New Phytologist

***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:

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

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

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Fig. S5 Multiple alignment of laccase proteins.

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Table S1 Primers used in this study.

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Methods S1 Additional experimental procedures.

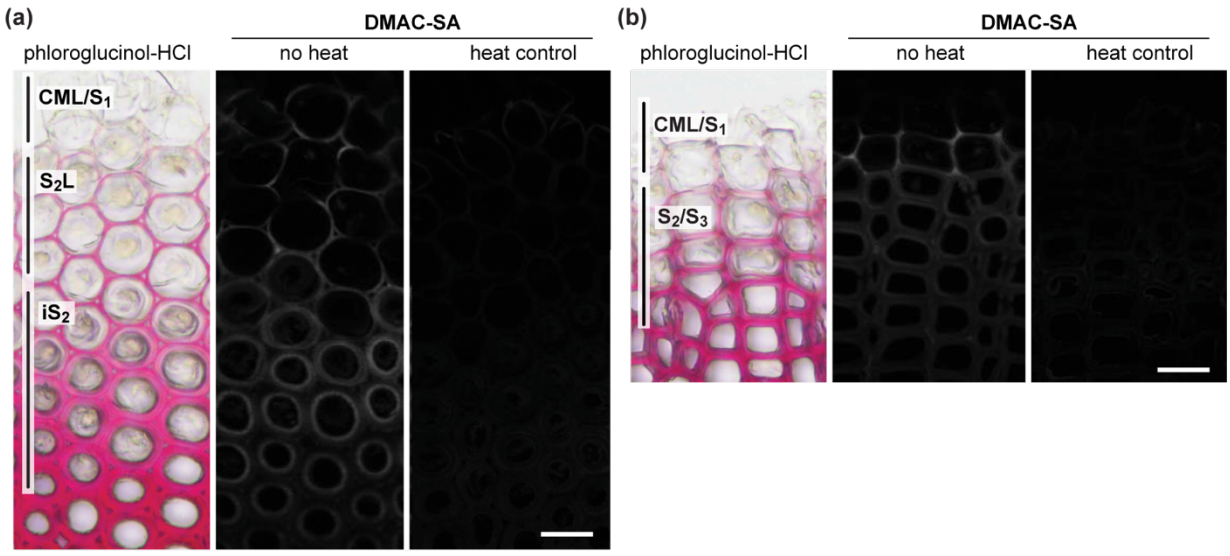

Fig. S1 Laccase-dependent incorporation of DMAC-SA into differentiating compression and normal wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood transverse sections were labeled with DMAC-SA in the presence of catalase, and then visualised as DMAC fluorescence with a confocal 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 µm.

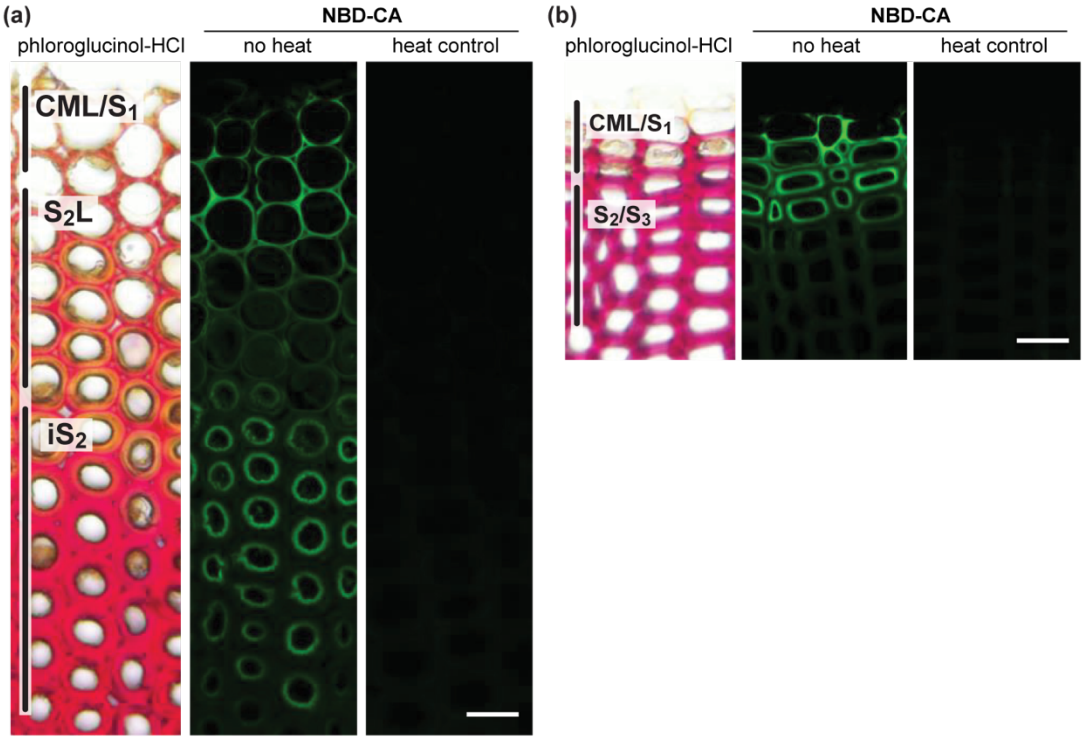

Fig. S2 Laccase-dependent incorporation of NBD-CA into differentiating compression wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood transverse sections were labeled with NBD-CA in the presence of catalase, and then visualised as NBD fluorescence with a confocal microscope. 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.

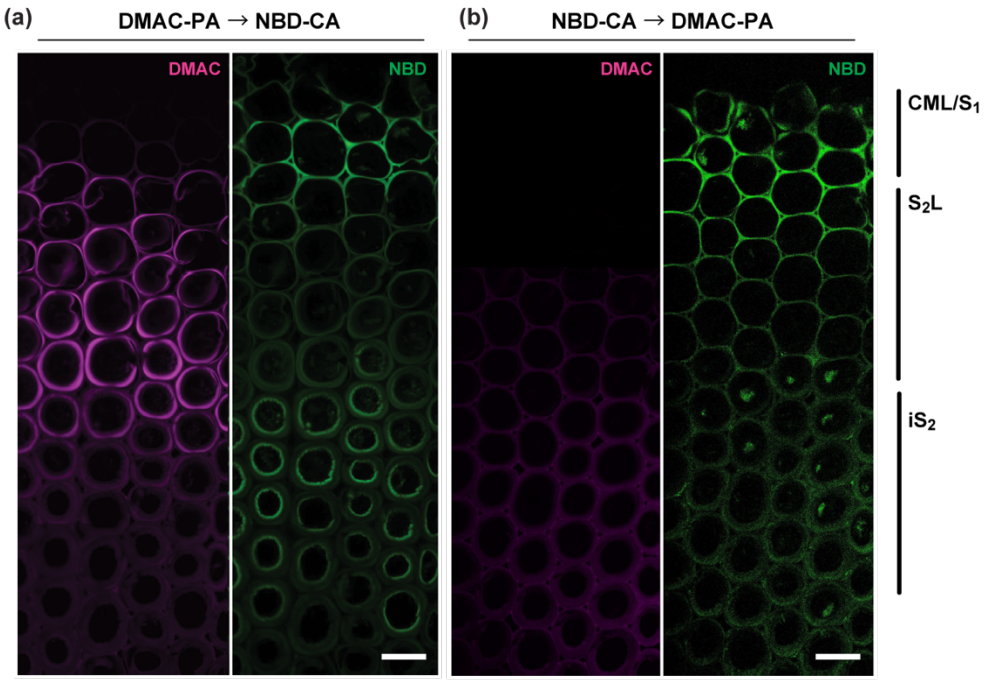

Fig. S3 Laccase-dependent incorporations of DMAC-PA and NBD-CA into differentiating compression wood tracheids of *C. obtusa* seedlings. Sections were incubated sequentially with DMAC-PA and then NBD-CA (DMAC-PA → NBD-CA; a) or in reverse order (NBD-CA → DMAC-PA; b), and then visualised as DNAC (magenta) and NBD (green) fluorescence. The preferential incorporation of DMAC-PA into S_2L 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 µm.

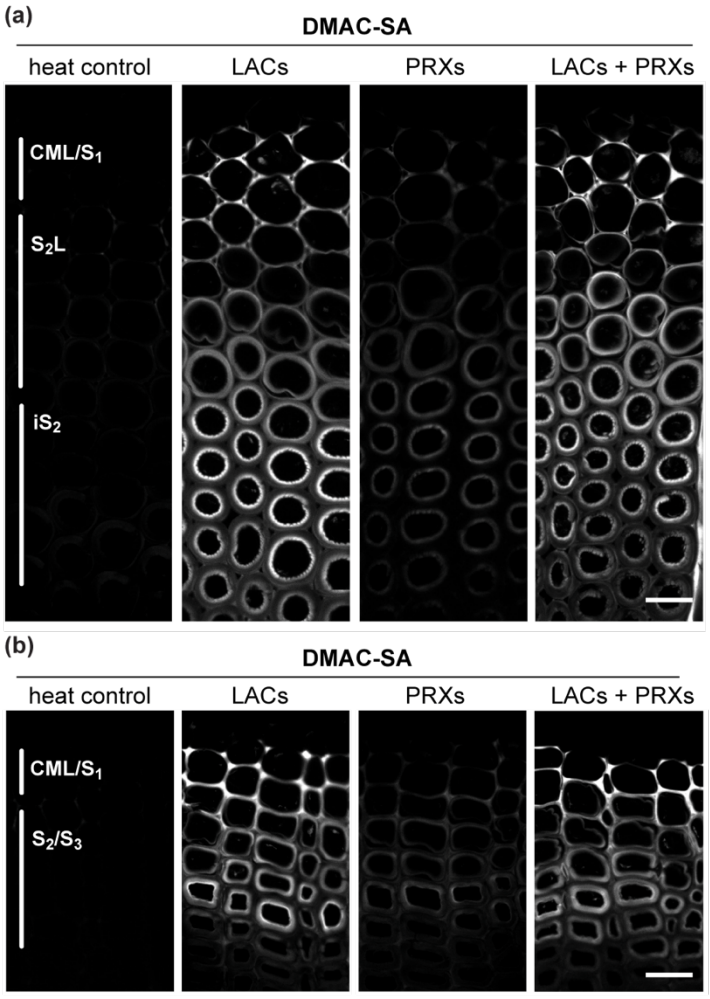

Fig. S4 Laccase- and peroxidase-dependent incorporations of DMAC-SA into differentiating compression and normal wood tracheids of *C. obtusa* seedlings. Compression (a) and normal (b) wood sections were labelled with DMAC-SA in the presence of catalase for detection of laccase/O_2 (LACs), glucose and glucose oxidase for peroxidase/H_2O_2 (PRXs), and with H_2O_2 for both laccase/O_2 and peroxidase/H_2O_2 (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.

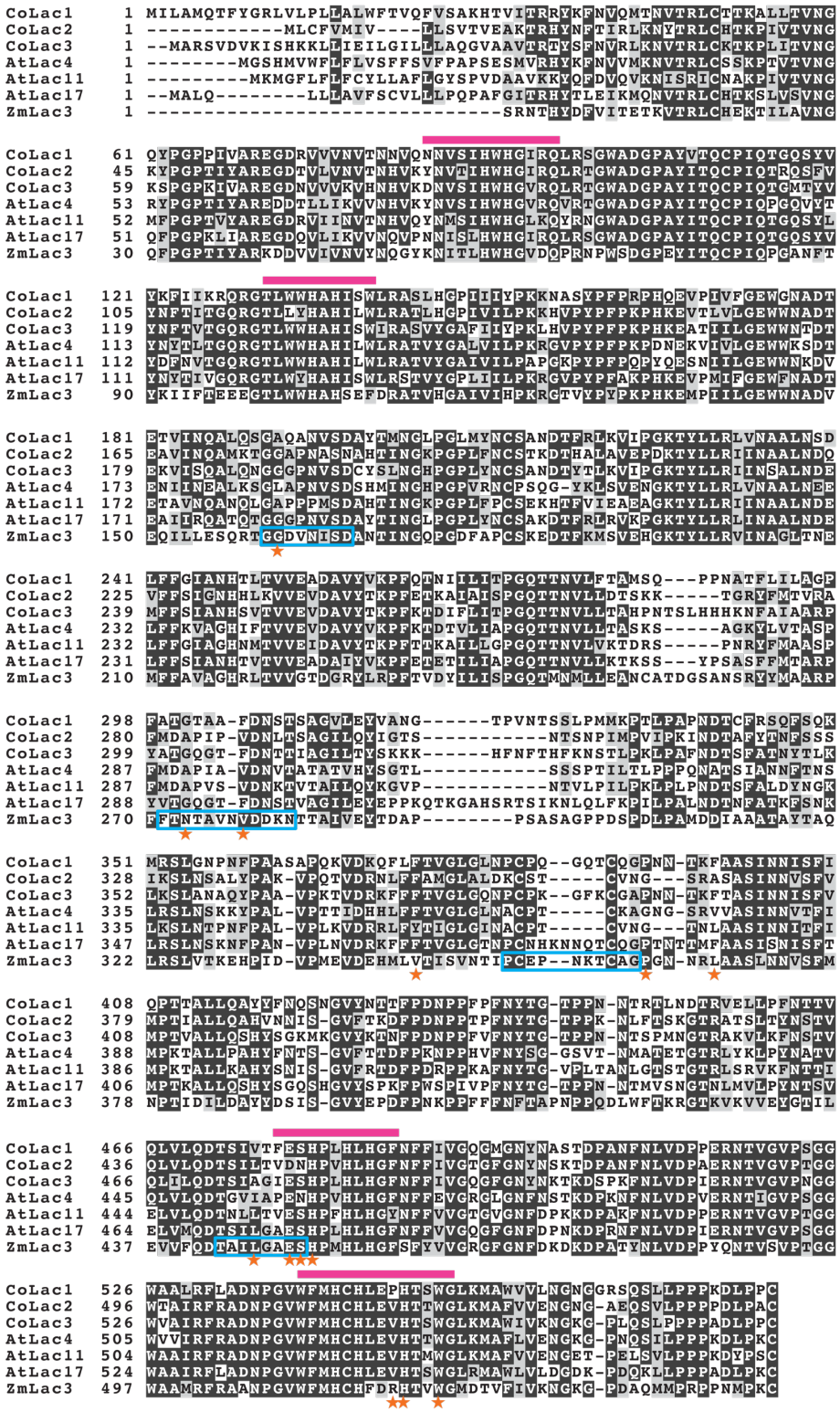

Fig. S5 Multiple alignment of laccase proteins. Amino acid sequences of the three *C. obtusa* laccases tested in this study (CoLac1, CoLac2 and CoLac3) were aligned with Arabidopsis and maize laccases. The copper-binding domain motifs (pink line), substrate-binding loops (blue box), and the residues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

Reference

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

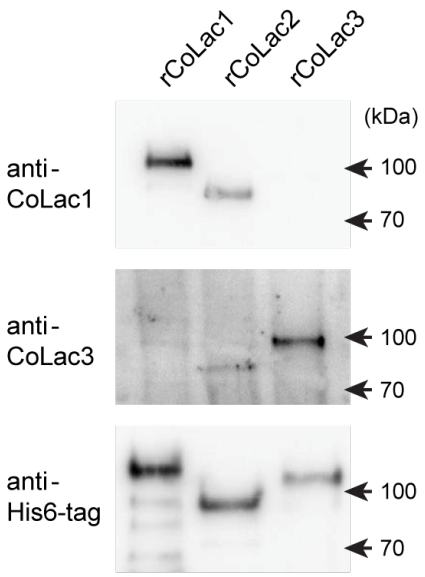

Fig. S6 Selectivity of anti-CoLac1 and anti-CoLac3 antibodies examined by immunoblotting against recombinant laccase proteins. Immunoblotting was performed with anti-CoLac1, anti-CoLac3 and anti-His6-tag antibodies against recombinant CoLac1, CoLac2 and CoLac3 proteins (rCoLac1, rCoLac2 and rCoLac3, respectively). Although there is no detectable cross-biding 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).

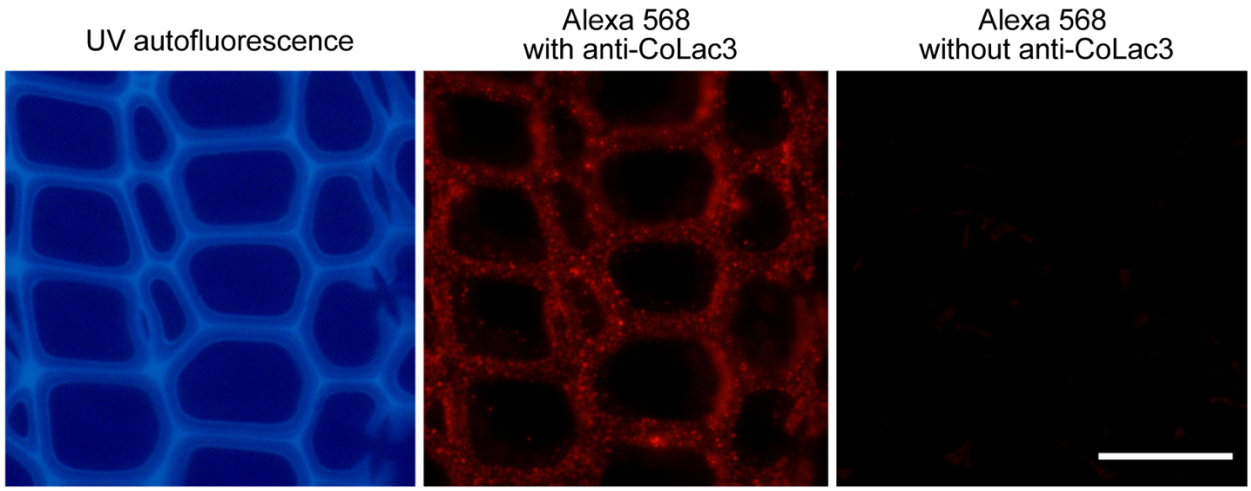

Fig. S7 Immunolocalisation of CoLac3 in cell walls of differentiating normal wood of *C. obtusa* seedlings. Sections were visualised by autofluorescence from deposited lignin (left) and Alexa 568 secondary antibody fluorescence from CoLac3 epitope (center). Control sections visualised without antibody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

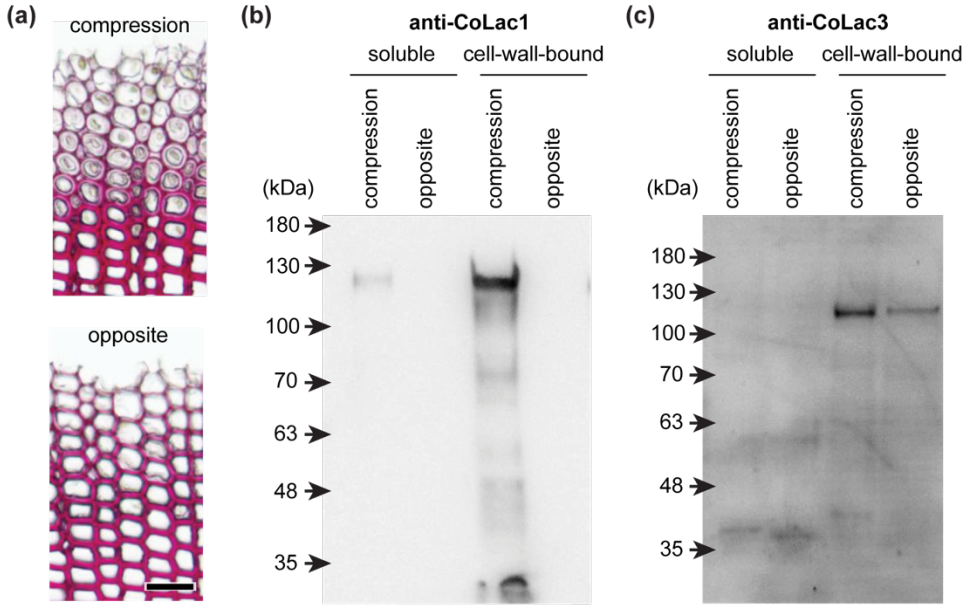

Fig. S8 Immunodetection of CoLac1 and CoLac3 in crude enzyme preparations prepared from differentiating compression and normal-wood-forming opposite tissues of a bent mature *C. obtusa* tree. Soluble and cell-wall-bound protein fractions were extracted from differentiating compression wood tissues and 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 µm). 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	CACACCCATCTCTGAACTCATTCAT
CoLac3 mRNA-R	Vector construction	GCATCCACCCCAACTTATTAGAAAA
CoLac3 5' RACE-R	5' RACE	CCTGGTGTGATGAGAAAGATGTCTG
Nested CoLac3 5' RACE-R	5' RACE	GTAGCAATCTGAGACGTTGGGTCCT
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	GCCAAAACTCCCAGCATTTA
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 ^{1}H–^{13}C 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 *Sac*II) (Hiraide *et al.*, 2014), and a full-length cDNA of *CoLac3* (PRI 201-AN, TaKaRa Bio; linearised with *Sac*I) 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-µm-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 μg mL^{-1} kanamycin and 500 μg mL^{-1} carbenicillin. Transformed cells were then further cultured in the modified LS medium further supplemented with 3 μM $CuSO_4$ 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 × *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 $CuSO_4$ 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 polyvinylidine 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 μg mL^{-1} protein) or those from *C. obtusa* wood tissues (10 μg 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 μg 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 [Δε (mM^{-1} 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.

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