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Supplementary Materials for

Developmental changes in lignin composition are driven by both monolignol supply and laccase specificity

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The PDF file includes:

Figs. S1 to S13
Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:

Datasets S1 to S3

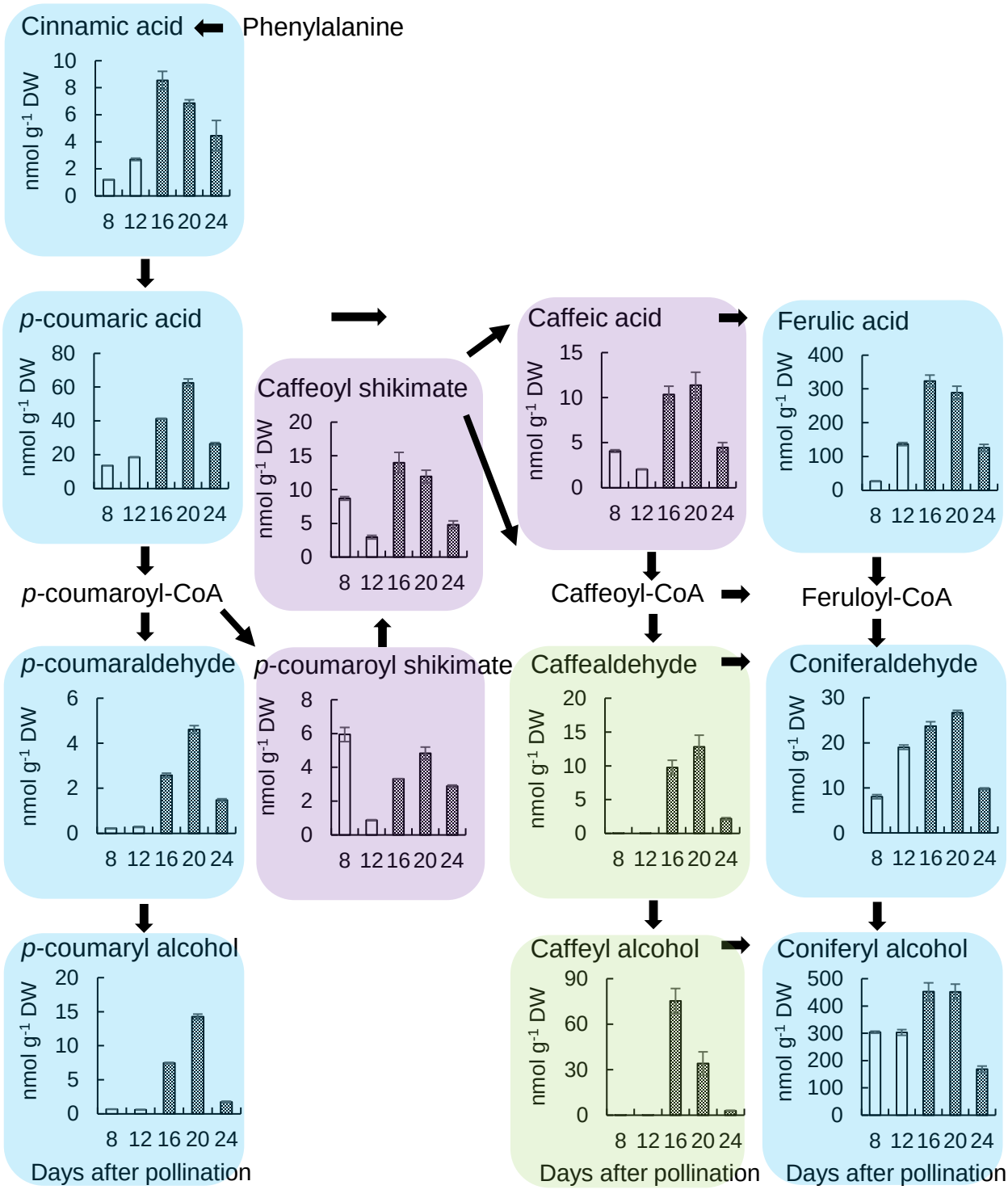


Fig. S1. Monolignol pathway metabolite levels in whole seeds of *Cleome hassleriana* at various stages of development. All the known precursors of G-lignin and predicted precursors of C-lignin, except for the CoA esters of caffeate and ferulate, were profiled by LC-MS analysis in extracts from whole seeds of *C. hassleriana* at the developmental stages shown. Approximately 100 mg of seeds harvested from each individual Cleome plant was counted as one biological replicate. Data are means ± SE derived from three biological replicates. The three distinct patterns of metabolite accumulation are highlighted in blue, purple and green, respectively.

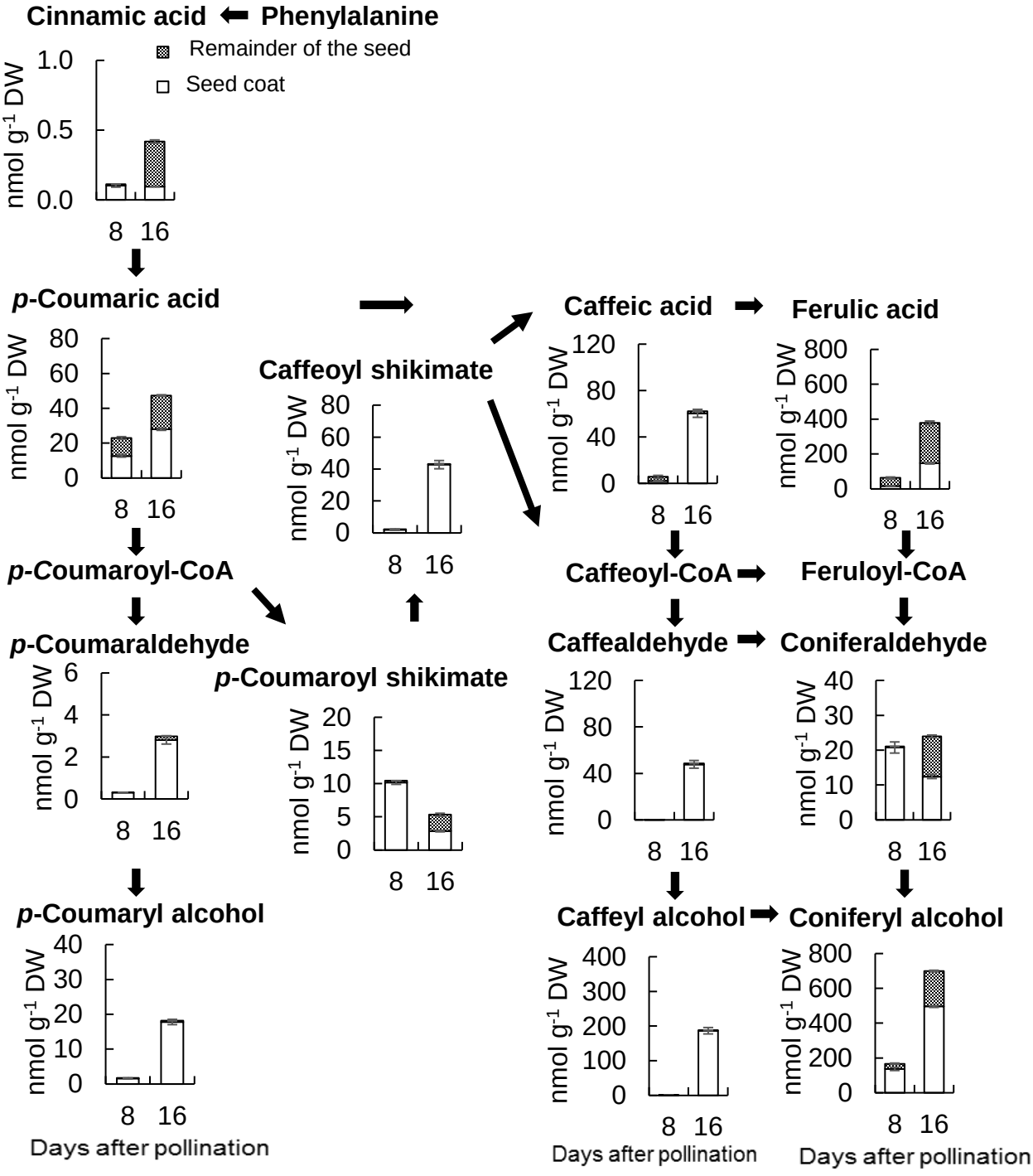
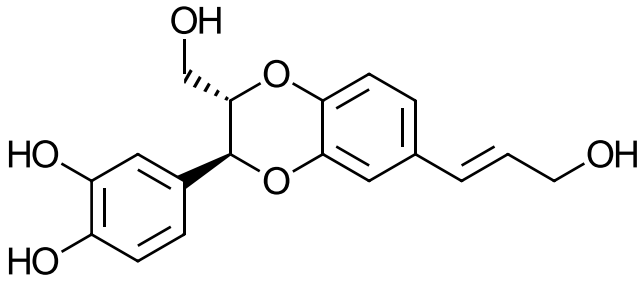
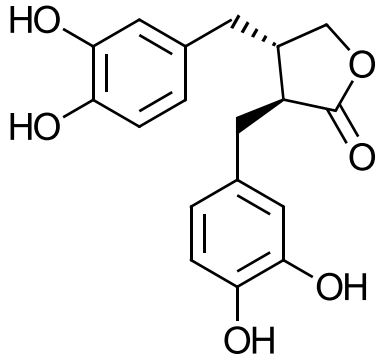


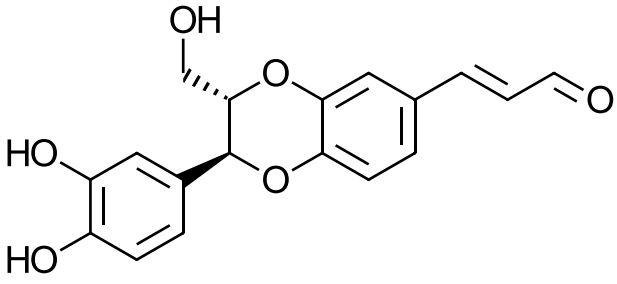
Fig. S2. Monolignol pathway metabolite levels in the seed coat and remainder of the seed at 8 and 16 DAP. All the known precursors of G-lignin and predicted precursors of C-lignin, except for the CoA esters of caffeate and ferulate, were profiled using LC-MS analysis in extracts from *C. hassleriana* seed coats and the remainder of the seeds at the developmental stages shown. Data were calculated as levels in per gram dry weight (DW) of whole seeds. Approximately 100 mg of seeds harvested from each individual Cleome plant was counted as one biological replicate. Data are means ± SE derived from three biological replicates.



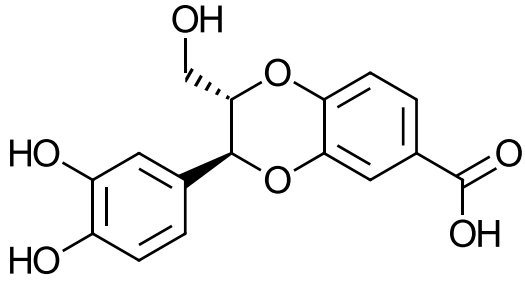
isoamericanol A



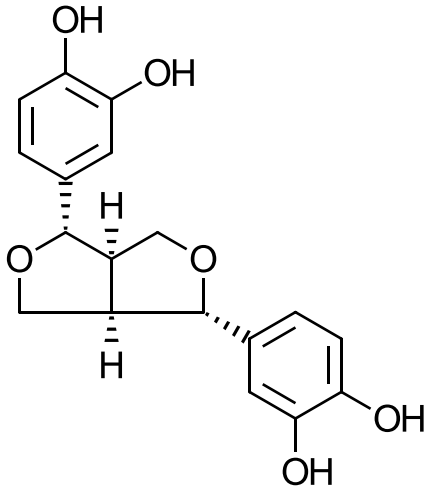
4',4''-Dihydroxyenterolactone



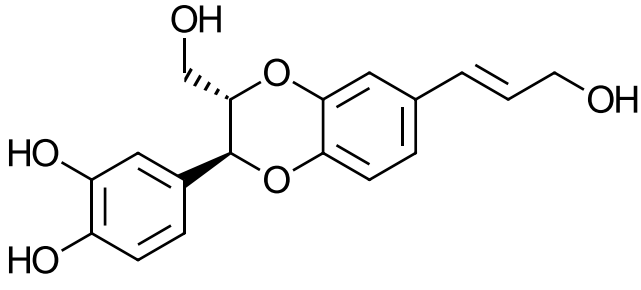
americanin A



iso-caffeyl alcohol-3,4-dihydroxybenzoic acid benzodioxane



(±)-3,3'-Bisdemethylpinoresinol



americanol A

Fig. S3. Structures of the six most abundant lignans in the *C. hassleriana* seed coat. Structures were determined by GC-MS analysis in comparison to chemically synthesized products and literature values.

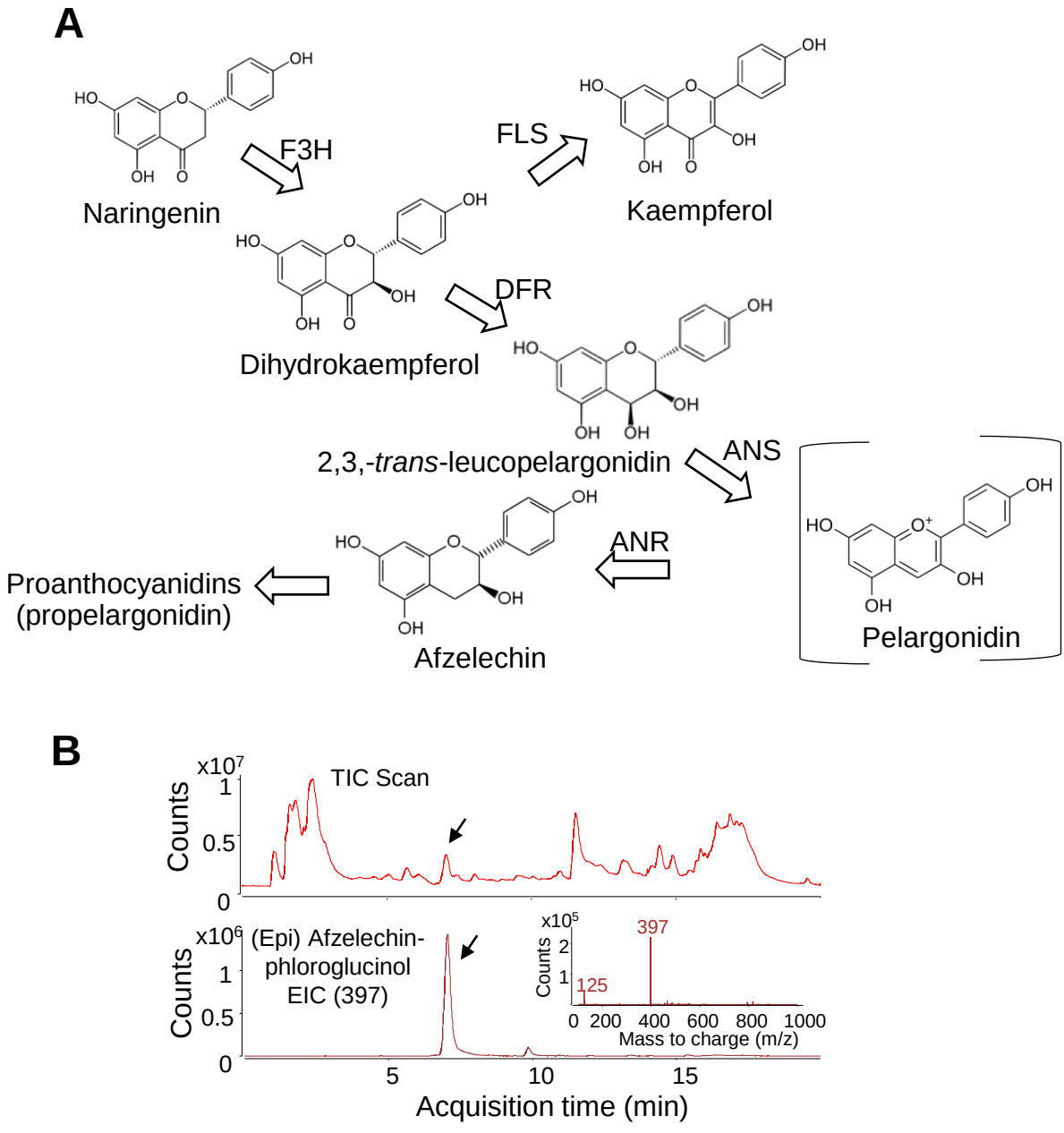


Fig. S4. Biosynthesis of the proanthocyanidin (PA) precursor afzelechin and determination of PA composition in the *Cleome hassleriana* seed coat. (A) Pathway consistent with metabolites identified in the seed coat. Enzymes are: F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase. (**B**) Identification of seed coat proanthocyanidins as afzelechin polymers by identification of the extension unit via phloroglucinolysis. Upper trace; total ion chromatogram (TIC) of insoluble PA after treatment with phloroglucinol. Lower traces; extracted ion chromatograms (EIC) scanned at m/z = 397 showing (epi) afzelechin-phloroglucinol, and mass spectrum of the peak showing the fragment m/z = 397.

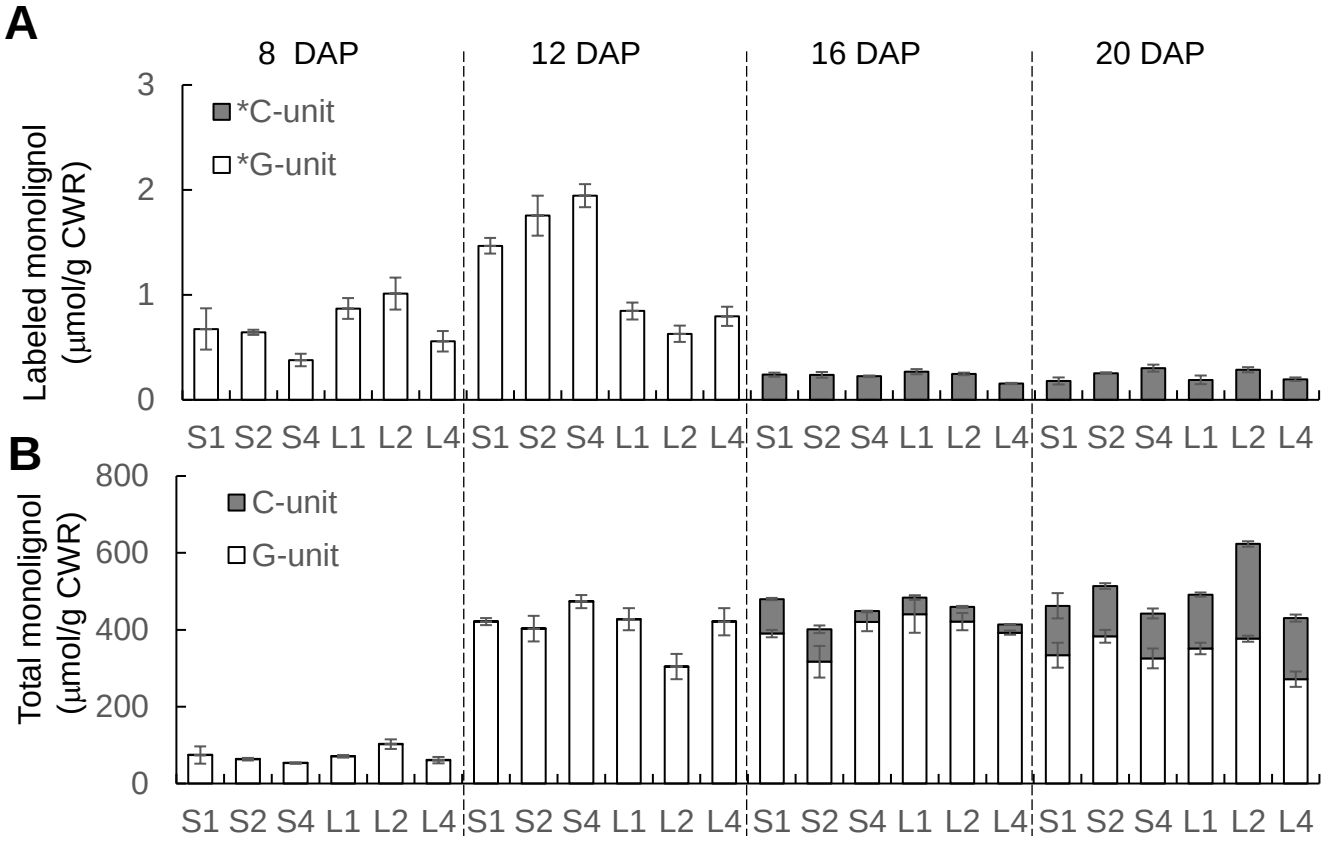


Fig. S5. Comparison of strategies for labeling *Cleome hassleriana* seeds with ^{13}C-phenylalanine for studying G- and C-lignin biosynthesis. (**A**) Labeled monolignol released by thioacidolysis. (**B**) Total monolignol thioacidolysis yield. Whole seeds at different developmental stages were fed with 100 µM $^{13}C_9$-phenylalanine (Phe). Seed coats were harvested 1, 2, or 4 days later for analysis. Labeled and total monolignol components of lignin were measured by thioacidolysis using GC-MS. DAP: days after pollination; S: Seeds were put hilum down into solid MS medium supplemented with $^{13}C_9$-Phe; L: seeds were transferred into liquid MS medium supplemented with $^{13}C_9$-Phe in a 6-well plate on a shaking bath; Numbers after S/L indicate days of feeding.

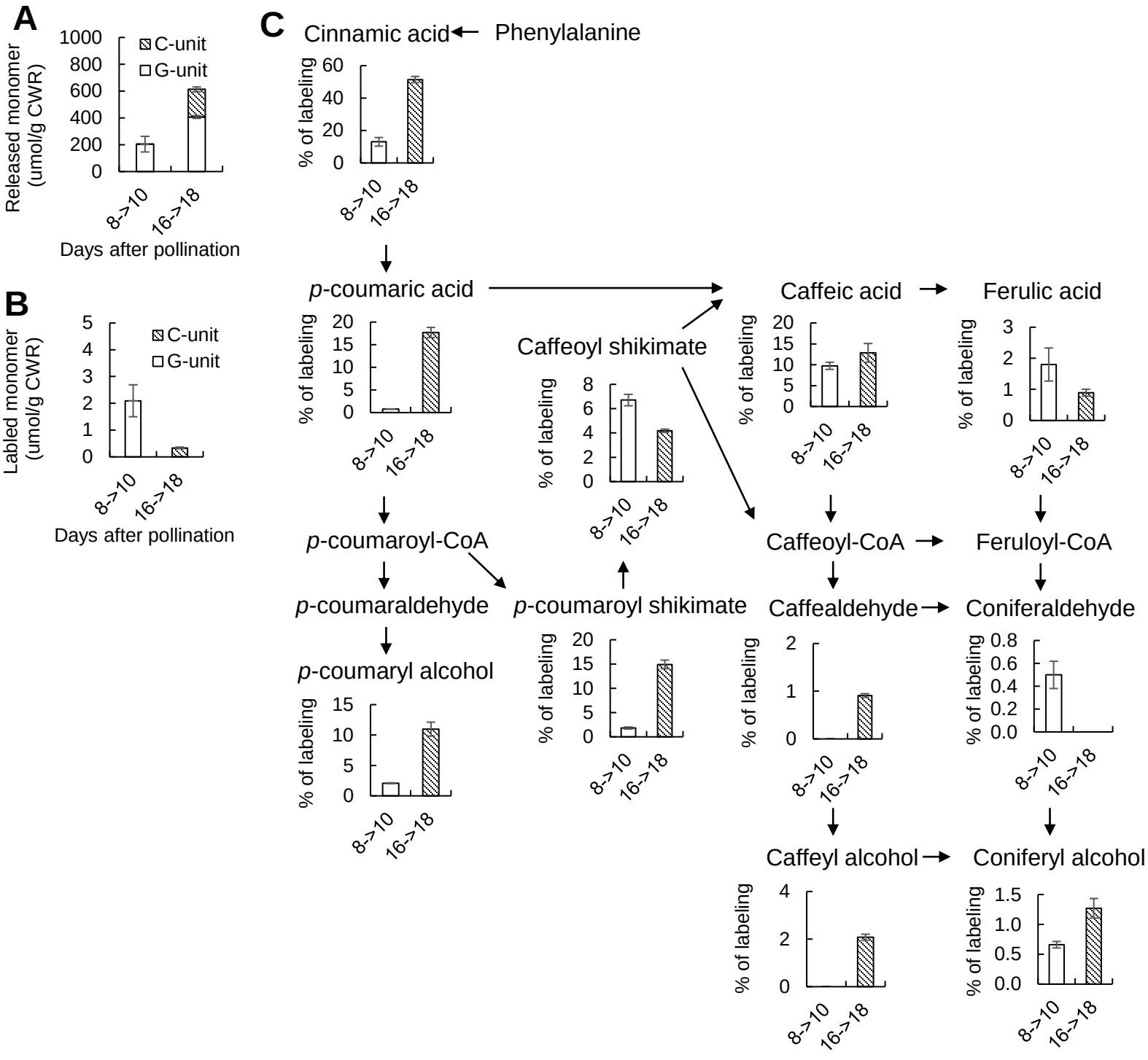


Fig. S6. Labeling of lignin and monolignol pathway intermediates with ^{13}C-cinnamic acid during *Cleome hassleriana* seed coat development. (**A**) Total lignin monomer thioacidolysis yields from isolated cell walls after feeding seed coats with $^{13}C_6$-cinnamic acid for the times shown (days after pollination). (**B**) Total thioacidolysis yields of labeled lignin monomers in the preparations in (**A**). Yields were calculated for the M + 6 ions as described previously (*12*). **(C**) Percentage labeling of individual pathway intermediates as determined by LC-MS analysis. Approximately 100 mg of seed coats isolated from seeds harvested from each individual Cleome plant was counted as one biological replicate. Data are means ± SE derived from three biological replicates.

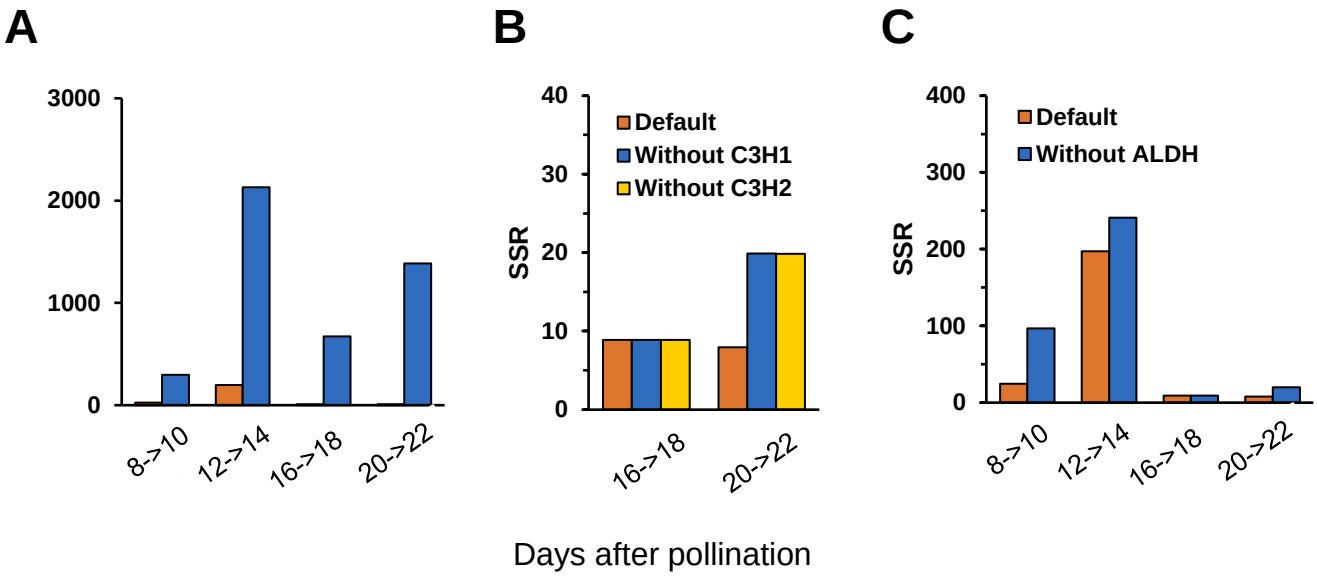


Fig. S7. Testing metabolic flux models for G and C monolignol biosynthesis.
(A) Comparison of sum of squared residuals (SSRs) of the metabolic flux model with (brown) and without (blue) the ad hoc reaction providing unlabeled coumarate.
(**B**) Comparison of SSRs obtained using default model against model without coumaraldehyde to caffealdehyde conversion (C3H1) and model without coumaryl alcohol to caffeyl alcohol conversion (C3H2).
(**C**) Comparison of SSRs obtained using default model against model without aldehyde dehydrogenase (ALDH) reaction.

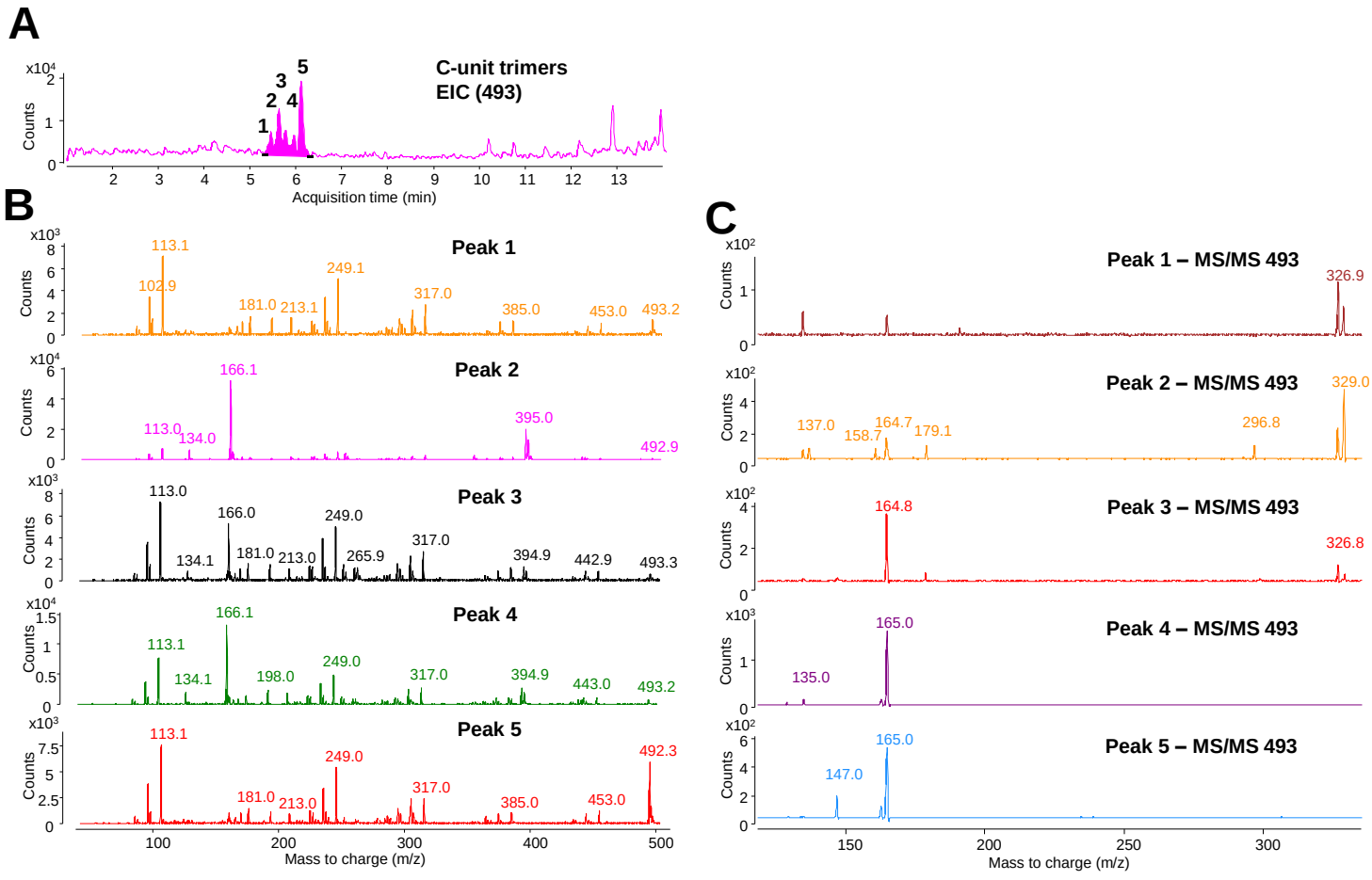


Fig. S8. Identification of caffeyl alcohol-derived trimers generated following incubation of Cleome seed coat cell wall protein extracts with caffeyl alcohol.

(**A**) Extracted ion chromatogram (EIC) showing all caffeyl-alcohol derived trimers at m/z = 493. (**B**) Mass spectra of peaks 1-5 in panel **A.** (**C**) MS/MS analysis of the peaks at m/z = 493 in panel **B.**

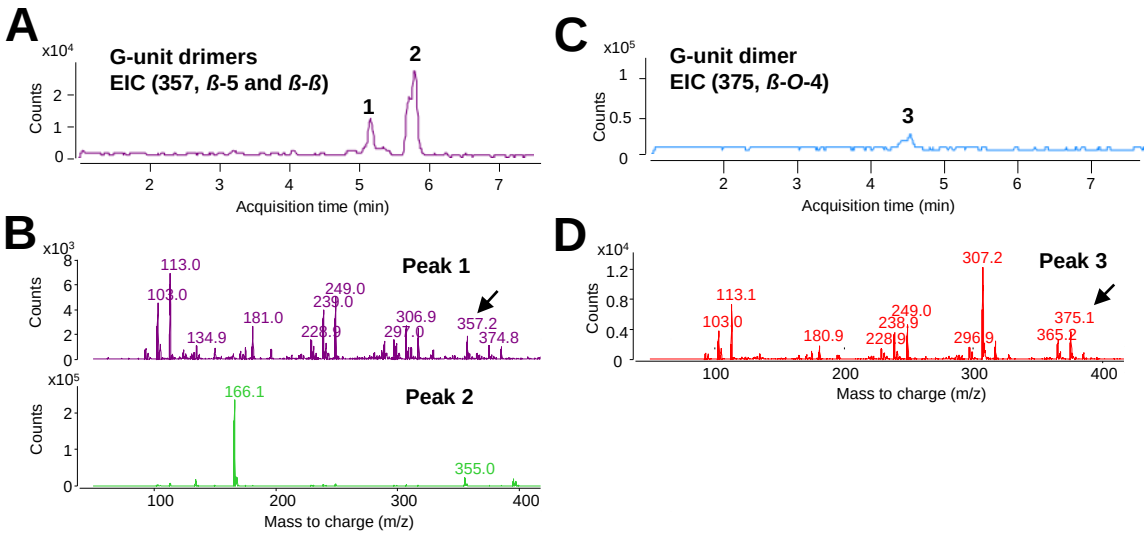


Fig. S9. Identification of coniferyl alcohol-derived dimers generated following incubation of Cleome seed coat cell wall protein extracts with coniferyl alcohol. (**A**) Extracted ion chromatogram (EIC) showing coniferyl alcohol derived dimers at m/z = 357. (**B**) Mass spectra of peaks 1 and 2 in panel **A.** (**C**) EIC showing coniferyl alcohol derived dimers at m/z = 375. (**D**) Mass spectrum of *β-O*-4 linked coniferyl alcohol dimer.

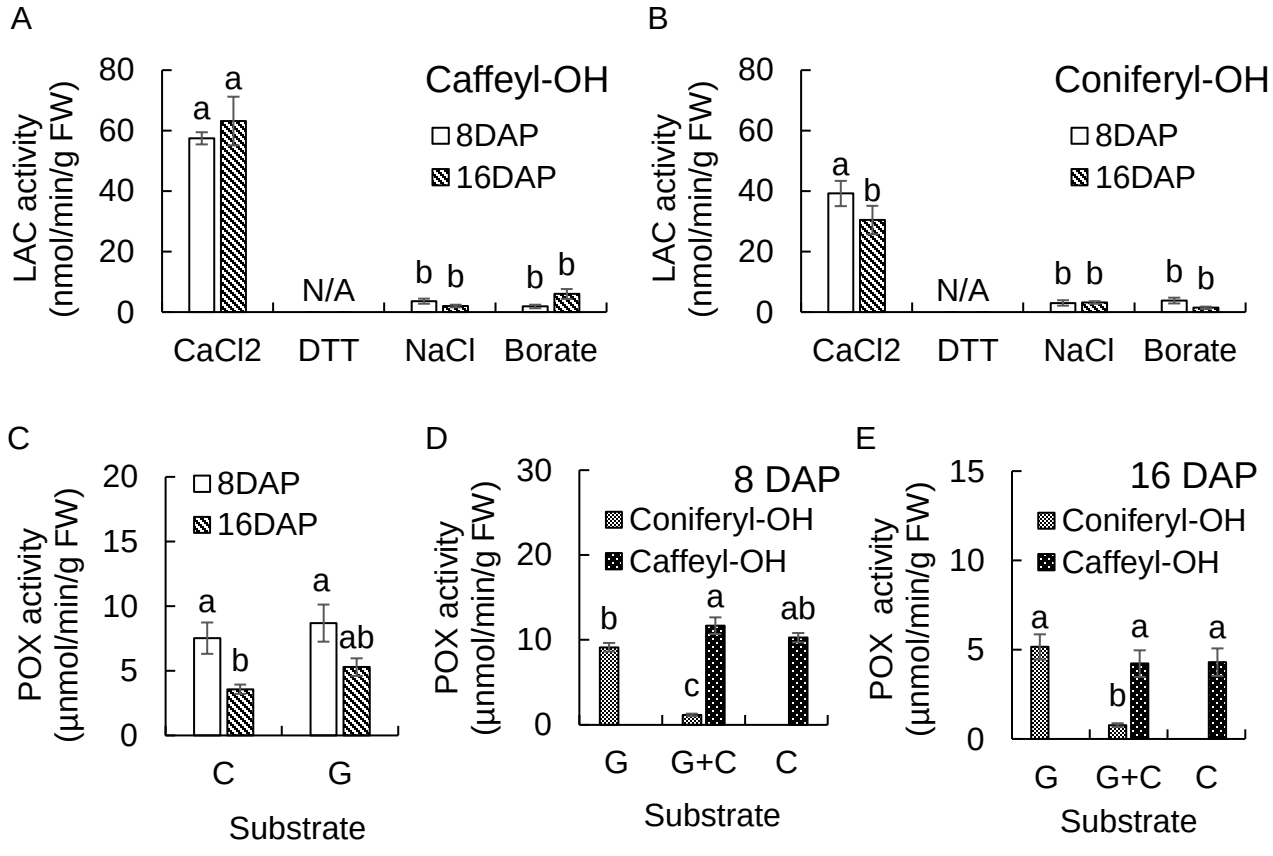


Fig. S10. Laccase and peroxidase activities in the *C. hassleriana* seed coat. (**A, B**) Laccase (LAC) activity against caffeyl and coniferyl alcohols in cell wall protein extracts from Cleome seed coat at 8 and 16 DAP. Cell wall proteins were extracted sequentially with 1 M $CaCl_2$, 2 mM DTT, 2 M NaCl, and 0.2 M borate. Laccase activities were measured in the presence of catalase to inhibit peroxidase as described in Methods. (**C**) Peroxidase (POX) activities against caffeyl and coniferyl alcohols in the $CaCl_2$ extracts from Cleome seed coat cell walls at 8 and 16 DAP. Peroxidase activity was measured in the presence of H_2O_2 as described in Methods. (**D,E**) Effects of caffeyl alcohol on the oxidation of coniferyl alcohol, and vice versa, by peroxidase enzymes in $CaCl_2$ extracts from Cleome seed coat cell walls at 8 and 16 DAP. Activity was calculated by substrate disappearance monitored by HPLC. Approximately 300 mg of seed coats isolated from seeds harvested from each individual Cleome plant were counted as one biological replicate. Data are means ± SE derived from three biological replicates. The different letters above the bars represent statistically significant differences determined by ANOVA (Duncan, $p \leq 0.05$) with SPSS Statistics (version 27; IBM).

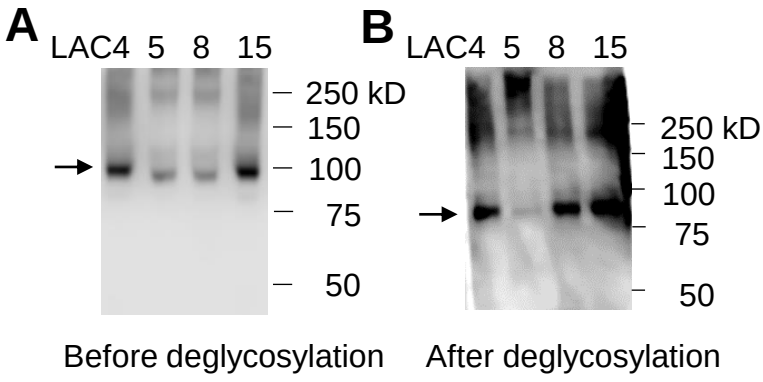


Fig. S11. Expression of laccases in *Nicotiana benthamiana*. ChLACs 4, 5, 8 and 15 were fused with GFP and 6x His tags using the vector pEarleyGate103. Recombinant LAC proteins purified from infiltrated tobacco (*Nicotiana benthamiana*) leaves were detected by western blot using anti-GFP antibody before (**A**) and after (**B**) deglycosylation by PNGase F. The molecular weights of the four ChLACs, GFP and 6x His are 62, 64, 65, 63, 27 and 1 kDa, respectively.

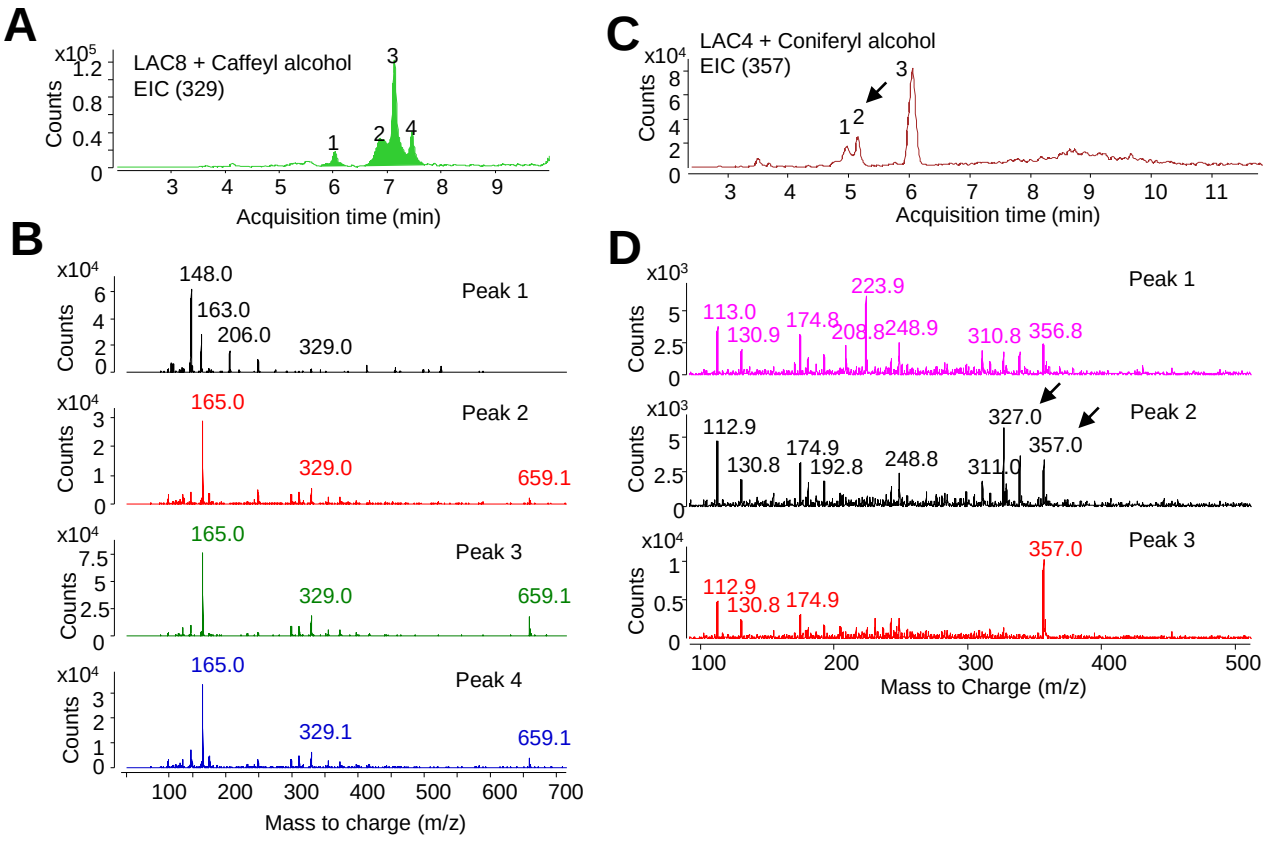


Fig. S12. Identification of caffeyl/coniferyl alcohol-derived dimers generated following incubation of recombinant ChLACs 8 and 4 with monolignols. (**A**) Extracted ion chromatogram (EIC) of caffeyl alcohol dimers at m/z = 329. (**B**) Mass spectra of peaks 1-4 in panel A. (**C**) Extracted ion chromatogram (EIC) of coniferyl alcohol dimers at m/z = 357. (**D**) Mass spectra of peaks 1-3 in panel C.

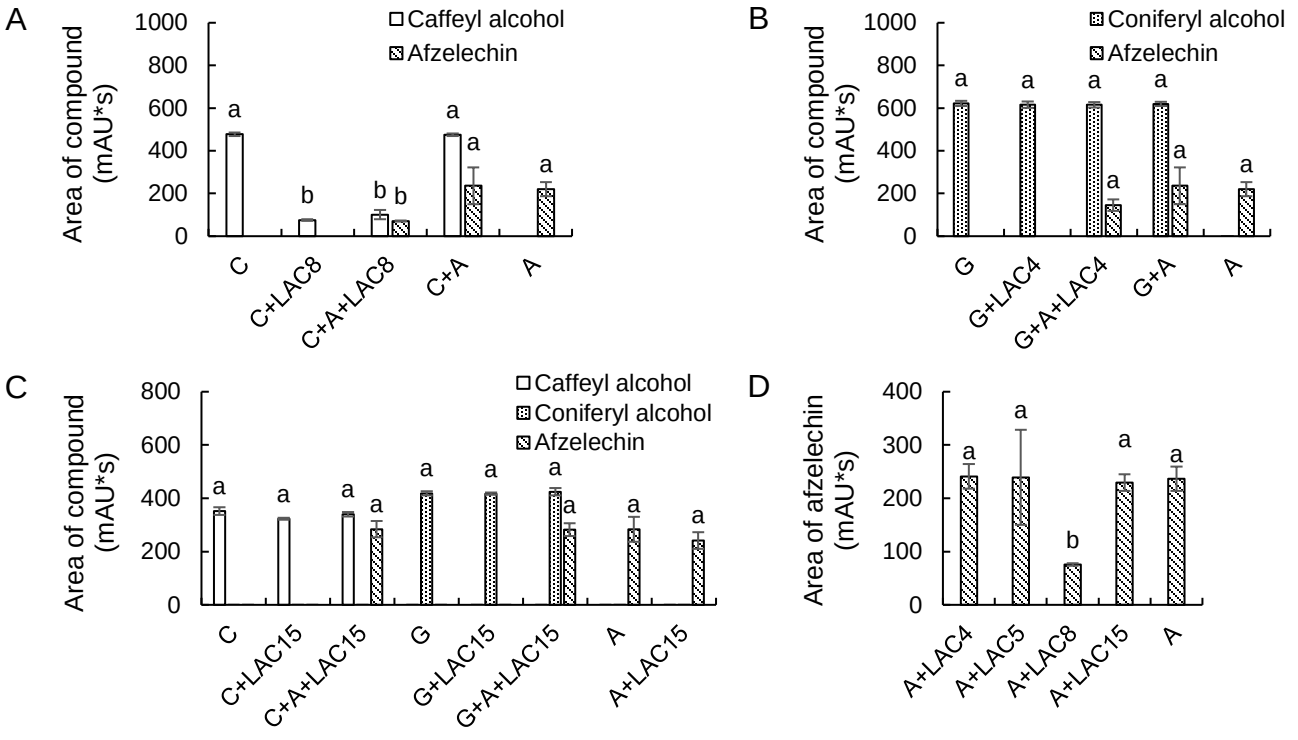


Fig. S13. Co-oxidation of monolignols and afzelechin by recombinant Cleome laccases. (**A**) Effects of afzelechin on the oxidation of caffeyl alcohol by ChLAC8. (**B**) Effects of afzelechin on the oxidation of coniferyl alcohol by ChLAC4. (**C**) Effects of afzelechin on the oxidation of caffeyl and coniferyl alcohols by ChLAC15. (**D**) Oxidation of afzelechin by ChLACs 4, 5, 8 and 15. Data are the areas of the compound peaks as determined on HPLC. C, caffeyl alcohol; A, afzelechin; G, coniferyl alcohol. Reactions (100 mL) contained 50 µM of each substrate and 300 ng of purified recombinant protein. Recombinant LAC protein purified from infiltrated tobacco (*Nicotiana benthamiana*) leaves harvested from three plants was counted as one biological replicate. Data are means ± SE derived from three biological replicates. The different letters above the bars represent statistically significant differences determined by ANOVA (Duncan, $p \leq 0.05$) with SPSS Statistics (version 27; IBM).

Annotation	8DAP	10DAP	12DAP	14DAP	16DAP	18DAP	Stem	Bark	Fiber	Pith
laccase-15	219	649	280	209	157	70	0	0	0	0
laccase-2-like	107	72	5	0	0	0	0	0	0	0
laccase-8-like	0	7	66	141	138	40	0	0	0	0
laccase-17.1	138	106	8	1	1	1	22	1	53	1
laccase-4	482	343	20	1	0	0	165	10	311	6
laccase-4-like	175	211	43	2	2	1	66	3	110	3
laccase-5	8	9	28	48	122	171	22	18	79	3
laccase-22-like	27	15	0	0	0	0	142	48	547	11
laccase-17.2	30	20	1	0	0	0	43	2	71	1

Table S1. Cleome laccases expressed in the seed coat. Data are FPKM values extracted from our previously deposited RNAseq data for developing Cleome seed coats and other tissues (*23*; NCBI Sequence Read Archive (SRA) repository, NCBI SRA accession no. SRX4923580-SRX4923609). Candidates with expression pattern consistent with involvement in C-lignin formation are boxed in red. DAP, days after pollination.

Table S2. List of oligonucleotide sequences used in this study.

Oligo name	Sequence (5' -> 3')
ChLAC4 F	ATGGGGCTTTACCGAGTCGGATTTC
ChLAC4 R	GCACTTGGGAAGATCGTTCGGTG
ChLAC5 F	ATGGCGACTCTCAAGAAATCCGTTTC
ChLAC5 R	ACAGACCGGCAAATCTGCAGGC
ChLAC8 F	ATGGCCAGTTTTGAGTGCTTTCTCATC
ChLAC8 R	ATATTGGTCGTACGTAGGATGGTTATAG
ChLAC15 F	ATGTCACTGCTCAGCGAAGCTCTTTC
ChLAC15 R	ACAAGTTGGCATGTGAGGCGGTG