood (b). On the other hand, CoLac3 was detected in cell-wall-bound protein fractions from both compression and opposite wood (c).

Table S1 Primers used in this study.

Primer name	Purpose	Sequence (5' → 3')
Nde I: CoLac1 ORF-F	Vector construction	ctgttgata_catatg_ATTCTCGCAATGCAGACATT
Sac I: hexa histidine: CoLac1 ORF-R	Vector construction	actgagctc_TCA_gtggtgatggtgatgatg_ACAGGGGGGAAGATCTTTGG
Nde I: CoLac2 ORF-F	Vector construction	ctgttgata_catatg_GCAGTACTCTCAAACACTATGATGC
Sac I: hexa histidine: CoLac2 ORF-R	Vector construction	attgagctc_TTA_gtggtgatggtgatgatg_GCATGCTGGAAGGTCCGGT
Nde I: CoLac3 ORF-F	Vector construction	ctgttgata_catatg_GCCAGAAGTGTGGATGTTAAG
Sac I: hexa histidine: CoLac3 ORF-R	Vector construction	attgagctc_TTA_gtggtgatggtgatgatg_GCAGGGAGGAAGATCAGCAG
CoLac1 mRNA-F	Vector construction	AGCTGAAGCTGAGATCAGATTGTTTCT
CoLac1 mRNA-R	Vector construction	CTGGCACAAAATTGGAAACAGTGA
CoLac2 mRNA-F	Vector construction	ATGTTGGCTTCTGGTTGTATGTTTG
CoLac2 mRNA-R	Vector construction	AACTCTGAGTGCTTCAACCATGGAG
CoLac3 mRNA-F	Vector construction	CACACCCATCTCTGAACTCATTCA
CoLac3 mRNA-R	Vector construction	GCATCCACCCCACTTATTAGAAAA
CoLac3 5' RACE-R	5' RACE	CCTGGTGTGATGAGAAAGATGTCTG
Nested CoLac3 5' RACE-R	5' RACE	GTAGCAATCTGAGACGTTGGTCCT
CoLac3 3' RACE-F	3' RACE	GCTCGTGAAGGAGATAATGTTGTGG
CoLac1 qPCR-F	qRT-PCR	ATCCTTCATTCAACCCACCA
CoLac1 qPCR-R	qRT-PCR	AGGGTCCGTGTGTTGTTAGG
CoLac2 qPCR-F	qRT-PCR	TCCAATCCCATAATGCCTGT
CoLac2 qPCR-R	qRT-PCR	CCCATTGCGAAGAACAGATT
CoLac3 qPCR-F	qRT-PCR	GCCAAAACCTCCAGCATTTA
CoLac3 qPCR-R	qRT-PCR	TACTGTCTTGGGCACTGCTG

Table S2 Chemical analysis data of *C. obtusa* cell wall samples.

	compression wood	normal wood
lignin content by thioglycolic acid assay		
lignin content (mg/g CWR)	283.3 ± 4.6*	272.7 ± 3.0
lignin composition by thioacidolysis		
total monomer yield (μmol/g CWR)	1126.8 ± 36.5**	1371.8 ± 37.2
H monomers (mol%)	15.4 ± 0.2**	< 0.1
G monomers (mol%)	84.6 ± 0.2**	99.9 ± 0.01
S monomers (mol%)	n.d.	n.d.
H/G monomer ratio (mol/mol)	0.18 ± 0.01**	< 0.01
polysaccharide composition		
arabinan (mg/g CWR)	9.0 ± 0.6**	11.6 ± 0.5
xylan (mg/g CWR)	46.3 ± 2.2	45.3 ± 3.6
mannan (mg/g CWR)	32.3 ± 1.0**	80.0 ± 8.6
galactan (mg/g CWR)	103.7 ± 11.5**	19.0 ± 2.2
amorphous glucan (mg/g CWR)	32.7 ± 1.6*	40.5 ± 1.9
crystalline glucan (mg/g CWR)	283.3 ± 4.6**	424.2 ± 7.5

Values refer to means ± standard deviations from three independent assays. Bold letter and asterisks indicate significant difference from normal wood control (Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$). CWR, cell wall residues; H, *p*-hydroxyphenyl; G, guaiacyl; S, syringyl; n.d., not detected.

Methods S1 Additional experimental procedures.

Histochemical staining

Phloroglucinol-HCl staining of wood stem sections was performed as described previously (Kim *et al.*, 2010). 3,3'-Diaminobenzidine (DAB) staining (Ranocha *et al.*, 1999; Hiraide *et al.*, 2016) was performed by incubating stem sections under the same conditions in the Materials and Methods for section labeling with fluorescence-tagged monolignols, except that the monolignol probe solution was replaced by 0.5 mM DAB in 20 mM Na-acetate buffer (pH 5.0).

Chemical analysis and nuclear magnetic resonance (NMR)

Compression and normal wood samples collected from ~3-year-old *C. obtusa* seedlings were pre-ground using a TissueLyser (Qiagen, Hilden, Germany), and then extracted with distilled water, 80% ethanol, and acetone to produce cell wall residue (CWR) samples (Wagner *et al.*, 2011). Thioglycolic assay (Suzuki *et al.*, 2009), analytical thioacidolysis (Yamamura *et al.*, 2011; Lam *et al.*, 2017) and sugar analysis (Lam *et al.*, 2017) for determination of lignin and polysaccharide composition of the CWR samples were conducted as described previously. For 2D NMR analysis, the CWRs (~150 mg) were further ball-milled and homogeneously dispersed in dimethylsulfoxide-*d*₆/pyridine-*d*₅ (4:1, v/v) NMR solvent (Kim and Ralph, 2010). The NMR analysis was performed with a Bruker Biospin Avance III 800US system (800 MHz; Bruker Biospin) equipped with a cryogenically cooled 5-mm TCI gradient probe. Adiabatic ¹H–¹³C heteronuclear single-quantum correlation (HSQC) NMR experiments were conducted using standard Bruker implementation (“hsqcetgpsp.3”) with acquisition parameters as described previously (Kim and Ralph, 2010; Tarmadi *et al.*, 2018). Data processing and analysis were conducted as described previously (Kim and Ralph, 2010; Tarmadi *et al.*, 2018) using Bruker TopSpin 4.0 (Mac) software (Bruker Biospin). Peak assignments were based on comparison with data from the literature (Kim and Ralph, 2010; Brennan *et al.*, 2012; Tarmadi *et al.*, 2018). For volume integration of lignin aromatic signals (Fig. 1c), C2–H2 correlations from G and C2–H2/C6–H6 correlations from H were used, and H signals were logically halved.

Protein sequence and gene expression analyses

Coding sequences of *CoLac1* and *CoLac2* were previously determined (Hiraide *et al.*, 2016). The full-length coding sequence of *CoLac3* was obtained by searching for laccase sequences against the *C. obtusa* RNA-Seq contig sequence dataset (DRA001036) reported by Sato *et al.* (2014) and a rapid amplification of cDNA ends (RACE)-PCR (Hiraide *et al.*, 2014) approach using primers listed in Table S2. Laccase protein sequences were aligned using ClustalW (Larkin *et al.*, 2007) and visualised with ExPASy BoxShade software (Artimo *et al.*, 2012). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and visualised with MEGAX (Kumar *et al.*, 2018). Quantitative real-time PCR (qRT-PCR) was performed on a Thermal Cycler Dice Real Time System II (TaKaRa) with cDNA samples from total RNAs extracted from differentiating tracheids of *C. obtusa* compression and normal wood (Hiraide *et al.*, 2014) and using gene-specific primers listed in Table S2. To determine the absolute transcript copy numbers, linearised plasmids harboring partial cDNA inserts of *CoLac1* and *CoLac2* (pGEM-T Easy, Promega; linearised with *SacII*) (Hiraide *et al.*, 2014), and a full-length cDNA of *CoLac3* (PRI 201-AN, TaKaRa Bio; linearised with *SacI*) were used to construct standard curves. The normalisation factor (NF) values for calibration of transcript abundance (Vandesompele *et al.*, 2002) were determined as described previously (Hiraide *et al.*, 2014).

Immunolocalisation

Anti-CoLac1 rabbit immunoglobulin G (IgG) antibody was obtained as described previously (Hiraide *et al.*, 2016). Similarly, anti-CoLac3 rabbit IgG was raised from a CoLac3-specific peptide (KGPLQSLPPPPAD). The specificities of anti-CoLac1 and anti-CoLac3 antibodies were confirmed by western-blot hybridisation with rCoLac1-3 (Fig. S6). For immunolocalisation, stem block samples from *C. obtusa* seedlings were fixed, embedded in LR White resin (Hiraide *et al.*, 2016), and sliced into 0.5- μ m-thick sections using a UltracutE ultramicrotome (Reichert-Jung). For immunolocalisation, the sections were first immersed in a blocking solution composed of 1% (w/v) bovine serum albumen (BSA) in Tris-buffered saline (pH 7.6; TBS) containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h, and then applied with anti-CoLac1 antibody diluted 20-fold, or anti-CoLac3 antibody diluted 4-fold in the blocking buffer for 2 days at 4°C. After rinsing three times for 15 min in TBS-T, the sections were treated with Alexa

Fluor 568 goat anti-rabbit IgG (H+L) antibody (Invitrogen) diluted 100-fold for immunolocalisation of CoLac1, or diluted 200-fold for immunolocalisation of CoLac3 in the blocking buffer for 3 h at 35°C. The sections were washed three times for 15 min in TBS-T, mounted in ProLong Diamond (Invitrogen), and then visualised using an Olympus BX50-54 fluorescence microscope with U-MWIG (Olympus) and U-MWU2 (Olympus) fluorescence cubes for Alexa568 fluorescence and lignin autofluorescence, respectively.

Expression of recombinant laccases in tobacco BY-2 cells

Recombinant laccases with His6-tags at their C-termini were expressed in tobacco BY-2 cells under the control of the *Cauliflower mosaic virus* 35S promoter (Sato and Whetten, 2006). The full-length coding sequences of *CoLac1*, *CoLac2*, and *CoLac3* were PCR amplified from a *C. obtusa* cDNA library (Hiraide *et al.*, 2014) using primers listed in Table S2. The PCR products were ligated with the PRI 201-AN-GUS vector (TaKaRa) by digestion with *Xba*I and *Sac*I to replace the β -glucuronidase (*GUS*) coding sequence with the target laccase sequences. The obtained binary vectors were used to transform tobacco BY-2 cells via *Agrobacterium tumefaciens* strain GV3101:pMP90 (Sato and Whetten, 2006). Transformed cells were screened on modified Linsmaier and Skoog (LS) medium (Nagata *et al.*, 1992) solidified with 0.4% (w/v) gellan gum and supplemented with 200 μ g mL⁻¹ kanamycin and 500 μ g mL⁻¹ carbenicillin. Transformed cells were then further cultured in the modified LS medium further supplemented with 3 μ M CuSO₄ to promote laccase expression (Sato and Whetten, 2006). After culturing for one week, cells were harvested, frozen in liquid nitrogen, and stored at -30°C until protein extraction. Collected cell samples were finely ground in liquid nitrogen, extracted with 1 mM EDTA and 0.2 mM PMSF in 50 mM Na-acetate (pH 5.0) on ice for 1 h, and then centrifuged (1000 \times g for 5 min). The supernatant was used as the soluble protein fraction. The remaining cell pellets were further washed with 1% (v/v) Triton X-100 in 20 mM Na-acetate buffer (pH 5.0), and then extracted with 1.5 M NaCl and 0.1 mM CuSO₄ in 20 mM Na-acetate buffer (pH 5.0) to obtain the cell-wall-bound protein fraction used for enzyme assays. The obtained protein extracts were concentrated by ammonium sulfate precipitation (80% saturation) and desalted by dialysis. Protein concentrations were determined by a standard Bradford method.

Preparation of crude protein extracts

Wood tissues from compression and opposite sides of *C. obtusa* stem samples were finely ground in liquid nitrogen, and then extracted sequentially with the extraction buffers to prepare the soluble and cell-wall-bound protein fractions, as described above, for preparation of the recombinant laccase protein extracts.

Western blotting

Proteins were separated by SDS-PAGE (7.5%) and transferred to a polyvinylidene difluoride membrane. The membranes were immersed in TBS-T with 2% (w/v) ECL Prime Blocking Reagent (GE Healthcare) for 1 h at room temperature to block nonspecific antibody binding. The membranes were incubated overnight with anti-His-tag antibody (clone: OGHis, MBL) diluted 5,000-fold and anti-CoLac1 antibody diluted 3,000-fold in 1% (w/v) BSA/TBS-T for hybridisation. For hybridisation with anti-CoLac3 antibody, the antibody was diluted 100-fold in the same buffer, and incubation time was extended to 36 h. After rinsing three times for 15 min in TBS-T, the membranes were immersed in 20,000-fold-diluted anti-mouse IgG, HRP-linked whole Ab sheep, or 5,000-fold-diluted anti-rabbit IgG, HRP-linked whole Ab goat (GE Healthcare) in 1% (w/v) BSA/TBS-T for 3 h at room temperature. The membranes were washed three times for 15 min in high-salt-concentration-TBS-T (containing 0.5 M NaCl and 0.02% [w/v] sodium dodecyl sulfate in TBS-T) and imaged. Chemiluminescence was developed using ECL Prime Western Blot Detection Reagent (GE Healthcare) and detected by a Fujifilm LAS 1000 image analyser.

Monolignol oxidation assay

For the enzyme assay, cell-wall-bound protein fractions prepared from the laccase protein preparations ($100 \mu\text{g mL}^{-1}$ protein) or those from *C. obtusa* wood tissues ($10 \mu\text{g mL}^{-1}$ protein) were incubated at 27°C with 20 mM Na-acetate buffer (pH 5.0), 50 μM monolignol substrate (CA or PA) (Freudenberg and Hübner, 1952; Matsushita *et al.*, 2019), or an *o*-dianisidine reference substrate, 10 $\mu\text{g mL}^{-1}$ catalase, and 100 μM CuSO_4 , in a final volume of 1 mL (Sato and Whetten, 2006). The consumption of substrate was periodically monitored by ultraviolet (UV) absorbance at 260 nm (CA and PA) and 460 nm (*o*-dianisidine) on a Shimadzu UV1600 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) (Sato and Whetten, 2006; Koutaniemi *et al.*, 2015). Absorbance change coefficients were experimentally

determined after complete reaction of each substrate by rCoLac1 [$\Delta\epsilon$ ($\text{mM}^{-1} \text{cm}^{-1}$): CA, -8.28 at 260 nm; PA, -13.56 at 260 nm; *o*-dianisidine, $+10.04$ at 460 nm].

Statistical analysis

One-way analysis of variance (ANOVA) with a post-hoc Tukey–Kramer’s test ($p < 0.05$) and Student’s *t*-test were performed using GraphPad Prism 8.4.0 (GraphPad Software Inc., San Diego, CA, USA).

Accession numbers

Sequence data used in this study can be found in the GenBank data library under accession numbers: CoLac3, LC494389; CoLac1, AB762662; CoLac2, AB762663; AtLac1, NP_173252 (AT1G18140); AtLac2, NP_180477 (AT2G29130); AtLac3, NP_180580 (AT2G30210); AtLac4, NP_565881 (AT2G38080); AtLac5, NP_181568 (AT2G40370); AtLac6, NP_182180 (AT2G46570); AtLac7, NP_187533 (AT3G09220); AtLac8, NP_195724 (AT5G01040); AtLac9, NP_195725 (AT5G01050); AtLac10, NP_195739 (AT5G01190); AtLac11, NP_195946 (AT5G03260); AtLac12, NP_196158 (AT5G05390); AtLac13, NP_196330 (AT5G07130); AtLac14, NP_196498 (AT5G09360); AtLac15, NP_199621 (AT5G48100); AtLac16, NP_200699 (AT5G58910); AtLac17, NP_200810 (AT5G60020); ZmLac3, AM086215; *C. versicolor* laccase, D13372.

References

- Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E et al. 2012.** ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research* **40**: W597–W603.
- Brennan M, McLean JP, Altaner CM, Ralph J, Harris PJ. 2012.** Cellulose microfibril angles and cell-wall polymers in different wood types of *Pinus radiata*. *Cellulose* **19**: 1385-1404.
- Freudenberg K and Hübner HH. 1952.** Oxyzimtalkohole und ihre Dehydrierungs-polymerisate (Hydroxy cinnamyl alcohols and their dehydrogenative polymerization). *Chemische Berichte* **85**: 1181–1191.
- Hiraide H, Yoshida M, Ihara K, Sato S, Yamamoto H. 2014.** High lignin deposition on the outer region of the secondary wall middle layer in compression wood matches the expression of a Laccase gene in *Chamaecyparis obtusa*. *Journal of Plant Biology Research* **3**: 87–100.

- Hiraide H, Yoshida M, Sato S, Yamamoto H. 2016.** In situ detection of laccase activity and immunolocalisation of a compression-wood-specific laccase (CoLac1) in differentiating xylem of *Chamaecyparis obtusa*. *Functional Plant Biology* **43**: 542–552.
- Kim H, Ralph J. 2010.** Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-*d*₆/pyridine-*d*₅. *Organic & Biomolecular Chemistry* **8**: 576-591.
- Kim JS, Awano T, Yoshinaga A, Takabe K. 2010.** Immunolocalization of β -1-4-galactan and its relationship with lignin distribution in developing compression wood of *Cryptomeria japonica*. *Planta* **232**: 109–119.
- Koutaniemi S, Malmberg HA, Simola LK, Teeri TH. 2015.** Norway spruce (*Picea abies*) laccases: characterization of a laccase in a lignin-forming tissue culture. *Journal of Integrative Plant Biology* **57**: 341–348.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018.** MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular biology and Evolution* **35**:1547-1549.
- Lam PY, Tobimatsu Y, Takeda Y, Suzuki S, Yamamura M, Umezawa T, Lo C. 2017.** Disrupting flavone synthase II alters lignin and improves biomass digestibility. *Plant Physiology* **174**: 972-985.
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R et al. 2007.** Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.
- Matsushita Y, Okayama M, Aoki D, Yagami S, Fukushima K. 2019.** Radical transfer system in the enzymatic dehydrogenative polymerization (DHP formation) of coniferyl alcohol (CA) and three dilignols. *Holzforschung* **73**: 189-195.
- Nagata T, Nemoto Y, Hasezawa S. 1992.** Tobacco BY-2 cell line as the 'HeLa' cell in the cell biology of higher plants. *International Review of Cytology* **132**: 1–30.
- Ranocha P, McDougall G, Hawkins S, Sterjiades R, Borderies G, Stewart D, Cabanes-Macheteau M, Boudet AM, Goffner D. 1999.** Biochemical characterization, molecular cloning and expression of laccases - a divergent gene family - in poplar. *European Journal of Biochemistry* **259**: 485–495.
- Saitou N, Nei M. 1987.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and Evolution* **4**: 406-425.
- Sato Y, Whetten RW. 2006.** Characterization of two laccases of loblolly pine (*Pinus taeda*) expressed in tobacco BY-2 cells. *Journal of Plant Research* **119**: 581–588.

Suzuki S, Suzuki Y, Yamamoto N, Hattori T, Sakamoto M, Umezawa T. 2009. High-throughput determination of thioglycolic acid lignin from rice. *Plant Biotechnology* **26**: 337-340.

Tarmadi D, Tobimatsu Y, Yamamura M, Miyamoto T, Miyagawa Y, Umezawa T, Yoshimura T. 2018. NMR studies on lignocellulose deconstructions in the digestive system of the lower termite *Coptotermes formosanus* Shiraki. *Scientific Reports* **8**: 1290.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**: research0034.0031.

Wagner A, Tobimatsu Y, Phillips L, Flint H, Torr K, Donaldson L, Pears L, Ralph J. 2011. CCoAOMT suppression modifies lignin composition in *Pinus radiata*. *Plant Journal* **67**: 119-129.

Yamamura M, Hattori T, Suzuki S, Shibata D, Umezawa T. 2012. Microscale thioacidolysis method for the rapid analysis of β -O-4 substructures in lignin. *Plant Biotechnology* **29**: 419-423.