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Research Report

p-Coumaroylation of lignin occurs outside of commelinid monocots in the eudicot genus *Morus* (mulberry)

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Abstract

The presence of *p*-coumarate (*p*CA) in plant cell walls is generally considered to be a trait present only in commelinid monocots. Here, we show that this long-held overgeneralizing assumption is incorrect and that mulberry trees (*Morus*) are eudicot plants that have lignins derived in part from monolignol *p*CA esters. As in commelinid monocots, the lignin-bound *p*CA acylates the sidechain γ-hydroxyl of both coniferyl and syringyl units. This discovery expands mulberry's potential applications to include being a source of *p*-coumaric acid, a supplier of nutritious berries, a forage crop, a decorative plant, and the main food source for silkworms.

Introduction

A distinctive characteristic of commelinid monocots is the presence of hydroxycinnamates, *p*-coumarate (*p*CA), and ferulate (FA), as key components in the cell wall (Harris and Hartley, 1976). These hydroxycinnamates produce a characteristic blue fluorescence under UV light, which serves as one of the diagnostic traits in identifying commelinid monocots from monocots that lack cell-wall-bound hydroxycinnamates (Harris and Hartley, 1980). Elucidation of how *p*CA and FA attach to the cell walls required extensive exploration of cell wall chemical compositions. Cell-wall-bound FA in commelinids is mostly present as arabino-FA subunits of glucuronoarabinoxylans (GAX) and diferulate crosslinks between hemicellulosic chains (Mueller-Harvey et al., 1986; Ralph, 2010). *p*CA also acylates GAX, but to a lower degree (Mueller-Harvey et al., 1986). Most of the cell-wall-bound *p*CA and a smaller fraction of the FA acylate the γ-hydroxy groups of lignin sidechain subunits (Ralph et al., 1994). Their derivation has been shown to be via lignification using pre-formed monolignol (ML) hydroxycinnamate conjugates (Ralph, 2010). ML acyl transferases couple *p*CA-CoA (or FA-CoA) to the γ-hydroxyl of MLs (S: sinapyl alcohol, G: coniferyl alcohol, and H: *p*-coumaryl alcohol) prior to exportation to the cell wall for lignification (Withers et al., 2012; Marita et al., 2014; Wilkerson et al., 2014). Although there is evidence for *p*CA and FA to be bound to the cell walls of many eudicot plant species (Hartley and Harris, 1981), the general consensus in the community is that commelinids are the only plants in which FA and/or *p*CA is bound to their hemicelluloses and/or lignin. Earlier studies investigating the presence of FA in eudicot species (e.g., in *Beta vulgaris*, sugar beet) found them to be associated with the pectin of ethanol-insoluble sugar beet pulp (Rombouts

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and Thibault, 1986) and, more recently, FA has been found to be part of the lignin of a variety of eudicot species (Karlen et al., 2016). To our knowledge, Kenaf (*Hibiscus cannabinus*) is the only eudicot species confirmed to produce cell walls with *p*CA bound to the lignin (Mottiar et al., 2022).

It has long been understood that there are hydroxycinnamates in the soluble metabolites of plant species, as they are key intermediates to many different biosynthetic pathways, those to anthocyanins, flavonoids, stilbenes, lignin, and coumarins to name a few (Reinprecht et al., 2013). As with all proof of chemical origin, it is challenging to establish how *p*CA and FA are attached to the cell wall when they are released and detected as free acids. Identification of diagnostic molecular fragments released from the cell wall is therefore required to provide insight into the origin of the FA or *p*CA. One of the most diagnostic techniques for identification of lignin-bound *p*CA and FA is derivatization followed by reductive cleavage (DFRC) (Lu and Ralph, 1999), a method that cleaves lignin β-ethers but leaves γ-esters intact; analytical thioacidolysis is also capable of releasing diagnostic ML-conjugates (Grabber et al., 1996), but partially cleaves the γ-esters. DFRC chemically depolymerizes lignin and releases ML-conjugates (ML-*p*CA or ML-FA esters) as partially saturated (7,8-dihydrocinnamate) diacetylated products (ML-DH*p*CA and ML-DHFA) (Regner et al., 2018). The DFRC assay has authenticated the incorporation of ML-conjugates into the lignin of a wide array of species (Karlen et al., 2016, 2018).

With an ever-growing portfolio of plant species identified and validated by botanical communities such as the World Floral Online (WFO) Plant List (https://wfoplantlist.org/plant-list, updated December 2021), it becomes more obvious that the study of lignin is limited to semi-random sampling of species based on commercial importance, cultural relevance, personal interest, authentic sample availability, or coincidental synergies with other projects in the researchers' networks. Assaying plant species by NMR and other chemical identification methods is time-consuming and requires extensive chemical confirmation to identify the presence of cell-wall components. Compiling a database of chemical compositional data and species phylogenetic relationships builds a more detailed understanding of conserved traits. To date, the wild-type specimens we have studied using two-dimensional heteronuclear single-quantum coherence nuclear magnetic resonance spectrometry (2D HSQC NMR), which have signals that could be assigned to *p*CA acylation of lignin, have all been commelinid monocots. As part of the characterization of two white mulberry (*Morus alba*) cultivars, "Kairyou nezumigaeshi" and a CAD-deficient mutant called "sekizaisou" (Yamamoto et al., 2020), we noted what appeared to be *p*CA in the HSQC NMR spectra of both cultivars. This observation was in contrast to earlier screening of black mulberry (*Morus nigra*) by UV-fluorescence and chemical characterization of diethyl ether extracts of alkaline saponification liquors, in which there was no evidence for cell-wall-bound *p*CA at the threshold of detection used (Hartley and Harris, 1981). Our signal was therefore: (1) From an unfortunate contamination of all the samples studied (which seemed unlikely); (2) associated with cell-wall polysaccharides (e.g. *p*CA associated with pectin in sugar beet); or (3) suggestive that our long-held belief that *p*CA acylation of lignin is an exclusive trait of commelinid monocots is incorrect. To answer this question, we performed a suite of chemical assays to elucidate the chemical composition of the lignin in five species of mulberry: *M. alba*, *M. nigra*, Indian mulberry (*Morus indica*), red mulberry (*Morus rubra*), and Texas mulberry (*Morus microphylla*), that represent the 20 *Morus* species currently recognized by the WFO Plant List and include the Asian and American species (Figure 1; Nepal and Ferguson, 2012). These results conclusively show that *p*CA is bound to the lignin of these *Morus* samples, and that *p*CA can be found acylating the lignin in certain eudicot trees, implying that the third option above is correct: that is, that *p*CA acylation of lignin is not a trait exclusive to commelinid monocots.

Results and discussion

Phenolic acids released by alkaline hydrolysis

The presence of *p*CA in *Morus* species has long been established with *p*CA levels in the fruit reaching 27.3 mg/100 g dry weight and up to 8.66 mg/100 g dry weight in leaves (Memon et al., 2010; Mahmood et al., 2012). Here, alkaline treatment released 15–23 mg *p*CA/100 g extract-free (EF) wood (Figure 1), with *M. alba* having the lowest and *M. microphylla* and *M. indica* having the highest levels. The trends were similar for *p*CA in the soluble metabolites extracted from leaf tissue of *M. nigra* (8.66 mg/100 g dry weight), but *p*CA was not detected from *M. alba* (Memon et al., 2010). As with the leaf extracts, cell-wall-bound FA was not detected in the alkaline hydrolysis liquor.

HSQC NMR characterization of the lignin

HSQC NMR of enzymatically isolated lignin confirmed the presence of *p*CA in all five mulberry samples (Figure 2 and Table 1). *Morus rubra*, *M. indica*, and *M. microphylla* all had similar S-content and 3% *p*CA on a peak volume basis ($^1/_2\mathbf{S}_{2/6}$ + $\mathbf{G}_2$ = 100%), whereas *M. nigra* and *M. alba* both had only 1% *p*CA. The S:G ratio was ∼60:40 (S/G: 1.42–1.54) for four of the species, with *M. nigra* having slightly higher S-lignin content (S:G 68:32), the data suggesting an association between higher G-content and higher *p*CA. *Morus indica* and *M. nigra* NMR spectra contained strong signals for hydroxycinnamaldehydes and hydroxybenzaldehydes (Kim et al., 2000, 2003). As observed in many hardwoods, the sidechain distribution in all five species heavily favored β-ether formation at 82–86% of quantified sidechains on an $\mathbf{A}_\alpha$ + $\mathbf{B}_\alpha$ + $^1/_2\mathbf{C}_\beta$ = 100% basis (Table 1 and Supplemental Figure S1).

Pyrolysis–GC–MS of isolated lignins in the presence of tetramethylammonium hydroxide

Pyrolysis of lignin in the presence of tetramethylammonium hydroxide (TMAH) efficiently trans-esterifies esters to their

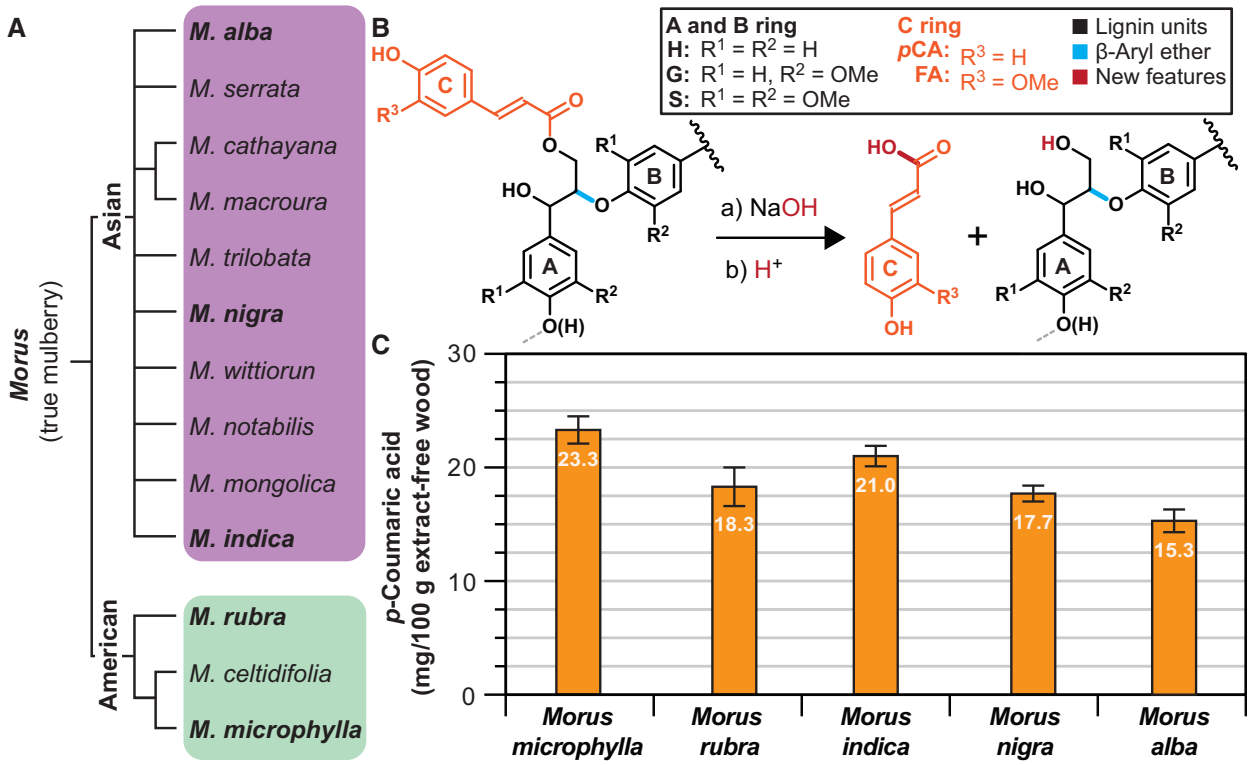

Figure 1 Phylogenetic tree of the *Morus* genus and quantification of cell-wall-bound *p*CA. A, Phylogenetic tree for the genus *Morus* adapted from Nepal and Ferguson (2012). B, Schematic for the saponification of γ-*p*CA esters, using the predominant β-aryl ether as an example lignin subunit. C, The amount (mg/100 g) of *p*-coumaric acid released from cell walls using 2 M NaOH at 90°C for 90 min. Error bars indicate SEM for $n = 3$ technical replicates.

methyl counterparts and methylates free hydroxy groups. This simplifies the pyrograms by reducing the number of thermal decomposition products formed when pyrolyzed in the absence of TMAH (Ralph and Hatfield, 1991; Clifford et al., 1995; Rencoret et al., 2018). The *p*CA subunits form methyl 4-methoxycinnamate in nearly quantitative yield, instead of thermally decomposing to 4-hydroxystyrene or other compounds as occurs under pyrolysis in the absence of TMAH (Ralph and Hatfield, 1991). The lignin of all five *Morus* species yielded the diagnostic methyl 4-methoxycinnamate (peak 22; Figure 3 and Table 2). The relative peak area of methyl 4-methoxycinnamate versus the summation of integrated TIC signals followed a similar trend as observed for the saponification and NMR, with *M. microphylla*, *M. rubra*, and *M. indica* having slightly higher intensities than *M. nigra*, and *M. alba* having the lowest relative peak area (Table 1).

DFRC of lignified cell-wall preparations

The DFRC product mixture of the five species revealed the presence of ML *p*CA conjugates, through the diagnostic ML dihydro-*p*CA (DH*p*CA) diacetate products (Figure 3). In chromatograms of both EF wood (Figure 3 and Table 1) and isolated lignins (Supplemental Table S1), the amount of DFRC-released ML-DH*p*CA followed the same trend as the incorporation levels of *p*CA shown in the other assays. The lignin-bound *p*CA content can be estimated by converting the quantified S-DH*p*CA and G-DH*p*CA products into lignin-bound *p*CA [mg *p*CA = (mg S-DH*p*CA/MW S-DH*p*CA + mg G-DH*p*CA/MW G-DH*p*CA) × MW *p*CA] with *M. microphylla* and *M. rubra* samples releasing around 40 mg/100 g EF cell wall (Table 1), *M. indica* releasing 28 mg/100 g EF cell wall, and both *M. nigra* and *M. alba* releasing 14 mg/100 g EF cell wall.

The DFRC analysis also revealed the presence of trace levels of G-FA conjugates (Figure 3). This discovery adds these *Morus* species to the list of plants that natively incorporate potential crosslinking species into the lignin polymer. In eudicot species, the FA conjugates predominantly reside on G-subunits, whereas in monocots the FA conjugates predominantly reside on S-subunits (Karlen et al., 2016).

Conclusion

The presence of the diagnostic ML-DH*p*CA conjugate in the DFRC product mixture of all five *Morus* species (Figure 3) confirms that cell-wall-bound *p*CA is on the lignin polymer. Therefore, the original observation of *p*CA signals in the HSQC NMR spectra of isolated lignin was correct (Yamamoto et al., 2020), and the trait is shared among *Morus* species. The phylogeny of the species indicates a break in the genus dividing *M. microphylla*, *M. rubra*, and *Morus celtidifolia* as American species and the rest as species diverged across Asia. The presence of *p*CA at similar levels in *M. indica*, *M. rubra*, and *M. microphylla* suggests that

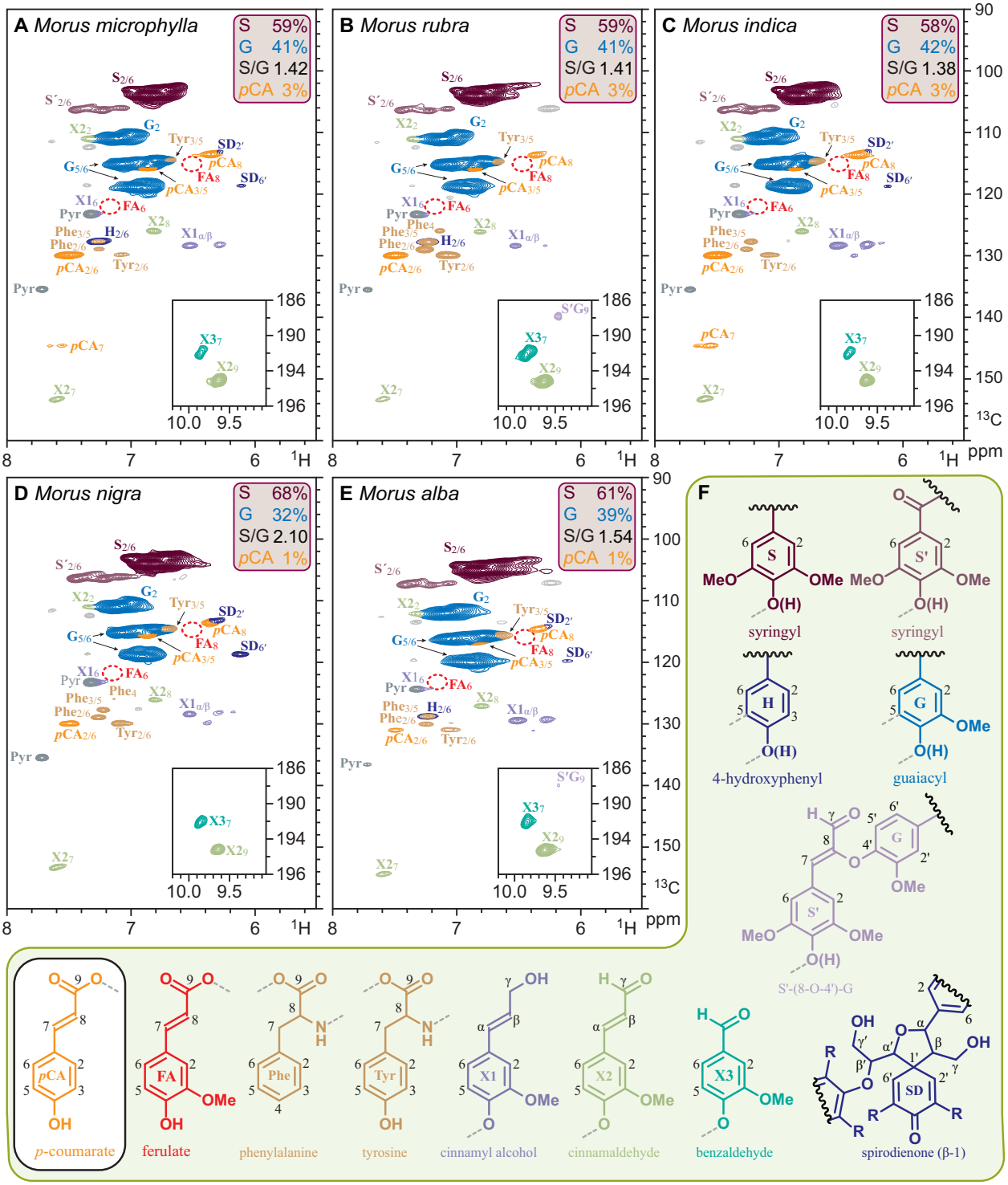

Figure 2 Aromatic regions of 2D ^{1}H–^{13}C correlation (HSQC) spectra. Spectra were obtained from *Morus* ELs isolated by cellulase treatment of ball-milled cell walls, solubilized in DMSO-d_6/pyridine-d_5 (4:1): A, *M. microphylla*; B, *M. rubra*; C, *M. indica*; D, *M. nigra*; and E, *M. alba*. F, Structures of lignin subunits, phenolate esters, and amino acids that are known to be present in plant cell walls. The substructure units and labels are color-coded to match their assignments in spectra A–E. The relative abundances were calculated on a $^{1}/_{2}\mathbf{S}_{2/6}$ + $\mathbf{G}_{2}$ = 100% basis using $^{1}/_{2}\mathbf{S}_{2/6}$, $\mathbf{G}_{2}$, and $^{1}/_{2}\mathbf{pCA}_{2/6}$.

there is little distinction in the *p*CA present in the species from America versus Asia.

With the discovery of *p*CA on their lignin, the *Morus* species join the small, but growing, list of plant species outside of the commelinid monocots to have lignin-bound phenolate esters. As many eudicot hardwood species and monocots (outside of the commelinids) lack lignin-bound *p*CA, we contend this is an example of convergent evolution and that *p*CA-acylated lignin provides some undetermined evolutionary advantage.

Table 1 Summary of *Morus* characterization data on a 100-g EF wood basis the values represent the mean ± SEM with technical replicates of $n = 3$ for the alkaline saponification assays and $n = 2$ for the DFRC assays

Characteristic	*M. microphylla*	*M. rubra*	*M. indica*	*M. nigra*	*M. alba*
Alkaline saponification (Figure 1)	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF
p-Coumaric acid	23.3 ± 1.2	18.3 ± 1.7	21.0 ± 0.9	17. 7 ± 0.7	15.3 ± 1.0
Ferulic acid	n.d.	n.d.	n.d.	n.d.	n.d.
NMR analysis of isolated lignin (Figure 2)					
S:G[a]	59:41	59:41	58:42	68:32	61:39
S/G ratio	1.42	1.41	1.38	2.10	1.54
*p*CA (%)	3.1	2.9	2.9	0.9	0.7
FA	n.d.	n.d.	n.d.	n.d.	n.d.
Hydroxycinnamaldehydes (%)	0.3	0.8	2.5	1.6	0.5
Hydroxybenzaldehydes (%)	0.2	0.6	1.6	1.5	0.3
Sidechains: A:B:C (β-O-4:β-5:β-β) (β-ether:phenylcoumaran:resinol)	86:6:8	84:10:6	82:10:8	84:7:9	83:9:8
Lignin composition by DFRC (Figure 3)					
MLs:	g/100 g EF	g/100 g EF	g/100 g EF	g/100 g EF	g/100 g EF
4-Hydroxycinnamyl alcohol (H_{OH})	0.25 ± 0.07	0.16 ± 0.06	0.03 ± 0.02	0.03 ± 0.01	0.10 ± 0.03
Coniferyl alcohol (G_{OH})	6.57 ± 1.82	6.02 ± 1.37	4.87 ± 2.28	5.38 ± 1.50	5.60 ± 1.44
Sinapyl alcohol (S_{OH})	13.37 ± 3.44	11.02 ± 2.66	9.73 ± 4.30	14.87 ± 3.77	10.91 ± 2.52
ML conjugates:	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF
Coniferyl *p*CA (G-*p*CA)	23.1 ± 0.0	15.1 ± 0.0	12.7 ± 0.0	11.0 ± 0.0	12.1 ± 0.0
Sinapyl *p*CA (S-*p*CA)	66.1 ± 21.0	76.7 ± 18.3	52.1 ± 29.4	21.0 ± 6.8	20.7 ± 6.3
Coniferyl FA (G-FA)	trace	trace	trace	trace	trace
	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF	mg/100 g EF
Total *p*CA by DFRC	38.7 ± 12.0	39.5 ± 9.6	28.0 ± 15.9	13.9 ± 4.6	14.4 ± 4.5

[a] $^1/_2S_{2/6} + G_2 = 100\%$ ML basis.

Table 2 Identified products from the GC-MS pyrograms (Figure 3) of isolated lignin samples pyrolyzed at 500°C in the presence of TMAH

#	R.T. (min)	Compound	Formula	MW	Source	*M. microphylla*	*M. rubra*	*M. indica*	*M. nigra*	*M. alba*
1	6.91	4-Methylanisole	$C_8H_{10}O$	122.2	H	0.51	0.57	0.25	0.36	0.29
2	8.10	Guaiacol	$C_7H_8O_2$	124.1	G	0.89	0.99	0.64	0.81	1.02
3	9.08	1,2-Dimethoxybenzenel	$C_8H_{10}O_2$	138.2	G	0.93	0.71	0.58	0.60	0.54
4	9.86	5-Methylguaiacol	$C_8H_{10}O_2$	138.2	G	0.71	0.47	0.38	0.58	0.40
5	10.17	4-Methylguaiacol	$C_8H_{10}O_2$	138.2	G	0.85	0.72	0.73	0.79	0.52
6	12.17	4-Ethylguaiacol	$C_9H_{12}O_2$	152.2	G	0.64	0.42	0.53	0.61	0.27
7	12.96	1,2,3-Trimethoxybenzene	$C_9H_{12}O_3$	168.2	S	1.03	0.70	0.70	0.96	0.59
8	14.14	Syringol	$C_8H_{10}O_3$	154.2	S	1.61	0.61	0.74	1.69	1.13
9	14.60	1,2,4-Trimethoxybenzene	$C_9H_{12}O_3$	168.2	G	0.49	0.70	0.46	0.53	0.53
10	15.40	1,2,3-Trimethoxy-5-methylbenzene	$C_{10}H_{14}O_3$	182.2	S	2.60	1.90	2.00	2.51	1.40
11	16.62	Methyl *cis*-isoeugenol	$C_{11}H_{14}O_2$	178.2	G	0.28	0.17	n.d.	n.d.	n.d.
12	16.71	4-Methylsyringol	$C_9H_{12}O_3$	168.2	S	1.40	0.67	1.10	1.60	0.52
13	17.87	3,4-Dimethoxybenzaldehyde	$C_9H_{10}O_3$	166.2	G	2.55	2.51	2.17	1.89	2.17
14	18.20	Methyl *trans*-isoeugenol	$C_{11}H_{14}O_2$	178.2	G	1.82	1.42	1.34	1.39	1.22
15	19.84	1,2,3-Trimethoxy-5-(2-propenyl)benzene	$C_{12}H_{16}O_3$	208.2		0.68	0.47	0.45	0.56	0.41
16	20.63	1-(3,4-Dimethoxyphenyl)ethanone	$C_{10}H_{12}O_3$	180.2	G	1.00	0.93	0.79	0.77	0.86
17	21.56	Methyl 3,4-dimethoxybenzoate	$C_{10}H_{12}O_4$	196.2	G	2.36	3.29	2.35	2.16	2.78
18	22.03	3,4,5-Trimethoxy-benzaldehyde	$C_{10}H_{12}O_4$	196.2		3.20	2.66	2.73	3.16	2.89
19	22.96	cis-2,6-Dimethoxy-4-propenylphenol	$C_{11}H_{14}O_3$	194.2	S	1.74	1.59	1.32	1.08	1.29
20	23.52	trans-2,6-Dimethoxy-4-propenylphenol	$C_{11}H_{14}O_3$	194.2	S	1.78	1.90	1.61	0.73	1.22
21	23.91	1,2,3-Trimethoxy-5-(2-propenyl)benzene	$C_{12}H_{16}O_3$	208.3		2.49	2.04	2.04	2.60	1.98
22	25.33	Methyl 4-methoxy-cinnamate	$C_{11}H_{12}O_3$	192.2	*p*CA	5.07	4.96	4.50	2.67	2.10
23	27.34	Methyl 3,4,5-trimethoxybenzoate	$C_{11}H_{14}O_5$	226.2	S	3.88	4.45	3.38	4.33	3.70
24	39.11	Methyl palmitate	$C_{17}H_{34}O_2$	270.5		2.22	3.74	3.81	2.66	3.85

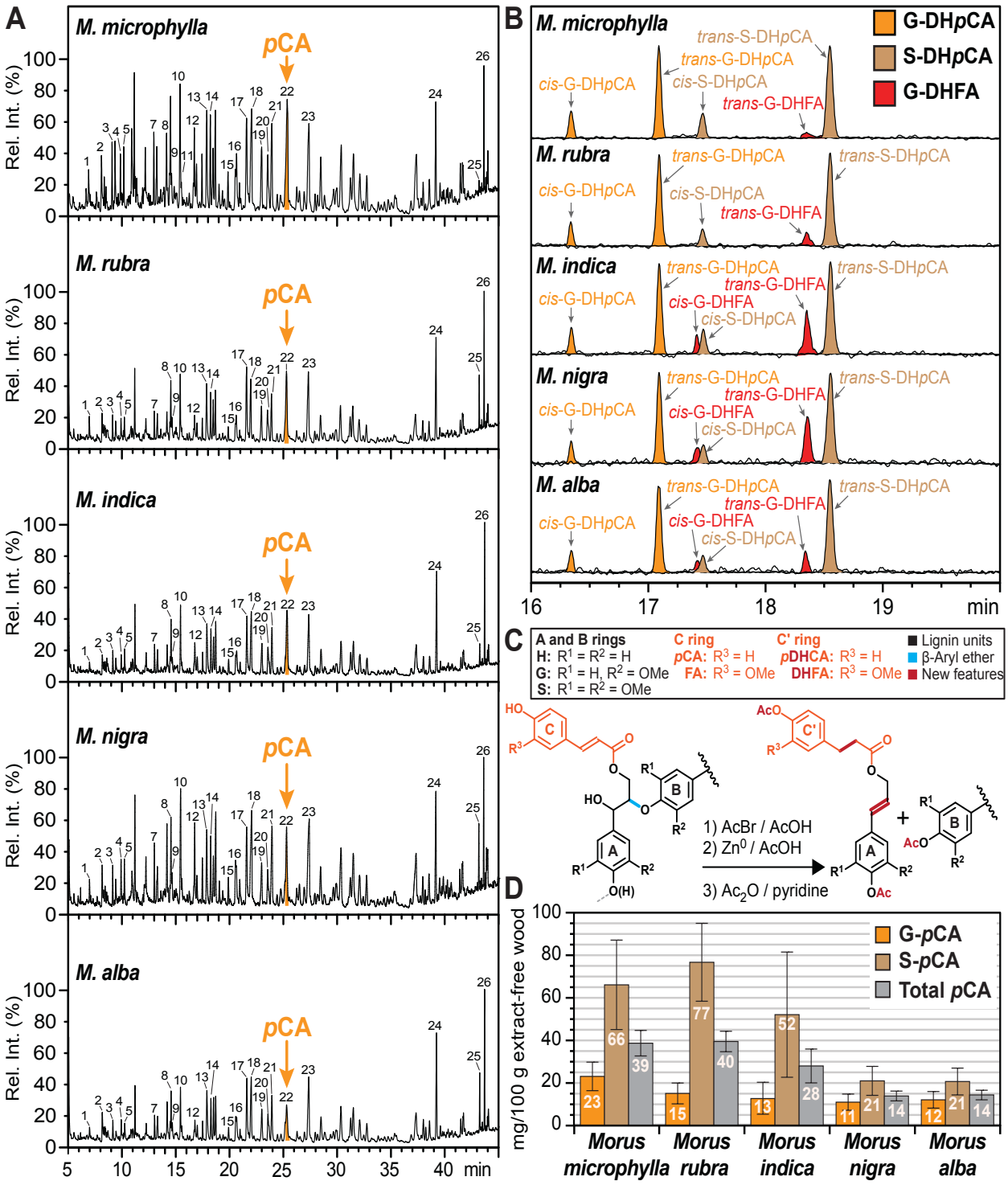

Figure 3 Chemical characterization by pyrolysis–GC–MS and DFRC confirming the presence of *p*CA. A, GC–MS pyrograms of *Morus* lignins pyrolyzed at 500°C in the presence of TMAH. Labeled peaks correspond to compounds identified and reported in Table 2. B, GC–MRM–MS chromatograms of the released products diagnostic for lignin-bound *p*CA (quantified as ML-DH*p*CA) with MRM transitions: G-DH*p*CA 370 > 131 *m/z* and S-DH*p*CA 400 > 161 *m/z*, and lignin-bound FA (quantified as DHFA) with MRM-transition G-DHFA 358 > 163 *m/z*. C, Schematic for the DFRC lignin depolymerization process. D, ML *p*CA abundance as determined by DFRC (detected as G-DH*p*CA and S-DH*p*CA then converted to G-*p*CA and S-*p*CA), and the calculated amount of lignin-bound *p*CA as determined from the DFRC product mixture. Error bars indicate SEM for $n = 2$ technical replicates.

Materials and methods

All chemicals used were purchased from Sigma-Aldrich unless otherwise specified.

Plant material and EF wood preparations

The black mulberry (*M. nigra*), Indian mulberry (*M. indica*), and Texas mulberry (*Morus microphylla*) samples were debarked, cut into small pieces, and freeze-dried. The white mulberry (*M. alba*) and red mulberry (*M. rubra*) samples were received as dried wood and were used as received. The EF samples were prepared as described in the Supplemental Methods.

Enzyme-lignin preparation

A fraction of the dried EF wood (750 mg) was ball-milled in 20 mL agate jars with 10 × 10 mm agate ball-bearings using a Fritsch Pulverisette 7 planetary ball mill operating at 600 rpm for 35 cycles of 10-min grinding, followed by 5-min breaks to avoid excessive heating. The ball-milled samples (650 mg) were suspended in 25.5 mM acetate buffer pH 5.0 (45 mL), crude cellulases (Cellulysin, Calbiochem, 20 mg) were added, and the samples were shaken at 250 rpm for 3 days at 35°C. They were then pelletized (10 min at 1,777 rcf on an Eppendorf 5810R). The acetate buffer was decanted and the enzymatic digestion was repeated. After two digestion cycles, the samples were washed three times by suspending the solids in the water, pelleting, and decanting the wash water. Finally, the samples were lyophilized to produce the enzyme-lignin (EL).

HPLC quantification of phenolic acids released by alkaline hydrolysis

EF wood (10–15 mg) was added to 2 mL screw-top vials (Sarstedt AG & Co., P/N: 72.694.600). The samples were spiked with *p*-anisic acid (312.6 µg) and treated with 2 M sodium hydroxide (1 mL). The samples were then heated to 90°C for 90 min. They were then acidified with 72 wt% sulfuric acid (100 µL) and placed on ice for 5 min to cool. The suspended solids were pelleted using an Eppendorf miniSpin Plus centrifuge operating at 14,000 rpm for 1 min. The supernatant was removed and filtered through a 0.2-µm Nylon filter into a 1.5-mL LC-vial, then analyzed on a Shimadzu Nexera X2 as described in the Supplemental Methods.

2D HSQC (^{1}H–^{13}C) NMR spectroscopy of isolated EL

The 2D HSQC NMR experiments (Kupče and Freeman, 2007) were performed on enzymatically isolated lignins (EL) as previously described (Kim et al., 2008). The lignin (10–20 mg) was dissolved in 500 µL of DMSO-d_6/pyridine-d_5 (4:1, v/v) and analyzed as described in the Supplemental Methods with a Bruker Biospin (Billerica, Massachusetts) NEO 700 MHz spectrometer equipped with a 5-mm QCI ^{1}H/^{31}P/^{13}C/^{15}N cryoprobe with inverse geometry (proton coils closest to the sample). The peak assignments were performed manually based on previously reported correlation peaks (Kim and Ralph, 2010; Kim et al., 2017). The aromatic signals are reported on a $^{1}/_{2}\mathbf{S}_{2/6}$ + $\mathbf{G}_{2}$ = 100% basis and the sidechains are reported on an $\mathbf{A}_{\alpha}$ + $\mathbf{B}_{\alpha}$ + $\mathbf{C}_{\beta}$ = 100% basis, in which **A** = β-ether, **B** = phenylcoumaran, and **C** = resinol.

Pyrolysis of EL in the presence of TMAH

About 100 µg of enzymatically isolated lignin (EL) was treated with 20 µL of 25 wt% TMAH in methanol. The sample was loaded into the Frontier Lab pyrolyzer and suspended above the furnace. The pyrolysis chamber was flushed with helium four times (pressurizing to 18.6 kPa each time) to purge out residual air. The sample was then released from the holder and dropped into the furnace to flash-pyrolyze at 500°C, directly injecting the pyrolysis mixture into the Shimadzu GCMS-2010 equipped with a RXi-5Sil MS column (Restek, 30 m × 0.25 mm × 0.25 µm). See the Supplemental Methods for the GC–MS operating parameters. Peak assignments were validated by comparison to the NIST 2011 GC–MS library and previous lignin pyrolysis literature (Ralph and Hatfield, 1991; Clifford et al., 1995; Rencoret et al., 2018).

Derivatization followed by reductive cleavage

Incorporation of ML conjugates (ML-*p*CA and G-FA) into the lignin was determined using the ether-cleaving ester-retaining DFRC method previously established for ML-OH, ML-*p*CA, and ML-FA conjugates (Lu and Ralph, 1999; Regner et al., 2018). See the Supplemental Methods for the DFRC protocol and the GC-multiple reaction monitoring (MRM) acquisition parameters described in Supplemental Tables S2 and S3.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sidechain regions of 2D ^{1}H–^{13}C correlation (HSQC) spectra.

Supplemental Table S1. DFRC product composition from *Morus* on a 100-g EL basis.

Supplemental Table S2. Chromatographic parameters for GC/MS/MS characterization of DFRC product mix.

Supplemental Table S3. Multiple-reaction-monitoring (MRM) parameters for GC/MS/MS characterization of DFRC product mixes.

Supplemental Methods.

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