4-Coumarate 3-hydroxylase in the lignin biosynthesis pathway is a cytosolic ascorbate peroxidase

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Lignin biosynthesis is evolutionarily conserved among higher plants and features a critical 3-hydroxylation reaction involving phenolic esters. However, increasing evidence questions the involvement of a single pathway to lignin formation in vascular plants. Here we describe an enzyme catalyzing the direct 3-hydroxylation of 4-coumarate to caffeate in lignin biosynthesis as a bifunctional peroxidase that oxidizes both ascorbate and 4-coumarate at comparable rates. A combination of biochemical and genetic evidence in the model plants Brachypodium distachyon and Arabidopsis thaliana supports a role for this coumarate 3-hydroxylase (C3H) in the early steps of lignin biosynthesis. The subsequent efficient O-methylation of caffeate to ferulate in grasses is substantiated by in vivo biochemical assays. Our results identify C3H as the only non-membrane bound hydroxylase in the lignin pathway and revise the currently accepted models of lignin biosynthesis, suggesting new gene targets to improve forage and bioenergy crops.
Because of its importance for plant vascular function and stress responses, and the economics of the food, paper, pulp, and biofuel industries, the biosynthesis of the cell wall polymer lignin is one of the most intensively studied areas of plant biochemistry. Lignin is predominantly composed of three monomers known as monolignols (p-coumaryl, coniferyl, and sinapyl alcohols), which are polymerized in the apoplast of vascular and fiber cells leading to the formation of the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of lignin, respectively. The 3-hydroxylation of the 4-coumarate moiety diverts flux from H toward G and S lignin, and this reaction is now generally accepted to occur at the level of the shikimate ester of 4-coumarate (Fig. 1). However, the facts that some grass species lack orthologs of caffeoyl shikimate esterase (CSE) and that reduced expression of some other pathway enzymes has less than expected to no phenotypic effect in grasses, along with paradoxical results of metabolomics and labeling studies in Arabidopsis, support the existence of an alternative pathway to the currently accepted route involving phenolic esters. A suggestion that the coumarate 3-hydroxylase (C3H) reaction is catalyzed by a complex of two membrane-bound cytochrome P450 enzymes, the coumaroyl shikimate 3-O-methyltransferase (CCoAOMT) caffeoyl CoA 3-O-methyltransferase, C3H hydroxylase, and cinnamoyl CoA reductase, CAD, cinnamyl alcohol dehydrogenase, and cinnamyl alcohol dehydrogenase, has been proposed by recent localization studies. Instead, early biochemical studies suggested that C3H was a soluble phenolase that required molecular oxygen and a reducing agent such as ascorbate to oxidize a wide variety of substrates, including free 4-coumarate. Although enzymes with similar properties were subsequently reported in monocot and dicot plants, in some cases with high specificity for 4-coumarate, C3H remains genetically and functionally uncharacterized.

In this study, we identify and characterize a bifunctional cytosolic ascorbate peroxidase as C3H, the missing link in the conventional phenylpropanoid pathway involving free phenolic acids.

Results

Identification and characterization of C3H. A previously described protocol with minor modifications (Supplementary Fig. 1) was used to assay C3H activity in crude protein extracts prepared from tissues of several plant species, and the highest activities were found in maize and Brachypodium (Fig. 2a), both of which lack CSE orthologs. The highly active maize root extract was subjected to further characterization (Fig. 2b). C3H activity was detected in the soluble but not the microsomal fraction. The activity was ascorbate dependent, completely inhibited by 2-mercaptoethanol, dithiothreitol and reduced glutathione, and...
decreased by 24% when incubated under N2. Addition of exogenous hydrogen peroxide completely abolished activity in crude extracts by causing depletion of ascorbate. NADH, but not NADPH, could partially replace ascorbate as co-factor. Using calibrated gel filtration chromatography, the molecular mass of the native maize root C3H was estimated to be 20–33 kDa (Fig. 2c). The presence of an earlier peak co-eluting at approximately 320 kDa with tyrosine ammonia-lyase (TAL) activity...
suggested that maize C3H may form part of a protein complex with other lignin pathway enzymes. The major band correlating with the C3H activity in the main peak of the partially purified C3H was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2d) followed by in-gel digestion and peptide mass fingerprinting (Fig. 2e). The band showed the expected size of C3H, and the sequence was matched to a 27.37 kDa cytosolic ascorbate peroxidase (APX1, GRMZM2G137839), with 10 peptides covering about 52% of the maize sequence (Fig. 2f).

In *Arabidopsis*, the APX family is represented by eight members, three cytosolic, three microsomal, and two chloroplastic. Using a phylogenetic approach, we identified putative C3H orthologs in a wide range of plant species (Supplementary Fig. 2). Purified and hemin reconstituted recombinant C3H from *Arabidopsis* and *Brachypodium* catalyzed both APX and C3H reactions in vitro with similar overall catalytic efficiencies (Supplementary Table 1). Recombinant C3H also hydroxylated l-tyrosine to form l-DOPA, with similar overall catalytic efficiencies (Supplementary Table 1).

Phenotypes of a *Brachypodium* c3h mutant. To provide genetic evidence for a role of C3H in lignification, we first searched for *Brachypodium* mutants available from the JGI collection (https://jgi.doe.gov)21. Line J25124 was identified as an activation tagged line with the T-DNA insertion in the last intron, 121-bp upstream of the stop codon of C3H (Bradi1g65820) (Fig. 3a). The activation tagged line J16575, which has the T-DNA insertion in the last exon of the APX3 gene (Bradi3g42340) encoding a microsomal APX was used as T-DNA control. Relative C3H transcript levels in the c3h lines were 30–40% lower than in the controls (Fig. 3b), and the extractable C3H activity was reduced (~40%), associated with reduced C3H protein amount determined by immunoblotting (Fig. 3b). The most plausible explanation for C3H downregulation in the c3h lines is the reported high frequency of methylation of the quadruple 35S enhancer sequence leading to its transcriptional silencing in T-DNA-based tagging populations22. The *Brachypodium* c3h lines showed a stunted bushy growth phenotype, delayed senescence, and few or non-viable seed (Fig. 3c). The lignin levels determined either by lignin autofluorescence, phloroglucinol staining, thioacidolysis, or reaction with acetyl bromide were significantly reduced when compared with controls, mainly from a reduction in lignin in fiber cells (high in S lignin) (Fig. 3c, d and Supplementary Table 3).

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Phenotypes of *Arabidopsis* c3h mutants. To investigate whether C3H plays a role in lignification in the dicot *Arabidopsis*, we first studied the SALK_000249 mutant line that has a T-DNA insertion in intron 7 of the C3H gene (At1g07890), 160-bp upstream of the stop codon (Fig. 4a). This line displays residual APX1 transcript levels (~15%–30%) and APX activity (~30%), but undetectable levels of C3H/APX1 protein as determined previously by immunoblotting.23,24 APX-deficient mutant lines have been widely characterized in relation to the role of APXs in H2O2 detoxification and multiple abiotic stresses in both monocots and dicots.25–27 The *Arabidopsis* c3h1/apx1 mutant showed similar lignin autofluorescence, phloroglucinol staining, and lignin thioacidolysis yield as wild-type plants (Fig. 4a, b). However, metabolomic and published transcriptomic25 analyses of responses to light stress in the c3h1/apx1 mutant revealed rapid reduction of caffeate levels, along with increased flavonoid glucoside levels and upregulated transcript levels of 4-coumarate:coenzyme A ligase (4CL) and flavonoid glucosyltransferases when compared with wild-type plants (Supplementary Fig. 7). These data support placement of C3H at the interface between 4-coumarate and caffeate in the initial steps of phenylpropanoid biosynthesis in *Arabidopsis*.

To further address the contribution of the acids pathway to lignification in *Arabidopsis*, we crossed the single cse2 and c3h1 mutants, generated their F1 progeny, and subsequently screened their F2 populations for cse2/c3h1 double mutants. After genotyping >300 F2 plants, no double mutants could be recovered. We were only able to identify genotypes either homozygous for c3h and heterozygous for cse (c3h1CSE2+/−) or homozygous for cse and heterozygous for c3h (cse2C3H1+/−) (Fig. 4c), which exhibited weakly reduced lignin levels (Supplementary Fig. 8a, b). In contrast to wild-type, the siliques of these c3h1CSE2+/− and C3H1+/−cse2 mutants contained a higher proportion of aborted seeds, close to the expected 3:1 ratio for segregation of the double homozygote (Fig. 4d, e and Supplementary Table 2). To overcome the problem of the lethal phenotype of the double homozygote, we generated RNA interference (RNAi) lines to target each gene independently in the opposite mutant background (Supplementary Fig. 9a, b). Both cse2 mutant/C3H1–RNAi and c3h1 mutant/CSE1–RNAi lines were recovered and exhibited growth defects and reduced lignin deposition when compared with their respective T-DNA mutant only controls (Fig. 4f–i, and Supplementary Table 3). Because the c3h1/apx1 mutant retains residual C3H activity, we obtained all other available c3h1 mutant alleles from the Arabidopsis Biological Resource Center (ABRC), including SALK lines 088596 (c3h2), 095678C (c3h4), and 143111 (c3h3) with the T-DNA insertions in the last intron, 3’-UTR region and fourth exon, respectively. In agreement with a previous report,28 no homozygous plants were found for c3h3, so heterozygous c3h3+/− plants were characterized along with homozygous c3h1, c3h2, c3h4, and wild-type controls (Supplementary Fig. 10). When compared with the c3h1/apx1 mutant, the other c3h1 mutant alleles showed downregulated C3H (10–30%) and CSE (30–45%) transcript levels, and increased anthocyanin content in leaves, increased numbers of xylem vessels, and reduced total...
Fig. 3 Phenotypic characterization of *Brachypodium* c3h mutants. **a** Activation tagging construct pJJ2LBA and diagram of the T-DNA insertion in line JJ25124 (http://jgi.doe.gov). **b** Relative expression by qPCR, extractable activity and protein level by immunoblotting in c3h mutants compared with wild-type and T-DNA control line JJ22251 (apx3). The antibodies raised against C3H showed no cross-reactivity and detect a band of 29.5 kDa. Line numbers are indicated in each lane. The full uncropped gel and blot shown are provided as a Source Data file. **c** Growth phenotype and transverse stem sections (UV- autofluorescence and phloroglucinol-HCl staining) of c3h mutants and apx3 and wild-type controls. **d** Total lignin (upper panel), S/G ratio (middle panel), and relative monolignol composition (lower panel) determined by thioacidolysis in c3h mutants and apx3 and wild-type controls. **e** Correlation plots for C3H activity with total lignin amount and individual lignin monomers. **f** Metabolite concentrations in mature stems of c3h mutants compared with apx3 and wild-type controls. Lignans were recognized from their fragmentation patterns. H/G lignan is 14.58 267 297 hydroxyphenyl guaiacyl lignan; G-lignan is 15.88 297 411 323 guaiacyl lignan, and S-lignan is 16.14 327 361 239 syringyl lignan glycoside (the first number is the retention time in min and the others are key mass-to-charge ratios, m/z). CWr cell wall residue. Error bars indicate mean ± SD, two-sided unpaired t-test. The R squared value ($R^2$) was calculated from the linear regression model using Excel. Data points for all biological replicates are shown.
siliques (segregation ratios estimated in Supplementary Table 2).

Visual phenotype and transverse stem sections (UV-auto fluorescence and phloroglucinol-HCl staining) of Arabidopsis c3h1 mutants in both Col-0 and Wassilewskija (Ws) backgrounds. c Screening for Arabidopsis c3h1/cse2 double mutants. T- and G- are T-DNA and gene specific primers used for genotyping. No c3h1/cse2 double mutants were obtained from over 300 F2 plants screened, and so cse2C3H1+/− (i.e. homozygous for cse2 and heterozygous for c3h1; lanes 4, and 11) and c3h1CSE2+/− (i.e., homozygous for c3h1 and heterozygous for cse2; lanes 5 and 14) were subsequently generated and their lignin content and composition estimated (Supplementary Fig. 8). The full uncropped gels are provided as a Source Data file. d Aborted seeds in c3h1CSE2+/− and cse2C3H1+/− mutant lines determined in 8 to 18 individual siliques (segregation ratios estimated in Supplementary Table 2). e Comparison of the self-fertilized F3 seeds of mutants and WT plants by light microscopy in young (middle panel) and mature (right panel) siliques. The left panel shows a magnification of the aborted ovules (circles) in cse2C3H1+/− mutants. f Visual phenotype and transverse stem sections (UV-autofluorescence and Mäule staining) of c3h1/CSEi-RNAi lines and c3h1 controls. g Total lignin amount and composition of c3h1/CSEi-RNAi lines and c3h1 controls. h Visual phenotype and transverse stem sections (UV-autofluorescence and Mäule staining) of cse2/C3H1-RNAi lines and cse2 controls. i Total lignin amount and composition of cse2/C3H1-RNAi lines and cse2 controls. Mäule staining protocol was used in f and h to assess lignin distribution and composition. CWr cell wall residue. Error bars indicate mean ± SD, two-sided unpaired t-test. Data points for all biological replicates are shown

Fig. 4 Phenotypic characterization of Arabidopsis c3h1 mutants. a Position of the T-DNA insertion, mature Arabidopsis plants and transverse stem sections (UV-autofluorescence and phloroglucinol-HCl staining) of c3h1 mutants and wild-type controls. b Lignin levels (upper panel) and relative monolignol composition (lower panel) determined by thioacidolysis of c3h1 mutants in both Col-0 and Wassilewskija (Ws) backgrounds. c Subsequent O-methylation of phenolic acids/esters. Theoretically, caffeate can be either converted to caffeoyl CoA by 4CL, with subsequent O-methylation by caffeoyl CoA 3-O-methyltransferase (CCoAOMT), or first be methylated by caffeic acid

stem lignin and S/G ratios (Supplementary Fig. 10a–d). These results are consistent with the phenotype observed above for the c3h lines in Brachypodium, and indicate that the homozygous c3h3−/− null mutation is lethal in Arabidopsis (Col-0).

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Fig. 5 Biochemical assays to study the fate of caffeate in Brachypodium and Arabidopsis. a Specific activities of individual caffeate 3-O-methyltransferase (COMT) and 4-hydroxycinnamate-CoA ligase (4CL) reactions in crude stem protein extracts from 1-month-old Brachypodium and Arabidopsis plants, performed at 10 and 50 μM caffeate concentrations (left panel), and double reactions performed by co-incubating caffeate with both cofactors required to perform the CoA activation (CoA + ATP) and 3-methoxylation (S-adenosyl methionine, SAM) reactions (right panel). The final common product of both parallel activities is feruloyl CoA, b Scheme of the studied reactions including cofactors and showing the proposed most favored pathways in the model monocot Brachypodium (red arrows) and the model dicot Arabidopsis (black arrows). The bar plot displays the activity ratios of competing enzymatic activities for both species and substrate (caffeate) concentrations calculated from the data shown in panel a. c Labeling patterns of lignin monomers in isotopic feeding experiments (m/z, mass-to-charge ratio). d Percentage of 13C-labeled ferulate incorporated into different monolignols (H-, G-, and S-units) and total lignin (T) in roots of Brachypodium and Arabidopsis seedlings. C3H 4-coumarate 3-hydroxylase, CSE caffeoyl shikimate esterase, CCoAOMT caffeoyl CoA 3-O-methyltransferase. Error bars indicate mean ± SD, two-sided unpaired t-test. n = 3

3-O-methyltransferase (COMT), with subsequent conversion to feruloyl CoA by 4CL (Fig. 1). To compare these alternatives in a grass and a dicot, we first determined the specific activities of COMT and 4CL for two different concentrations of caffeate in crude stem protein extracts from actively growing Brachypodium and Arabidopsis plants, performed at 10 and 50 μM caffeate concentrations (left panel), and double reactions performed by co-incubating caffeate with both cofactors required to perform the CoA activation (CoA + ATP) and 3-methoxylation (S-adenosyl methionine, SAM) reactions (right panel). The final common product of both parallel activities is feruloyl CoA, whereas in extracts from Arabidopsis the main products were caffeoyl CoA and feruloyl CoA. Furthermore, the specific activity of maize root C3H towards 4-coumarate was higher than that of the competing reaction catalyzed by 4-hydroxycinnamoyl coenzyme A ligase (4CL) (Supplementary Table 4). The efficient methylation of caffeate by COMT in grains might therefore drive the monolignol pathway through the C3H reaction (Fig. 5b). Consistent with the greater importance of the acids pathway in monocot grasses compared with dicots, we observed higher incorporation of 13C-labeled ferulate into the G-units of lignin in Brachypodium when compared with Arabidopsis (Fig. 5c, d). Furthermore, the lack of homologs of CSE, which is by-passed by the C3H reaction, seems to be phylogenetically associated with monocot plants (Supplementary Fig. 11). Arabidopsis CSE orthologs show ~80% identity among dicots, ~60% identity in non-commelinid monocots, and ~45% identity in most grasses. However, it remains unclear why rice and switchgrass retained CSE-like genes with 62% and 59% identity, respectively.

Discussion
Our data demonstrate that a pathway to monolignols involving free phenolic acids is functional in both monocot and dicot model species. Notably, C3H is the only reported hydroxylase involved in phenylpropanoid biosynthesis that is not a membrane-bound cytochrome P450. Parallel pathways to the caffeoyl derivatives used as building blocks of S and G monolignols (Supplementary Fig. 12) may provide redundancy, and therefore robustness, to the early steps in the lignin pathway, especially as 4-coumaroyl CoA in the esters pathway is also the precursor for stress and developmentally induced flavonoid biosynthesis. These findings revise the current model of the monolignol pathway in plants to include a cytosolic route that, in grasses that possess TAL activity, bypasses both membrane-associated cytochrome P450 reactions involved in H and G lignin synthesis, and place the 3-O-methylation of monolignol precursors by COMT at the level of caffeic acid. This “soluble” pathway may be associated with detoxification of stress-induced reactive oxygen species and link the basic metabolic process of lignin biosynthesis with different stress responses, plant defense, and acclimation pathways. Further
experimental evidence under multiple stress conditions will be necessary to better understand the cross-talk between phenylpropanoid metabolism and oxidative stress in plant cells.

Because of its bifunctionality, it is difficult to differentiate genetically the two activities of C3H/APX as causative of the lignin biosynthesis. The evolutionary acts as an effector of RAC1, a GTPase involved in generation of active oxygen species during defense in rice. The evolutionary implications of these associations remain to be explored.

Methods

Enzyme activity assays.

The crude protein extracts and reaction mixtures used to assay C3H activity were prepared using modified protocol described previously. Crude ammonium sulfate extracts were prepared by grinding in liquid N2 and homogenizing 2 g of fresh plant tissues in 6 ml of 50 mM sodium phosphate buffer pH 6 in the presence of 1 g polyvinylpyrrolidone (PVPP) and 1 g of 0.5 mM acid-washed glass beads. Samples were incubated overnight in a tube rotator at 4 °C. After filtration through four layers of Miracloth (Millipore) and centrifugation for 1 min at 500 g to remove the PVPP and glass beads, ammonium sulfate was added to a final concentration of 0.5 g ml−1. After centrifugation, the pellet was resuspended in one half the original volume in 50 mM phosphate buffer pH 6. The homogenates were assayed directly. The reaction mixtures were optimized to a total volume of 100 µl containing 100 µl of 10 mM sodium phosphate buffer (pH 7.5, 5 µl of 1 M sodium phosphate buffer, 15 µl of the crude protein extracts equilibrated a 0.7 µg µl−1, and was incubated for 2 h at 30 °C with shaking and stopped with 10 µl of acetic acid. Incubating the reaction with phosphate buffer pH > 8 and preparing the crude protein extracts with antioxidants such as dithiothreitol or 2-mercaptoethanol or the stock solutions with dimethyl sulfoxide completely inhibited C3H activity. Stock solutions were made with 50 mM phosphate buffer pH 6 and 4-coumarate was dissolved by sonication and heat (10 min at 96 °C) with shaking.

Previously described protocols were used to assay TAL1, COMT, and 4CL activity. Statistical analysis was done using 0.5–1 g of ground stem tissues. The actively growing shoots of 

Brachypodium and Arabidopsis plants was resuspended in 2.7 ml of extraction buffer (100 mM Tris–Cl pH 7.5, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM diithiothreitol) in the presence of 1 g PVPP. The suspension was incubated in a tube rotator at 4 °C for 45 min. The supernatant was recovered after centrifugation (12,000 x g for 5 min) and desalted using a PD-10 column (GE Healthcare) according to the manufacturer’s instructions. The protein concentrations were estimated with the Bio-Rad protein assay. In order to test COMT-4CL and 4CL-CCoAOMT reactions simultaneously, caffeic acid was co-assayed directly. The reaction mixtures were optimized to a total volume of 100 µl containing 100 µl of 10 mM sodium phosphate buffer, 100 µM caffeic acid, 100 µM acetoacetate, 5 µl of 20 mM L-ascorbate, 7.5 µl of 1 mM sodium phosphate buffer, 15 µl of the crude protein extracts equilibrated a 0.7 µg µl−1, and was incubated at 37 °C for 15 min. The solutions were centrifuged at 10,000 g for 15 min at 4 °C. The supernatants were concentrated 25-fold and electrospray ionization-tandem mass spectrometer (LC-ESI-MS/MS) (Agilent Technologies) with a C18 reversed phase capillary column, 0.3 mm x 150 mm, (3.5 µm particle size). The LC separation consisted of solvent A: H2O:0.1% formic acid and solvent B: methanol:0.1% formic acid with the following gradient: 10 min 3% acetonitrile, 10 min 30%, 20 min 70%, 20 min 100% with a flow rate of 4 µl min−1 for 60 min. The peptides eluted were analyzed by ESI-Q-TOF2 mass spectrometer.

Phylogenetic analysis.

Protein sequences of C3H were BLAST searched using the NCBI GenBank database. The phylogenetic analysis included protein sequences from six monocots (Brachypodium distachyon, Oryza sativa, Setaria italica, Sorghum bicolor, Panicum virgatum and Zea mays), five dicots (Arabidopsis thaliana, Glycine max, Medicago truncatula, Pisum sativum and Populus trichocarpa), one lycophyte (Selaginella moellendorfii), and 7,580 proteins (Arabidopsis thaliana, Brachypodium distachyon, Oryza sativa, Setaria italica, Sorghum bicolor, Panicum virgatum and Zea mays) were injected into a capillary liquid chromatography-electrospray ionization-tandem mass spectrometer (LC-ESI-MS/MS) (Agilent Technologies) with a C18 reversed phase capillary column, 0.3 mm x 150 mm, (3.5 µm particle size). The LC separation consisted of solvent A: H2O:0.1% formic acid and solvent B: methanol:0.1% formic acid with the following gradient: 10 min 3% acetonitrile, 10 min 30%, 20 min 70%, 20 min 100% with a flow rate of 4 µl min−1 for 60 min. The peptides eluted were analyzed by ESI-Q-TOF2 mass spectrometer.

Monoisotopic masses from the tryptic digests were used to identify the corresponding proteins by searching the plant databases in NCBI and SwissProt using the MASCOT search algorithm (http://www.matrixscience.com). The sensitivity of the method was determined using a commercial standard protein (BSA) digestate (Sigma-Aldrich) by trypsin digestion at 1 pmoI µl−1.

Recombinant protein expression and kinetics.

The full-length Brachypodium (Bradi65820) and Arabidopsis (At1g07890) C3H complementary DNA (cDNA) sequences were obtained from Phytozome v12.0 and amplified by RT-PCR (Phusion HiFi polymerase; New England BioLabs) from cDNA extracted from stem tissues of Arabidopsis thaliana (Col-0). Recombinant protein expression and purification were performed as previously described using the expression plasmid (5′-CACCATGGCGGAGAAGCTACCCCGGACG-3′ and 5′-TCCGAACTGGGTGA TGTGGAAGCTTAA-3′ for Brachypodium and 5′-CACCATGAGCAAGAACACCAAACGC-3′ and 5′-CTGAGCTTGGTTGTGTGTGCATGCTTAA-3′ for Arabidopsis) in Rosetta DE3 E. coli (Novagen) expressing T7 RNA polymerase. Recombinant C3H was purified by nickel affinity chromatography and used for the solution optimization. The C3H protein was then expressed directly from the PCR by RNA from seedling, stem or leaf tissues. Based on the tissue-specific expression pattern in the Arabidopsis eFP Browser (http://bar.utoronto.ca/), APA3 is mainly expressed in mature pollen and at very low levels in other tissues when compared with APX1. Total RNA was isolated using Trizol reagent (Invitrogen) and first-strand cDNA was synthesized using the oligo(dT)18 primer (5′-CACAATGGCGGAGAAGCTACCCCGGACG-3′ and 5′-CTGAGCTTGGTTGTGTGTGCATGCTTAA-3′) and random hexamers (Invitrogen). The cDNAs were cloned into pENTR-D Topo and subsequently into pDEST17 vector by LR recombination reaction resulting in a 6xHis–C3H fusion construct. 6xHis-tagged C3H protein was expressed in E. coli strain Rosetta grown at 37 °C in Luria Bertani (LB) medium containing 0.1 mg ml−1 carbenicillin. After reaching an optical density of 600 nm of 0.7–0.9, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown at 16 °C for 18 h. Cells from 25 ml culture were harvested by centrifugation and resuspended in 2 ml of extraction buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl and 50 mM imidazole). All the following steps were carried out in ice using an ultrasonic homogenizer (Model-120, Fisher Scientific). The lysates were recovered by centrifugation (16,000 g) for 20 min. Ni-NTA beads (Qiagen) were added and the suspension incubated at 4 °C for 30 min under constant inversion, and the unbound proteins were washed three times with 1 ml of extraction-washing buffer. Target proteins were eluted with 250 µl of elution solution (50 mM Tris–HCl buffer pH 8.0, 500 mM NaCl, and 250 mM imidazole) and their purity was verified by SDS–PAGE.

Preparation of recombinant C3H was performed following procedures developed for soybean cystolic APX with some modifications. Both purified CrAPX and recombinant Brachypodium C3H were buffer exchanged in 50 mM sodium phosphate buffer pH 6, and washed over a 10 kDa Amicon concentrator to a final volume of 4 ml. Bovine hemin (10 mg) was dissolved in 1 ml of 10 mM NaOH and brought to a final volume of 10 ml with 50 mM phosphate buffer pH 6. The hemin solution (1 ml) was slowly added in drops to the C3H solutions (4 ml) with gentle stirring. After 15 min at 4 °C the solutions were centrifuged at 12,000 g for 10 min to remove denatured reconstitution products. The concentration of the supernatants (reconstituted holoenzymes) were determined using the PAGE gel stained with Coomassie Blue Reagent (Bio-Rad).
Bradford assay and directly used for kinetic assays. Enzyme kinetics were performed using 500 ng of the reconstituted protein in the optimized 100 µl reaction described above including 10 µl of 0.3% H2O2 solution and increasing concentrations (50–2000 µM) of the substrate 4-carboxylic CH3 for CHH, ascorbate for APX and tyrosine for tyrosine hydroxylase enzymatic activities. Both tyrosine hydroxylase and CH3 activities were determined by HPLC as described above, whereas APX activity toward ascorbate (E290 = 2.8 nm⁻¹ cm⁻¹) was characterized spectrophotometrically using a Biorekt SynergyMax microplate reader following. Background oxidation was measured by combining substrate and H2O2 without reconstituted CH3. Since APX has been observed to display non-Michaelis–Menten kinetics31,32, the kinetic constants, kcat and KM, were determined by using the solver function of Excel to determine the best-fit parameters from the Hill equation (\(v = \frac{V_{max} \cdot [S]^{n}}{K_{n} + [S]^{n}}\), where \(n\) is the initial rate, \(n\) is a qualitative indication of the degree of cooperativity, \(K_{n}\) is the substrate concentration at which the velocity is half-maximal, and \(V_{max}\) is the maximum velocity. When \(n = 1\), the Hill equation reduces to the more usual Michaelis–Menten equation (\(v = \frac{V_{max} \cdot [S]}{K_{M} + [S]}\)).

**Protein modeling.** Comparative protein homology modeling was carried out with SWISS-MODEL to generate models for Brachypodium and Arabidopsis CH3 using a template the crystal structure of soybean APX/CH3 (PDB ID: 1v0h). The template structures were captured with an EVOS FL Cell Imaging System (Thermo Fisher) equipped with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). To remove genomic DNA contamination, total RNA was treated with DNase for 30 min at 37 °C following the TURBO DNA-free® Kit (Fisher scientific). First-strand cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s protocol. The cDNA samples were diluted 20-fold and used as qRT-PCR templates. Arabidopsis cse (At1g52760) primers 5’-CTCTTTGTTGCGGTATAGGC-3’ (F1) and 5’-CAGTAACTCCTCTCTTGTTTCCAC-3’; Arabidopsis cse (At1g52760) primers 5’-GGGAATCCATCAAAACAGGTTG-3’ and 5’-CAACCAGTTCCTGCTAGTG-3’ were used for amplification.

**Real time qPCR.** Total RNA was extracted from mature stem tissue of Brachypodium T-DNA and Arabidopsis RNAi lines above using Triol (Thermo Fisher). Total RNA was converted to cDNA using the Superscript III First-Strand Synthesis Kit (Invitrogen). The expression clones were introduced into the pB7GWIWG2(I) vector (https://gateway.psb.ugent.be/) by LR recombination reaction. This binary vector provides tolerance to Basta (phosphinothricin) as a selectable marker, in contrast to the background SALK mutant cse or cis rearranged with the marker NPTII, which confers resistance to kanamycin. After transformation by sequencing, the expression clones were introduced into A. thaliana (strain CV129-5B1) using Agrobacterium tumefaciens strain GV3101 (pMP90RK) by electroporation. Single cse1 and cse2 mutant plants were transformed using floral dip, selected in soil with Basta (7.5 mg ml⁻¹) genotyped by qRT-PCR and characterized for lignin phenotype. Seeds were vernalized as described above and transferred to a growth chamber for 8 weeks at a temperature of 22 °C, 70% humidity and a light intensity of 100 µE m⁻² s⁻¹, with a 16 h light/8 h dark photoperiod.

**Plant material and growth conditions.** Brachypodium T-DNA line J25124 (IIL000024891) was obtained from the JGI T-DNA collection (https://www.jgi.doe.gov) and identified as an activation tagged line transformed with the pJL22B vector and with the T-DNA insertion in the last intron with positive orientation, 121-bp upstream of the stop codon of the cse gene. Line J25124 (IIL000024891), the parent of the T-DNA mutant population, was used as the wild-type control. Brachypodium T-DNA line J22251 (IIL000018556) was used as a T-DNA control transformed with the same pJL22B activation tagging vector and positive T-DNA orientation, 632-bp downstream of the stop codon of a microsomal ascorbate peroxidase 3 (APX3, Brachypodium T-DNA line J22251 (IIL000018556), the 121-bp, on the individual T-DNA generation lines in which the activation tagging was induced by application of brassinolide at 0, 6, 1, 3, and 7 days on non-induced samples at 1 and 7 days as previously described.

**Western blotting and tissue printing.** Crude mature stem protein extracts were prepared as above for determination of CH3 activity and protein levels in Brachypodium cse T-DNA and Arabidopsis RNAi line above using Triol (Thermo Fisher).

**Histochimical staining and microscopy.** Mature stems from Brachypodium and Arabidopsis plants above were cut and the bottom 2 cm embedded in 70% glycerol. Slices of 100 µm thickness were cut using a HM 650 V Vibratome Blade Microtome (Thermo Fisher Scientific). The samples were stained with either 0.1% of 2,3,5-triphenyltetrazolium chloride (MTT) or 0.1% of 2,3,5-triphenyl tetrazolium chloride (MTC) in ethanol: 12 N HCl in a 1:2 ratio. For Mäule staining, the cross-sections were cut into 1 mm thick sections with a razor blade and gently blotted dry with absorbent paper. The section was then pressed firmly onto nitrocellulose membrane for 15–20 s. Detection of anti-CH3 IgG binding was carried out using goat anti-rabbit (IgG) serum conjugated to alkaline phosphatase and visualized using a substrate bromochloroindophosphate/nitroblue tetrazolium.
the seed phenotype characterization in Arabidopsis were taken with an AxioCam MRC 5 camera attached to a Zeiss Axio Zoom.V16 stereo zoom microscope.

**Metabolic profiling.** For untargeted metabolite profiling in mature Brachypodium stem tissues, lyophilized, ground samples (~10 mg) were weighed into centrifuge tubes and extracted with 2 ml of 80% ethanol and 50 µl of internal standard sorbitol (1 mg mL⁻¹). Samples were extracted overnight in a tube rotator at room temperature and then centrifuged at 1900 × g for 20 min. The supernatant was transferred into scintillation vials and stored at −20 °C. A 1 ml aliquot was dried under a nitrogen stream, dissolved in 0.5 ml acetonitrile, and silylated to generate trimethylsilyl (TMS) derivatives. Samples (1 µl) were injected into a GC-MS Agilent 5975C inert XL operated in electron impact (EI; 70 eV) ionization mode as described previously. Metabolite peaks were extracted using a characteristic mass-to-charge (m/z) ratio to minimize integration of co-eluting metabolites. The extracted peaks of known metabolites were scaled back to the total ion current (TIC) using previously calculated scaling factors. Peaks were quantified by area integration and normalized to the quantity of internal standard recovered, amount of sample extracted, derivatized, and injected. A user-created database (>2400 spectra) of EI fragmentation patterns of TMS-derivatized compounds and the Wiley Registry 10th Edition/NIST 2014 Mass Spectral Library were used to identify the metabolites in the samples. Unidentified metabolites were designated by their retention time and key m/z ratios.

For targeted profiling in leaves of Arabidopsis during the light stress time-course experiment, plants grown in a peat pellet were exposed to 1000 µmol m⁻² s⁻¹ light intensity at 22 °C for a period of 0, 20, 60, and 90 s as previously described. Five biological replicates, each composed of leaves pooled from at least 90 different plants, were harvested and extracted with 2 ml of 80% ethanol and 50 µl of internal standard sorbitol (1 mg mL⁻¹). Samples were extracted using a water bath at 85 °C for 30 min at 40 °C. Once the samples were dried, 150 µl of a 1:1 mixture of methanol and chloroform (50 µl at 3.02 mg ml⁻¹) was added as an internal standard. Methylene chloride (3 ml) was added to the samples and the vials capped tightly and gently vortexed to ensure mixing. Solvent/water layers were separated by centrifugation at 500 g for 2 min. The lower organic layer was then transferred to new 15 ml screw-capped vials and two scoops of sodium sulfate added to the samples, which were allowed to sit overnight to absorb any remaining water. Three milliliters of the dry solvent were transferred to 4 ml screw-capped vials and dried under a nitrogen stream for 30 min at 40 °C. Once the samples were dried, 150 µl of a 1:1 pyridine:BSTFA (N,O-Bis(trimethylsilyl)trimfluoroacetamide) solution was added, vortexed, and incubated for 30 min at 40 °C for derivatization prior to GC analysis.

Samples were vortexed again, transferred to GC vials and run directly on a Hewlett-Packard 7890A gas chromatograph with a 5975C series mass selective detector (column: Agilent DB-5 ms, 60 m × 0.25 mm × 0.25 µm film thickness). The inlet, main GC oven and FID were held at 250 °C with a column flow of 0.6 ml min⁻¹. The Low Thermal Mass (LTM) column temperature program began at 130 °C for 2 min and was ramped at 150 °C min⁻¹ to 325 °C. Mass spectra were recorded in electron impact mode (70 eV) with 60–650 m/z scanning range. The ions studied were the following: 299 for the thioethyalted syleryng monomer TMS (S-unit), 269 for the thioethyalted coniferyl monomer TMS (G-unit), 239 for the thioethyalted coumaryl monomer TMS (H-unit), and 57 for the internal standard docosane. The silylated monomers are unstable and were not stored at room temperature for more than 24 h prior to injection.

**Isotopic labeling experiments.** 13C₆-labeled furfural was synthesized following a previously described protocol with minor modifications. Knoevenagel–Debner condensation was performed between 13C₆-labeled vanillin (100.0 mg, 0.64 mmol) and malonic acid (133.3 mg, 1.28 mmol) in ethanol (1 ml) using piperidine (10 µl) as catalyst. The reaction took place under reflux at 78 °C for 8 h. The mixture was poured into ice, acidified with hydrochloric acid (pH ~4), filter under vacuum, washed with chilled water (10 ml), and re-crystallized in ethanol: water (1:1, v/v) to furnish 78% yield of furfural. The purity of the labeled compound was assessed by LC/MS and 1H NMR. For the labeling experiments, wild-type Brachypodium and Arabidopsis plants were first grown in culture tubes containing ½ Gamborg’s B5 salt mixture and 0.5% Phytagel under continuous light conditions. After germination, seedlings were transferred to culture tubes containing 20 ml of the same medium fed with 0.1 mM 13C₆-furfural and harvested after three weeks. Control experiments using unlabeled furfural were conducted in parallel. Separated roots were ground in liquid nitrogen and stored at −80 °C until use. Samples were extracted for thioacidolysis as described above and the percentage of label incorporated into the lignin monomers was calculated as follows:

\[ \text{% }13 \text{C incorporated} = \frac{\text{peak area labeled}}{\text{peak area labeled} + \text{peak area unlabeled}} \times 100 \]

Peak areas of the thioacidolysis products of lignin (H-, G-, and S-units) were identified, and the incorporation into total lignin estimated using the sum of the three individual peak areas.

**Statistics.** Statistical analysis of the results was performed by unpaired, two-tailed t-test. A 95% confidence interval was used for statistics and α = 0.05 was considered significant. All statistical tests were performed using Prism software.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available within the paper and its Supplementary Information, or are available from the corresponding author upon reasonable request. The source data underlying Figs. 2a, b, d, 3b, d, f, 4b, c, d, g, i, 5a, d, Supplementary Figs. 5, 6, 7, 8a, b and Supplementary Tables 1, 2, 3 and 4 are provided as a Source Data file.

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Author contributions

R.A.D. and J.B. conceived and designed the study. L.E-T. performed 4CL and COMT enzymatic assays and the labeling experiments. M.D.P. synthesized isotopic labeled ferulate. J.C.S.-Y. genotyped the Arabidopsis T-DNA lines. F.K.C., R.M., N.E. and T.J.T. performed the metabolomic analysis. R.X. helped with the co-expression analyses. B.J.V. performed peptide sequencing. R.M. and L.S. provided the Arabidopsis homozygous c3h mutants and anti-(C3H)-monoclonal antibodies, and helped during the generation of the Arabidopsis RNAi lines. J.B. generated and analyzed all other data, and J.B. and R.A.D. wrote the manuscript.

Additional information

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