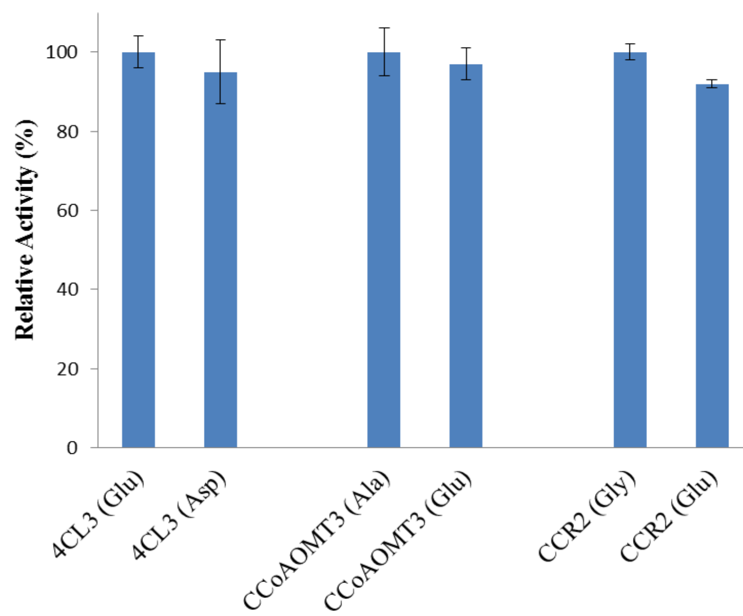

Supplemental Figure 1. Single nucleotide polymorphisms (SNP) in the monolignol pathway enzymes of *P. trichocarpa* Nisqually-1 do not alter their enzyme activities. The error bars represent standard error of three technical repeats.

Supplemental Figure 2. Xylem specific expression of GUS activity in transgenic *N. tabacum* transformed with *pBI121-4CLXP* construct.

Supplemental Table 1. The expression and purification systems used for the production of the 21 recombinant proteins in the monolignol biosynthetic pathway of *P. trichocarpa*

Enzyme family	Gene name	GenBank accession number	Expression System	Purification System
PAL	*PAL1*	EU603319	*E. coli*	GST tag
	PAL2	EU603321	*E. coli*	GST tag
	PAL3	EU603318	*E. coli*	GST tag
	PAL4	EU603322	*E. coli*	GST tag
	PAL5	EU603320	*E. coli*	GST tag
C4H	*C4H1*	EU603304	*s. cerevisiae*	Microsome
	C4H2	EU603302	*s. cerevisiae*	Microsome
4CL	*4CL3*	EU603298	*E. coli*	HIS tag
	4CL5	EU603299	*E. coli*	HIS tag
HCT	*HCT1*	EU603313	*E. coli*	GST tag
	HCT6	EU603314	*E. coli*	GST tag
C3H	*C3H3*	EU603301	*s. cerevisiae*	Microsome
CCoAOMT	*CCoAOMT1*	EU603307	*E. coli*	HIS tag
	CCoAOMT2	EU603309	*E. coli*	HIS tag
	CCoAOMT3	EU603308	*E. coli*	HIS tag
CCR	*CCR2*	EU603310	*E. coli*	GST tag
CAld5H	*CAld5H1*	EU603312	*s. cerevisiae*	Microsome
	CAld5H2	EU603311	*s. cerevisiae*	Microsome
COMT	*COMT2*	EU603317	*E. coli*	HIS tag
CAD	*CAD1*	EU603306	*E. coli*	GST tag
	CAD2	EU603305	*E. coli*	GST tag

Supplemental Table 2. The SNP allelic variants of the monolignol biosynthetic enzymes in *P. trichocarpa*

Gene	Amino Acids	Scaffold	Base	Genome Sequence	RNA-Seq Sequences
PAL1	Lys/Lys	scaffold_6	10034330	T	Y
PAL2	Leu/Leu	scaffold_8	2053326	T	Y
	Thr/Thr	scaffold_10	19775321	T	Y
	Ala/Ala	scaffold_10	19775807	C	Y
	Lys/Lys	scaffold_10	19775861	A	R
	Tyr/Tyr	scaffold_10	19775906	C	Y
PAL5	Thr/Thr	scaffold_10	19781996	A	C
	Asn/Asn	scaffold_10	19782824	C	T
	Gln/Gln	scaffold_10	19783271	A	G
C4H1	Leu/Leu	scaffold_13	15378110	G	R
	Phe/Phe	scaffold_13	15381180	G	Y
C4H2	Gly/Gly	scaffold_19	15705883	T	W
	Phe/Leu	scaffold_19	15707492	A	Y
4CL3	Thr/Thr	scaffold_1	5571743	T	Y
	Glu/Asp	scaffold_1	5571941	C	S
	Tyr/Tyr	scaffold_1	5572191	C	Y
	Lys/Lys	scaffold_1	5572238	C	Y
4CL5	Gly/Gly	scaffold_3	17691787	A	R
C3H3	Pro/Pro	scaffold_6	2004783	A	R
	Ser/Ser	scaffold_6	2004789	T	Y
CCoAOMT1	Tyr/Tyr	scaffold_9	8859033	A	R
	Lys/Lys	scaffold_9	8859249	T	Y
CCoAOMT3	Ala/Glu	scaffold_8	8961572	G	K
CCR2	Gly/Glu	scaffold_3	17211828	G	R
CAld5H1	Leu/Phe	scaffold_5	8721692	G	R
	Thr/Pro	scaffold_5	8722148	T	K
CAld5H2	Ala/Ala	scaffold_7	13783347	A	M
	Leu/Leu	scaffold_7	13784182	C	Y
	Ser/Ser	scaffold_7	13784196	T	Y
	Leu/Met	scaffold_7	13784320	T	W
	Glu/Glu	scaffold_7	13784361	G	R
COMT2	Val/Val	scaffold_12	371215	G	S
	Ser/Ser	scaffold_12	372954	T	Y

The rows not highlighted show the synonymous allelic variants where the SNPs do not alter the amino-acid sequences. The highlighted rows show the non-synonymous allelic variants where the SNPs cause a change in the amino-acid sequences. Non-synonymouse SNP allelic variants were identified for *4CL3, CCoAOMT3, CCR2, CAld5H1* and *CAld5H2*. These allelic variants were subsequently cloned, expressed recombinantly, and their activities assayed to study if these allelic variants have the same or different activities. The scaffold numbers and SNP locations are based on *P. trichocarpa* genome V2.

Supplemental Table 3. The optimal reaction conditions and cofactors for the reaction and inhibition kinetic assays of the 10 monolignol biosynthetic enzyme families (Liu et al., 2012).

Monolignol Enzyme Family	Cofactors	Optimum Assay Condition
PAL	–	50 mM Bis–Tris buffer (pH 8.5), 45 °C
C4H	2 mM NADPH	50 mM sodium phosphate buffer (pH 6.0), 30 °C
4CL	2 mM MgCl2, 2 mM ATP, 0.2 mM CoA	50 mM Tris–HCl buffer (pH 7.5), 37 °C
HCT	0.5 mM shikimic acid, 2 mM NADPH	50 mM MES buffer (pH 5.9), 37 °C
CCR	2 mM NADPH	50 mM MES buffer (pH 6.1), 45 °C
C3H	2 mM NADPH	50 mM sodium phosphate (pH 7.0), 20 °C
CCoAOMT	0.25 mM SAM, 2 mM MgCl2	50 mM Tris–HCl buffer (pH 7.5), 30 °C
CAld5H	2 mM NADPH	50 mM Citrate buffer (pH 5.4), 30 °C
AldOMT	0.25 mM SAM, 2 mM MgCl2	50 mM Tris–HCl buffer (pH 7.5), 30 °C
CAD	2 mM NADPH	50 mM MES buffer (pH 6.1), 24 °C

REFERENCE

Liu, J., Shi, R., Li, Q., Sederoff, R.R., Chiang, V.L. (2012). A standard reaction condition and a single HPLC separation system are sufficient for estimation of monolignol biosynthetic pathway enzyme activities. Planta **236:** 879-885.

Supplemental Table 4. The PC-IDMS derived absolute quantification of the monolignol enzymes in SDX of *P. trichocarpa*. PAL4|5 indicates the sum of PAL4 and PAL5 protein abundance (Shuford et al., 2012) because they cannot be differentiated by PC-IDMS.

Monolignol enzymes	**Concentration (nM)**
4CL3	1195
4CL5	142.2
C3H3	123.5
C4H1	109.3
C4H2	62.44
CAD1	436.1
CAD2	8.978
CAld5H1	117.5
CAld5H2	125.9
CCoAOMT1	583.9
CCoAOMT2	408.5
CCoAOMT3	82.96
CCR2	159.8
COMT2	8219
HCT1	170
HCT6	46.96
PAL1	40.93
PAL2	221.5
PAL3	30.18
PAL4|5	223.2

REFERENCE

Shuford, C.M., Li, Q., Sun, Y., Chen, H., Wang, J., Shi, R., Sederoff, R.R., Chiang, V.L., Muddiman, D.C. (2012). Comprehensive Quantification of Monolignol-Pathway Enzymes in Populus trichocarpa by Protein Cleavage Isotope Dilution Mass Spectrometry. J. Proteome Res. **11:** 3390-3404.

Supplemental Table 5. Primers used in this study.

Primer Name	Primer Sequence
4CLP-F	CAAGCTTAAAGAGAAGTTAGGTCACTCCTCC
4CLP-R	TGGATCC CATTATGGCGTCCATTGCGGGCT
PAL739479-s	CCTTACCACGTAGTGGCAAGGATTTGTGGCTCTTCCTTTTC
PAL739479-a	AAATCCTTGCCACTACGTGGTAAGGG**TA**GAGCCAAAACAAG
*PAL739479-*s*	AAATACTTGCCACTAGGTGGTATGGATGGAGCTACTAACAG
*PAL739479-*a*	CCATACCACCTAGTGGCAAGTATTTCATCTGTCTCTGCTCC
PAL820245-s	CCTTTTAACGTAAGAGCTACGATTTGTGGCTCTTCCTTTTC
PAL820245-a	AAATCGTAGCTCTTACGTTAAAAGGGTAGAGCCAAAACAAG
*PAL820245-*s*	AAATAGTAGCTCTTAGGTTAAATGGATGGAGCTACTAACAG
*PAL820245-*a*	CCATTTAACCTAAGAGCTACTATTTCATCTGTCTCTGCTCC
i7-SF	ACTAGT CCATTTTGGCATAGAGGATCATCG
i7-M1	TCGAGCCTCCTCTGACAACTCC
i7-M2	AGTTGTCAGAGGAGGCTCGATTACGTTAAAGCAGCTCAAAAGT
i7-SR	GAGCTC GACAGAGTTGATTTCTCGTTCG
i7-ASF	GACTGTCGACCCATTTTGGCATAGAGGATCATCG
i7-ASR	TGCAGGATCCGACAGAGTTGATTTCTCGTTCG

s=sense strand; a=antisense strand; *=amiRNA* sequence (Shi et al., 2010)

Supplemental Method 1. Chemical and biochemical synthesis of monolignol precursors for enzymatic assays

Phenylalanine, cinnamic acid, 4-coumaric acid, caffeic acid, ferulic acid, sinapic acid, coniferaldehyde, coniferyl alcohol, sinapaldehyde and sinapyl alcohol were purchased from Sigma Aldrich (St. Louis MO, USA). For all compounds except the alcohols, product ion spectra were acquired for the $[M+H^+]^{1+}$ molecular ion of each synthetic standard using a TSQ Quantum triple-stage quadruple mass spectrometer (Thermo Scientific, San Jose, CA). For the alcohols, product ion spectra were acquired for the $[M–OH^-]^{1+}$ molecular ion. Collision energy of 10 eV and 1.5 mtorr of argon were used in acquiring all spectra, except for the acyl-CoA derivatives, which utilized collision energy of 30 eV.

Synthesis of 5-hydroxyferulic acid: Pyridine (1 mL), piperidine (5 µL), and aniline (10 µL) were added successively to a stirred mixture of 5-hydroxyvanillin (50 mg, 1 mmol) and malonic acid (75 mg). The resulting solution was stirred at 25 °C for 1 week. After acidification with 1 N HCl to pH 2, the resulting mixture was extracted with 10 mL of ethyl acetate and then purified over a silica column, yielding 63 mg 5-hydroxyferulic acid. MS *m/z* (%) 211 ($[M+H^+]^+$, 15), 193 (100), 169 (1), 160 (18), 133 (8).

4-Coumaraldehyde, 4-coumaryl alcohol, caffealdehyde, caffeyl alcohol, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol were chemically synthesized in our lab as described in Li et al., (2001). Briefly, 4-acetoxy-benzaldehyde (1 mmol) and (1, 3-dioxolan-2-yl-methyl)-triphenylphosphosphonium bromide (1 mmol) were dissolved in CH_2Cl_2 (20 mL). Solid K_2CO_3 (1 mmol) and 18-crown-6 (0.01 mmol) were added. The reaction mixture was kept at room temperature for 6 hr, and filtered to separate the organic phase from the solid phase. Aqueous HCl (10%, 10 mL) was added to the organic portion and stirred in the mixture at 25°C for 4 hr. The mixture was then diluted with 10 mL H_2O and extracted three times with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ and saturated aqueous NaCl solutions successively, then dried over $MgSO_4$ under vacuum. A silica gel column was used for purification. The 4-acetoxycoumaraldehyde (0.1 g) was dissolved in 20 mL of 0.2 M KOH in 95% EtOH, and stirred for 2 hr under N_2. The solvent was removed under vacuum, and the mixture diluted with 10 mL H_2O and extracted with ethyl acetate (10 mL ×3). The combined organic layers were dried over Na_2SO_4 and evaporated to give 4-coumaraldehyde (61 mg, 41%

yield). 4-Coumaryl alcohol was synthesized from 4-acetoxycoumaraldehyde according to the procedure described by Daubresse et al. (1994). Briefly, 4-acetoxycoumaraldehyde (0.1 g) was dissolved in MeOH along with KH_2PO_4. $NaBH_4$ (50 mg) was slowly added at 0°C. Cold water was added and MeOH was removed under vacuum and the mixture was extracted with CH_2Cl_2, washed with H_2O, dried and evaporated to give 4-acetyloxy-2-propen-1-ol (90 mg). 4-Acetyloxy-2-propen-1-ol (50 mg) was dissolved in 20 mL of 0.2 M KOH in 95% EtOH, and stirred for 2 hr under N_2. The solvent was removed under vacuum, and the mixture diluted with 10 mL H_2O and extracted with ethyl acetate (20 mL ×3). The combined organic layers were dried over Na_2SO_4 and purified on a silica gel column to give 4-coumaryl alcohol (62 mg, 41% yield).

Caffealdehyde and caffeoyl alcohol, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol were synthesized using the same procedure with overall yield of 40–53%. 4-Coumaraldehyde: MS *m/z* (%) 149 ($[M+H^+]^+$, 100), 131 (32), 121 (7), 107 (3), 103 (7), 93 (1), 91 (1), 77 (1), 55 (4). Caffealdehyde: MS *m/z* (%) 165 ($[M+H^+]^+$, 45), 147 (100), 119 (14), 91 (6), 55 (1). 5-Hydroxyconiferaldehyde: MS *m/z* (%) 195 ($[M+H^+]^+$, 85), 177 (100), 167 (3), 163 (11), 149 (6), 145 (2), 135 (1), 131 (5), 121 (1), 117 (1), 107 (1), 106 (1), 103 (5), 55 (1). 4-Coumaryl alcohol: MS *m/z* (%) 133 $[M–OH^-]^{1+}$, 100), 115 (1), 105 (21), 103 (2), 79 (2), 77 (1). Caffeyl alcohol: MS *m/z* (%) 149 $[M–OH^-]^{1+}$, 100), 131 (96), 103 (31), 77 (2), 73 (1). 5-Hydroxyconiferyl alcohol: MS *m/z* (%) 179 $[M–OH^-]^{1+}$, 70), 164 (1), 161 (4), 147 (100), 133 (2), 119 (9), 91 (4).

4-Coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA were enzymatically synthesized from each acid (Beuerle and Pichersky 2002). Purified *P. trichocarpa* 4-coumarate: CoA ligase-3 (4CL3) (Shuford et al. 2012) recombinant protein from *Escherichia coli* was used to biochemically synthesize 4-coumaroyl-CoA and caffeoyl-CoA. Briefly, 6 mg acid, 4 mg coenzyme A hydrate (CoA), and 14 mg ATP were dissolved in a total volume of 40 ml of 50 mM Tris-HCl (pH 7.5) buffer containing 2.5 mM $MgCl_2$. 0.3 mg of purified protein was added to the mixture to start the reaction. After 30 min at 37 °C, 1.6 g ammonium acetate was added to stop the reaction. The resulting mixture was purified by using an SPE cartridge (Chromabond C_{18} ec, Macherey-Nagel). Yields of 1.7 mg 4-coumaroyl-CoA, 2.3 mg caffeoyl-CoA and 1.5 mg feruloyl-CoA were obtained, which represented 36%, 48%, and 36% yield respectively, based on CoA used in the reaction. The purity and identity of all synthesized products were confirmed by

tandem MS. Product ion spectra were acquired directly for the $[M+H^{+}]^{+}$ molecular ion of each compound on a TSQ Quantum Triple Quadrupole mass spectrometer (Thermo Scientific) at a collision energy of 10 eV and 1.5 mtorr of argon. 4-Coumaroyl-CoA: MS *m/z* (%) 914 ($[M+H^{+}]^{+}$, 56.5), 768 (8.7), 505, (9.9), 428 (40.0), 407 (100), 341 (6.3), 305 (21.1), 261 (10.9). Caffeoyl-CoA: MS *m/z* (%) 930 ($[M+H^{+}]^{+}$, 60.2), 768 (20.5), 521 (8.8), 428 (43.3), 423 (100), 410 (7.0), 341 (11.5), 321 (20.6), 261 (14.8). Feruloyl-CoA: MS *m/z* (%) 944 ($[M+H^{+}]^{+}$, 56.0), 768 (7.0), 535 (11.7), 437 (100), 428 (48.8), 410 (7.4), 335 (19.4), 261 (13.3), 177 (8.3). 5-Hydroxyferuloyl-CoA: MS *m/z* (%) 960 ($[M+H^{+}]^{+}$, 5), 768 (3), 551 (3), 453 (100), 428 (41), 410 (3), 351 (19), 341 (8), 261 (19), 193 (8). Sinapoyl-CoA: MS *m/z* (%) 974 ($[M+H^{+}]^{+}$, 7), 768 (5), 467 (100), 428 (40), 365 (21), 341 (6), 261 (17), 207 (14).

4-coumaroyl shikimic acid and caffeoyl shikimic acid were enzymatically synthesized using 6 mg 4-coumaroyl-CoA, or 6 mg caffeoyl-CoA and 2 mg shikimic acid in a volume of 20 mL of potassium phosphate buffer (pH 7). The reaction was started by the addition of purified HCT6 (Shuford et al., 2012) (0.5 mg). After incubating for 20 min at 30 °C, the product was extracted three times with 20 mL of ethyl acetate. The organic layer was recovered, then dried over Na_2SO_4 and evaporated to give 1.5 mg 4-coumaroyl shikimic acid (65% yield) or 1.2 mg caffeoyl shikimic acid (54% yield). 4-Coumaroyl shikimic acid: MS *m/z* (%) 321 ($[M+H^{+}]^{+}$, 18.2), 303 (3.5), 165 (5.6), 147 (100), 139 (1.6), 119 (2.3) . Caffeoyl shikimic acid: MS *m/z* (%) 337 ($[M+H^{+}]^{+}$, 20.2), 319 (3.0), 181 (4.1), 163 (100), 145 (2.8), 145 (2.8), 139 (1.4), 117 (1.0).

REFERENCES

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Suzuki, S., Harding, S.A., and Chiang, C.L. (2001). The last step of syringyl monolignol biosynthesis in angiosperm dicots is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell **13:** 1567-1585.

Beuerle, T., Pichersky, E. (2002). Purification and characterization of benzoate:coenzyme A ligase from Clarkia breweri. Arch. Biochem. Biophys. **15:** 258-264.

Shuford, C.M., Li, Q., Sun, Y., Chen, H., Wang, J., Shi, R., Sederoff, R.R., Chiang, V.L., Muddiman, D.C. (2012). Comprehensive Quantification of Monolignol-Pathway Enzymes in

Populus trichocarpa by Protein Cleavage Isotope Dilution Mass Spectrometry. J. Proteome Res. **11:** 3390-3404.

Supplemental Method 2. Generation of transgenic *P. trichocarpa* with xylem specific PAL downregulation

Assembly of a xylem-specific transformation construct: To generate a transformation construct with xylem-specific expression, a 1.5kb promoter region of *4CL3* (POPTR_0001s07400) was amplified from *P. trichocarpa* genomic DNA with primers *4CLP-F* and *4CLP-R* (see Supplemental Table 5 online) and replaced the 35S promoter in the transformation construct *pBI121* and *MIR408-pBI121* (Shi et al., 2010) to generate *pBI121-4CLXP* and *MIR408-pBI121-4CLXP*. The xylem specific expression of this *4CL* promoter was validated using transgenic *Nicotiana tabacum* transformed with *pBI121-4CLXP* (Horsch et al., 1985) and GUS staining (Li et al., 2011; see Supplemental Figure 2 online).

Generation of transgenic *P. trichocarpa* with xylem-specific individual downregulation of *PAL1* or *PAL2*: To generate transgenic *P. trichocarpa* plants downregulated in either *PAL1* or *PAL2* expression, amiRNAs (21 nt) targeting *PAL1* and *PAL2* were designed using online program Web MicroRNA Designer (http://wmd.weigelworld.org), and amplified using primer sets *PAL739479* for *PAL1* and *PAL820245* for *PAL2* (see Supplemental Table 5 online). The amplified sequences were integrated into the *Ptr-MIR408-pBI121-4CLXP* construct to generate *4CLP-PAL1a* and *4CLP-PAL2a*. These constructs were transformed into *P. trichocarpa* as described by Song et al., 2006.

Generation of transgenic *P. trichocarpa* with xylem specific downregulation of three *PALs* (*PAL2, PAL4* and *PAL5*): To assemble the transformation construct *4CLP-PAL245i*, that target the downregulation of *PAL2, PAL4* and *PAL5*, a 284bp fragment was amplified from *PAL4* cDNA (Shi et al. 2010) with primers *i7-SF* and *i7-M1* (see Supplemental Table 5 online), and a 158bp fragment was amplified from *PAL2* cDNA (Shi et al. 2010) with primers *i7-M2* and *i7-SR*. Using these two fragments as template, overlapping PCR (Warrens et al. 1996) was performed to generate a sense fragment with primers *i7-SF/i7-SR* and an antisense fragment with primers *i7-ASF/i7-ASR*. These sense and antisense fragments were sequenced and cloned into *pCR2.1-GL* (Li et al. 2011) at *SpeI/SacI* and *SalI/BamHI*, respectively. The resulting antisense:GL:sense fragment was cloned into *pBI121-4CLXP* at *BamHI/SacI* to generate *4CLXP-PAL245i*. This

REFERENCES

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz D., Rogers, S.G., Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227:** 1229–1231.

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Harding, S.A., Chiang, V.L. (2001). The last step of syringyl monolignol biosynthesis in angiosperms in regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell **13:** 1567-1586.

Shi, R., Yang, C., Lu, S., Sederoff, R., Chiang, V.L. (2010). Specific down-regulation of *PAL* genes by artificial microRNAs in *Populus trichocarpa*. Planta **232:** 1281-1288.

Song, J., Lu, S., Chen, Z.Z., Lourenco, R., Chiang, V.L. (2006) Genetic Transformation of *Populus trichocarpa* Genotype Nisqually-1: A Functional Genomic Tool for Woody Plants. Plant Cell Physiol. **47:** 1582-1589.

Warrens, A.N., Jones M.D., and Lechler, R.I. (1996). Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene **186:** 29–35.