Monolignol ferulate conjugates are naturally incorporated into plant lignins

Steven D. Karlen,1,2 Chengcheng Zhang,3 Matthew L. Peck,3 Rebecca A. Smith,1,2 Dharshana Padmakshan,1 Kate E. Helmich,1,2 Heather C. A. Free,4,5 Seonghee Lee,6 Bronwen G. Smith,5 Fachuang Lu,1,2 John C. Sedbrook,7 Richard Sibout,8 John H. Grabber,9 Troy M. Runge,1,10 Kirankumar S. Mysore,11 Philip J. Harris,4 Laura E. Bartley,2∗ John Ralph1,2∗

Angiosperms represent most of the terrestrial plants and are the primary research focus for the conversion of biomass to liquid fuels and coproducts. Lignin limits our access to fibers and represents a large fraction of the chemical energy stored in plant cell walls. Recently, the incorporation of monolignol ferulates into lignin polymers was accomplished via the engineering of an exotic transference into commercially relevant poplar. We report that various angiosperm species might have convergently evolved to natively produce lignins that incorporate monolignol ferulate conjugates. We show that this activity may be accomplished by a BAHD feruloyl–coenzyme A monolignol transferase, OsFMT1 (ATS), in rice and its orthologs in other monocots.

INTRODUCTION

One of the major adaptations of terrestrial plants to life on land is their ability to produce lignin for structural strength and defense (1). Stochastically synthesized through stepwise radical coupling of 4-hydroxycinnamyl alcohols (called monolignols, primarily coniferol and sinapyl alcohols), lignin is a polymer with aryl ether and various other C–O–C and C–C interunit connections (2, 3). Fragmentation of lignin allows polysaccharidase enzymes to access and convert cell wall polysaccharides to monomeric sugars or facilitates cell wall deconstruction to cellulose, hemicelluloses, and lignin fragments. These components are used in economically important processes, including production of paper and other fiber products, second-generation biofuels, and other bioproducts (4, 5). Lignin fragmentation often requires high temperatures and/or harsh chemical treatments to cleave even its weakest interunit bonds (6, 7), However, if chemically labile ester bonds are introduced into the lignin polymer, as can be accomplished by augmenting the prototypical monomers with monolignol ferulate (ML-FA) conjugates (Fig. 1A), then lignin fragmentation can occur under mild pretreatment conditions (Fig. 1B) (8–10). The findings here provide evidence that “zip-lignins,” lignins derived, in part, from ML-FA’s, have developed naturally via convergent evolution in diverse angiosperm lineages (Fig. 2).

Initial work on the incorporation of ML-FAs into plant lignin was carried out by engineering poplar trees (Populus alba × Populus grandidentata) to express a gene from Chinese angelica [Angelica sinensis (As), a dicotyledonous Chinese medicinal plant] encoding a feruloyl–coenzyme A (CoA) monolignol transferase (AsFMT) (8). The AsFMT enzyme couples monolignols with feruloyl-CoA, an intermediate in the monolignol biosynthetic pathway (fig. S1), to produce ML-FAs. AsFMT is a member of a family of proteins found in plants and fungi termed BAHD acyltransferases (AT; Fig. 3) (11). AsFMT-expressing poplar trees produce ML-FAs and use them in lignification, resulting in improved cell wall saccharification following mild base pretreatment (8).

The lignin assay, derivatization followed by reductive cleavage (DFRC) (12), was used to confirm that ML-FAs were integrally incorporated into the lignin of AsFMT-expressing poplar trees (8). DFRC cleaves β-ether bonds to release lignin fragments (12), while leaving the ester linkages of incorporated ML-FAs intact. Hence, DFRC-releasable coniferol and sinapyl dihydroferulate diacetates (ML-DHFAs; Fig. 1C) are diagnostic for the ML-FAs incorporated into lignin and their release level is proportional to the amount of ML-FAs in the lignin (8). However, because DFRC releases just a fraction of the incorporated ML-FAs as ML-DHFAs, only the relative level of conjugates in the lignin can be determined (8). The threshold to detect DFRC-released ML-DHFAs by gas chromatography–multiple reaction monitoring–mass spectrometry (GC-MRM-MS; table S1) is ~0.01 mg/g of acetyl bromide soluble lignin (ABSL) (see Materials and Methods). These experimental conditions revealed that wild-type (WT) poplar trees already release low levels (0.3 mg/g of ABSL) of the ML-DHFAs from their lignins (8), indicating that poplar plants naturally synthesize ML-FA conjugates and use them in lignification.

RESULTS

Plants accumulating ML-FAs in extractives also use them for lignification

Armed with the new knowledge that plants naturally incorporate ML-FAs and an analytical method to diagnostically detect their incorporation into the polymer with suitable sensitivity, we reexamined the lignin of three plants known to produce ML-FAs in their extractives: Chinese angelica (A. sinensis, family Apiaceae), kenaf (Hibiscus cannabinus, family Malvaceae), and balsa (Ochroma pyramidale, family Malvaceae). Performed on isolated lignins, the DFRC assay showed that these plants also used ML-FAs in their lignification (table S2).

Phylogeny of plants using ML-FAs in lignification

The abovementioned discovery prompted a survey to determine whether the utilization of ML-FAs in lignification was a trait of eudicots or all
The symbol leaving the esters intact. (FA can be formed when ML-FAs are incorporated into Karlen et al angiosperms so whether it was a trait ubiquitous to all lignifying plants. We performed the DFRC assay on a set of plants representing the spermatophytes or “seed plants,” including 13 gymnosperms and 54 angiosperms (Fig. 2A). The assayed gymnosperms showed no evidence of ML-FAs, whereas low levels were present in the lignin of many, but not all, of the angiosperms (Fig. 2B and tables S3 to S5). Some of the assayed eudicots showed detectable levels of the diagnostic ML-DHFAs, predominantly derived from coniferyl ferulate (G-FA). Many of the monocots, especially the recently evolved commelinids, which include the major cereal crop plants, tested positive for ML-FAs, with sinapyl ferulate (S-FA) as the main conjugate.

Origin of ML-FAs and transferase specificity
The discovery of ML-FA production in this diversity of plant species, and notably in plants that use other types of monolignol conjugates for lignification, prompted us to investigate whether ML-FAs arise from a lack of specificity in other transferases or from dedicated FMT enzymes. The “non-specific transferases” hypothesis is most readily addressed in grasses, namely, in rice [Oryza sativa (Os)] and Brachypodium [Brachypodium distachyon (Bd)], in which p-coumaroyl-CoA monolignol transferases (PMTs), as well as BAHD ATs, have already been identified [AT4 = OsPMT1 (13); Bradi2g36910 = BdPMT1 (14); and Bradi2g36980 = BdPMT2 (15)].

To test the specificity of PMT, we compared the amount of DFRC-releasable ML-DHFAs between a Brachypodium line with native BdPMT1 expression and a sodium azide–generated Bdpm1 missense mutant line that produced lignins devoid of p-coumarates (14). Levels of DFRC-released ML-DHFA were similar between the Brachypodium WT (0.14 mg/g of ABSL) and mutant lines (0.15 mg/g of ABSL) (Fig. 4 and table S6), indicating that BdPMT1 activity does not influence ML-FA production and, thus, ML-FAs cannot be attributed to the low specificity of BdPMT1.

Furthermore, introduction of the OsPMT1 gene from rice into the eudicot Arabidopsis (Arabidopsis thaliana), which does not natively incorporate detectable levels of monolignol conjugates of any kind into its lignin (<0.01 mg/g of ABSL), resulted in transformants with p-coumarates esterified to lignin at a level that was easily quantified by DFRC through the release of monolignol dihydro-p-coumarates (ML-DHPCAs) (16). However, both the OsPMT1-expressing and WT Arabidopsis lines did not produce any
detectable ML-DHFAs (Fig. 4 and table S6). These two experiments strongly suggest that ML-FA conjugates incorporated into commelinid monocot lignins are not the result of low PMT specificity.

**Identification of a putative new FMT in commelinids**

To test the hypothesis that ML-FA conjugates are the product of dedicated FMT enzymes, we examined the annotated genomes of members of the grass family Poaceae, in which ML-FA conjugates are ubiquitous, for proteins with close homology to AsFMT and OsPMT1. We find that these proteins are in distinct BAHD subclades (Fig. 3, clades III and V, respectively). Although rice and other commelinids lack homologs of OsPMT1 (56% identity) and thus seemed to be a reasonable candidate for an enzyme having FMT activity.
Here, we provide molecular genetic evidence consistent with the hypothesis that OsAT5 is an FMT. The OsAT5-D1 line has a transfer DNA (T-DNA) insertion with multiple copies of a 35S promoter sevenfold increase in released ML-DHFAs (Fig. 4B and table S6) compared with negative segregants lacking the T-DNA insertion (fig. S3B). In contrast, the expression of more distant genes flanking the insertion site remained unchanged (fig. S3). As hypothesized, OsAT5-D1 plants exhibited an approximately fivefold increase in the DFRC-releasable ML-DHFA but no increase in ML-DHpCA; by comparison, the best AsFMT poplar lines showed an approximately 66% increase in released ML-DHFAs (Fig. 4B and table S6) (8). The increase in ferulate is not associated with arabinoxylans, a well-known location of ferulate incorporation in grass cell walls (18), because ferulate released by mild acid hydrolysis remained unchanged (fig. S5).

To confirm the association between increased expression of OsAT5 and increased lignin-associated ferulate esters, we generated and characterized three additional overexpression lines, in which expression was confirmed in rice plants with the known location of ferulate incorporation in grass cell walls (18).

Species codes for locus identifiers with order classifications in parentheses are as follows: AT, Arabidopsis thaliana (Brassicales); Bradi, Brachypodium distachyon (Poales); C7Z876, Eucryp, Eucrypsus grandis (Myrtales); Glyma, Glycine max (Fabales); GSMUA, Musa acuminata (Zingiberales); GRMZM, Zea mays (Poales); LOC_Os, O. sativa (Poales); Medtr, Medicago truncatula (Fabales); Pavir, Panicum virgatum (Poales); PDK, Phoenix dactylifera (Arecales); POTR, Populus trichocarpa (Malpighiales); and Sb, Sorghum bicolor (Poales).

### Fig. 3. Phylogenetic reconstruction of BAHD acyl-CoA ATs is consistent with the convergent evolution of the two feruloyl-CoA monolignol transferases, OsAT5/FMT and AsFMT. Maximum likelihood phylogeny of AsFMT, OsAT5, and biochemically characterized BAHD proteins (11). Branch values are based on 1000 bootstraps. Protein IDs are National Center for Biotechnology Information GenBank identifiers or genome locus identifiers.
was driven by the ZmUbi promoter (Fig. 4B and fig. S4). All showed increased DFRC-releasable ML-DHFA (Fig. 4B and table S6), compared to the negative segregant controls, establishing that OsAT5 acts on the monolignols. By all indications, then, OsAT5 is a native FMT enzyme in rice, that is, OsAT5 = OsFMT1.

Having identified OsAT5 as a potential grass FMT gene, we compared the OsAT5 sequence to known BAHD AT gene sequences in other commelinid monocots. BAHD proteins were previously identified in the draft genomes of banana and date palm (both nongrass commelinids), on eorhizomols in the eudicots (fig. S2B). The presence of OsAT5-encoding genes correlates with detectable levels of DFRC-releasable ML-DHFAs, suggesting that the commelinid monocot FMT genes may have evolved as a trait of this group.

DISCUSSION

Upon examination of the phylogeny of the assessed plant species (Fig. 2), some patterns emerged. For example, plants known to produce significant levels of other monolignol conjugates also incorporate ML-FAs into their lignins. These include the commelinids, which incorporate monolignol p-coumarates into their lignins and also have ferulates acylating the arabinoxylan hemicellulosases (18), and plants that use monolignol p-hydroxybenzoates in lignification, such as palms (Arecaceae), willows (Salix), and poplars/aspen (Populus). A weaker link exists between plants incorporating ML-FAs and those known to use monolignol acetates, which include kenaf and sisal (19), several other monocots (20–22), and various hardwoods (23, 24). However, the introduction of ML-FAs into Arabidopsis via transgenesis, which appears to natively lack them, indicates that the ability to use monolignol conjugates does not appear to be isolated to those species that have evolved to do so, highlighting the plasticity of cell wall lignification.

The evidence for BAHD proteins with FMT activity (fig. S2) suggests that the activity has arisen at least twice; that is, it has convergently evolved. For example, the two nonhomologous FMT protein sequences, OsFMT from a commelinid monocot and AsFMT from a eudicot, have little sequence similarity (20%). Furthermore, the model that shows that FMT activity is an ancestral trait of angiosperms is inconsistent with the apparent absence of DFRC-releasable ML-DHFAs in most of the noncommelinid monocots and many of the eudicots, although it cannot be ruled out. Whether an AsFMT ortholog is responsible for the incorporation of ML-FAs into eudicot lignins is unclear from the phylogenetic reconstructions. Analysis of the genomes of the ML-FA—producing eudicot tree species Eucalyptus globulus and Populus trichocarpa reveals a large number of clade III BAHD ATs that are absent from the commelinids, none of which have great similarity to AsFMT, as the closest poplar homologs are POPTR_0001s31750 and POPTR_0004s01720, with 36% identity (fig. S2A).

The convergent evolution and subsequent proliferation of plants that incorporate ML-FA conjugates into their lignins indicate that, potentially, there is a biological advantage for the production of this lignin structure. Regardless of the actual driving forces selecting for them, the diversity and environmental success of plants with native zip-lignins show that they have no apparent general disadvantages in terms of plant defense or structural stability.

Finally, our findings further refute the contention by some researchers that lignins are derived only from three monolignols. It has been increasingly evident over the past 20 years that many other compounds bio-synthesized by plants are used as monomers in lignification (2, 3, 25, 26). As demonstrated here, ML-FA conjugates must now be added to the list of authentic lignin precursors. In practical terms, our discovery unveils new approaches to increasing levels of readily cleavable ester bonds in the lignin backbone, either by breeding or by transgenic methods similar to those used to introduce AsFMT into poplar (8). Further work is also needed to explore the effects of ML-FA—containing lignins on processes such as carbon sequestration and biomass utilization.
MATERIALS AND METHODS

DFRC procedure for ML-DHFA conjugates released from cell wall lignins

Incorporation of ML-FAs into the lignin was determined using the ether-cleaving, ester-retaining DFRC method previously established for ML-pCA and ML-FA conjugates (8, 14, 27, 28). Extract-free cell wall samples (50 mg) or enzyme lignin samples (50 mg) were stirred in 2-dram vials fitted with polytetrafluoroethylene pressure release caps with acetylated sodium acetate:acetic acid [1:4 (v/v), 4 ml]. After heating for 3 hours at 50°C, the solvents were removed by SpeedVac (50°C, 35 min, 1.0 torr, 35 torr/min; Thermo Scientific SPD131DDA). Crude films were suspended in absolute ethanol (0.5 ml), dried on SpeedVac (50°C, 15 min, 6.0 torr, 35 torr/min), and then suspended in dioxiane/acidic acid/water [5:4:1 (v/v), 5 ml]) with nanopowder zinc (250 mg). The vials were then sealed, sonicated to ensure suspension of solids, and stirred in the dark at room temperature for 16 to 20 hours. Additional nanopowder zinc was added as required to maintain a fine suspension of zinc in the reaction mixtures. The reaction mixtures were then quantitatively transferred with dichloromethane (DCM; 6 ml) into separatory funnels charged with a saturated ammonium chloride (10 ml) and an internal standard (diethyl 5,5'-difluorobutane, 54.0 μg). Organics were extracted with DCM (4 × 10 ml), combined, dried over anhydrous sodium sulfate, and filtered, and the solvents were removed via rotary evaporation (water bath at <50°C). Free hydroxyl groups on DFRC products were then acetylated for 16 hours in the dark using a solution of pyridine and acetic anhydride [1:1 (v/v), 5 ml], after which the solvents were removed via a rotary evaporator to yield crude oil films.

To remove most of the polysaccharide-derived products, acetylated DFRC products were loaded onto solid phase extraction (SPE) cartridges (Supelco Supelclean LC-Si SPE tube, 3 ml, product no. 505048) with DCM (2 × 1.0 ml). After elution with hexanes/ethyl acetate [1:1 (v/v), 8 ml], solvents were removed by rotary evaporation, and the products were transferred in stages with GC-MS-grade DCM to final sample volumes of 200 μl into GC-MS vials containing a 300-μl insert. Samples were analyzed on a triple-quadrupole GC-MS/MS (Shimadzu GCMS-QP8030) operating in MRM mode using synthetic standards for authentication and calibration. The GC program and acquisition parameters are listed in table S1, and the result of the DFRC assay is listed in tables S2 to S4.

Detection threshold for DFRC-released ML-DHFAs

When DFRC products are analyzed via a triple-quadrupole GC-MS, operating in MRM mode, the detectable amount of releasable ML-DHFA is ~0.01 mg/g of ABSL. This estimated threshold of detection is partially based on instrument limitations but is mainly limited by the presence of a large amount of polysaccharides, lignin fragments, and the DFRC-released monoglucolins that create a complex matrix from which the desired ML-DHFA products need to be extracted. Scaling up the DFRC reaction increases not only the amount of ML-DHFA products formed but also the amount of the other matrix components. We have found that the crossover point for improved sensitivity with increasing biomass is ~50 to 100 mg of plant cell walls.

Estimating the ML-FA incorporation levels

Most ML-FAs incorporated into lignin form structures that are not cleaved by DFRC and are not released as measurable ML-DHFAs products. Therefore, biomimetically lignified cell walls prepared with known amounts of ML-FAs were subjected to DFRC to estimate the efficiency of ML-DHFA production from ML-FAs incorporated into plant lignins. For this purpose, primary maize cell walls containing bound native peroxidases were lignified by the dropwise addition of separate solutions of dilute hydrogen peroxide and a 1:1 mixture of coniferyl and sinapyl alcohols substituted with either G-FA (0, 8.4, 15.4, and 26.7 weight % (wt%)) or S-FA (0, 9.0, 16.5, and 28.4 wt%) (10). The artificially lignified cell walls were then analyzed by DFRC, and the results were plotted as the weight % of ML-FA used to prepare the cell walls versus the weight % of ML-DHFA released per gram of ABSL. Regression constants for the released ML-DHFAs were determined by fitting the data to a linear model (y = mx, where y = wt% ML-DHFA in cell wall dehydrogenation polymer and x = wt% ML-DHFA released; G-FA: m = 53, R² = 0.99; S-FA: m = 34, R² = 0.99). These results indicate a direct relationship between DFRC-releasable ML-DH conjugates and the quantity of ML-FA in lignin. However, we stress that this approach provides only a rough means of estimating the quantity of ML-FAs incorporated into native plant lignins.

Applying these results to the yields of ML-DHFAs from surveyed plant species suggests that ML-FA incorporation into lignin averaged 1.8 wt% and ranged from 0.2 to 6.6 wt% (tables S2 to S4). The average weight % of ML-FA in eudicots (2.0 wt%) was slightly higher than the average for the monocots (1.7 wt%). The monocots on average released more S-DHFA than G-DHFA (77:23) as compared to the eudicots that mostly released G-DHFA (S-DHFA/G-DHFA, 1:99).

Procedure to determine ABSL content

The ABSL contents were measured in 1-cm quartz cuvettes on a Shimadzu UV-1800 spectrophotometer at λ = 280 nm and ε280 = 20.0, as previously described (29, 30).

Preparation of cell wall and enzyme lignin samples

Cell wall samples were prepared by one of four methods (A to D) described below.

Method A: Gymnosperm and angiosperm samples were freeze-dried and shaker-milled into fine powder (Retsch MM 400). Samples were then solvent-extracted sequentially with water (3 × 45 ml), 80% ethanol (3 × 45 ml), and acetone (2 × 45 ml) by first suspending the sample in solvent, sonicating for 20 min, pelleting by centrifugation (8800g for 20 min; Sorvall Biofuge Primo Centrifuge), and, finally, decanting the supernatant. The extract-free pellet was then dried under vacuum for analysis.

Method B: Tissues with lignified walls were identified histochemically [phloroglucinol–HCl and ultraviolet fluorescence microscopy (31)]. The lignified tissues were isolated and ground (ROCKLABS Ltd.) in 3-(N-morpholino)propanesulfonic acid–KOH buffer [20 mM (pH 6.8)]. The homogenates were centrifuged (1000g for 10 min); resuspended in buffer; filtered through a nylon mesh (11-μm pore size); washed successively with buffer, ethanol, methanol, and n-pentane; and air-dried. Starch granules were detected and removed from the preparations from Phoenix canariensis, using the method of Carnachan and Harris (32).

Method C: Hardwood eudicot samples were Wiley-milled to pass through a 40-mesh screen, air-dried, and extracted three times with a 9:1 mixture of acetone and water. Three additional extractions with methanol were used when required to remove intensely colored extractions. After air-drying, the samples were again Wiley-milled to pass through a 40-mesh screen.

Method D: Samples of mature stems were processed to destarched alcohol-insoluble residues (AIR), following a reported procedure (17). Enzyme lignins were prepared from ball-milled materials, as previously described (33, 34). Briefly, ball-milled extract-free materials (1 g;
prepared using method A) in 50-ml centrifuge tubes were incubated at 35°C and mixed in a shaker at 225 rpm for 3 days with 40 ml of sodium acetate buffer (pH 5.0) and 40 mg of crude cell cultures (CELLULYSIN, EMD Biosciences). After incubation, solids were pelleted by centrifugation (8800g for 20 min; Sorvall Biofuge Primo Centrifuge) and washed with acetate buffer (2 × 40 ml). Washed solids were then treated again with the crude cell cultures (40 mg) for 3 days and then pelleted and washed with reverse osmosis water (3 × 40 ml). The resulting pelleted solids were dried on a freeze dryer to yield ~10% of the dry weight of the original ball-milled cell wall material.

**Phylogenetic analysis of BAHD ATs**

Phylogenetic analysis was conducted in two stages. First, to determine the evolutionary relationship between OsAT5 and AsFMT proteins, we conducted a maximum likelihood phylogenetic reconstruction of three characterized rice ATs (OsAT5, OsAT4/PMT, and OsAT10) and AsFMT with a set of 46 biochemically characterized BAHD enzymes that had previously been divided into five clades, I to V (11), and three PFO2458-containing proteins from fungi and one from moss (Physcomitrella patens) to serve as an outgroup. We used MEGAS2.2.2 (35) to infer and visualize maximum likelihood phylogenies with the following parameters: amino acid substitutions according to the Jones-Taylor-Thornton model, γ distribution of mutation rate among sites, a distribution shape parameter of 5, and gaps treated by partial deletion, allowing site coverage as low as 95%. From these analyses (Fig. 3), we concluded that AsFMT and OsAT5 are not evolutionarily related. Rather, OsAT5, OsAT4, and OsAT10 are closely related to the MsBanAAT in BAHD clade V and belong to a subclade of BAHD proteins, previously referred to as the “Mitchell clade.” By contrast, AsFMT is a member of clade III.

Second, we separately analyzed clade III and the Mitchell clade across diverse species and focused on the genera represented in the DFRC database. We searched the following genome annotation sources and versions, which were current at the time of the analysis: maize (Zea mays), MaizeGDB version 2; switchgrass (Panicum virgatum), Phytodrome version 1.1; palm (Phoenix dactylifera), Weill Cornell Medicine–Qatar PDK sequence version 3; eucalyptus (Eucalyptus grandis), Phytodrome version 1.1; and banana (Musa acuminata), Banana Genome Hub DH-Pahang v1. For comparison with previous analyses, we also included sequences from the following sources: Arabidopsis (A. thaliana), The Arabidopsis Information Resource version 10; soybean (Glycine max), Phytodrome version 7.0; Medicago truncatula, Mt3.5; sorghum (Sorghum bicolor), Phytodrome version 7.0; rice (O. sativa), Michigan State University version 6.1; and B. distachyon, Phytodrome version 7.0 (17). Phylogenetic analyses were then conducted with the Pfam domain protein sequences (fig. S2B).

We determined the BAHD clade of each predicted protein via comparison with the D’Auria set using Clustal2 (37) and omitted sequences that lacked the region surrounding the highly conserved active-site motif HXXXD. From the maximum likelihood phylogenetic reconstructions for these initial single species (100 bootstraps), we identified proteins most closely related to those in the Mitchell clade and clade III. For clade III, we identified 12 proteins from eucalyptus, 9 from Arabidopsis, 1 from palm, and none from rice, maize, or banana, suggesting that clade III is not as widely represented in commelinids as in dicots (fig. S2A). For the Mitchell clade, we identified 22 predicted proteins from banana, 20 from rice, 17 from maize, 23 from switchgrass, 13 from sorghum, 16 from Brachypodium, 11 from palm, and 1 from eucalyptus. This builds on the previous results by providing evidence that this clade of genes not only is more abundant in grasses than in eudicotyledonous plants (17) but also includes numerous members in other commelinid monocots. The four subgroups (a to d) of Mitchell clade “i” (within clade V), which have both grass and banana and/or palm proteins, are consistent with there being at least four members of this gene family in the last common ancestor between the grasses and other commelinids. We then carried out maximum likelihood phylogenetic reconstructions with the commelinid-expanded Mitchell clade and related protein sequences and the dicot-expanded clade III. We used 1000 bootstraps for the Mitchell clade tree, with the outgroup consisting of the Arabidopsis spermidene dicoumaroyl transferase and spermidene dinosaponyl transferase proteins and a group of closely related enzymes that function in taxol biosynthesis. For the clade III tree, we ran 500 bootstraps, and the outgroup consisted of two clade II proteins.

**Gene expression of OsPMT in A. thaliana**

The OsPMT gene from rice was introduced into Arabidopsis (A. thaliana), which does not natively incorporate monolignol conjugates of any kind into its lignin, and targeted to secondary cell wall–forming cells (proCELLULOSE SYNTHASE 7::OsPMT) (16). These plant lines successfully produced monolignol p-coumarates and incorporated them into their lignins.

**Rice lines overexpressing OsFMT (OsAT5)**

Rice plants were grown in a greenhouse in Turface Athletics medium/vermiculite (1:1) mix supplemented three times per week with fertilizer (Jack’s Professional LX 15-5-15 4Ca 2Mg) at temperatures from 29° to 32°C during the day and from 24° to 25°C during the night. After germination in water, 7-day-old seedlings were transplanted to the greenhouse. Natural day lengths of less than 13 hours were supplemented with artificial lighting.

We characterized two classes of OsFMT/OsAT5 overexpression rice mutants, an activation-tagged line and three independent Ubiquitin1 promoter lines. Depending on the assay, we characterized the second (T2) and/or third (T3) generation of selfed progeny of activation-tagged homozygous mutant and WT segregant rice line of PPG2-2A-20021, referred to herein as OsAT5-DI. The T1 generation of this line, which is in the Dongjin cultivar background, was previously reported to have an increased in wall-associated ferulates in the mature leaf sheaths (17). We characterized mutant progeny of 2A-20021.10 and WT segregant progeny of 2A-20021.11. DFRC data are from the T3 generation (mutant 2A-20021.10.7.45.2 and WT 2A-20021.11.2.40.2). Trifluoroacetic acid (TFA) fractionation data are from the T2 generation (mutant 2A-20021.10.2.2 and WT 2A-20021.11.7.2), as are gene expression data. In this notation, the period separates generation identities, and the numbers indicate the number of the parental plant of the analyzed progeny. Genotyping, as previously described by Bartley et al. (17), was used to isolate homozygous lines and for spot-checking to confirm future generations.

To generate independent lines overexpressing OsAT5, a pUC-based plasmid containing the full coding sequence for OsAT5 was synthesized de novo. We then recombinated the gene into the pCAMBIA1300-Ubi-GW-Nos construct (38) to produce pCAMBIA1300-Ubi4AT5. This binary vector contains a Gateway cassette, flanked by the maize Ubi1 promoter, the 3′-terminator of nopaline synthase from Agrobacterium tumefaciens, and the Hpt2 gene that confers resistance to hygromycin. The rice cultivar Nipponbare was used for transformation after introducing the overexpression
vector pCAMBIA1300-UbiAT5 into the A. tumefaciens strain EH105. The transformation procedure was similar to the method described by Nishimura et al. (39). Briefly, embryogenic calli were obtained from 3-week-old immature embryos of Nipponbare. EH105 suspended in an optical density of 0.1 at 600 nm (approximately 3 × 10⁶ colony-forming units/ml) was cocultivated with calli for 90 s and placed on sterile filter paper to remove excess Agrobacterium. The infected calli were incubated for 60 hours in the dark and, later, were washed and reincubated on regeneration media for 4 weeks under continuous light (3.5 klux). Regenerated plants were grown in deep plastic containers for 1 week, transferred to pots for growth in the greenhouse under the conditions described above, and genotyped for Hpt2 and the Ubi₃₅::AT5 construct (table S7). We characterized the T₁ progeny of Ubi₃₅::AT5 lines—9, 10, and 17—along with negative segregant WT plants for each line. Several additional lines were set aside because of low T₀ seed set or apparent lack of expression of the transgene, assayed as described below.

OsAT5 overexpression was assessed using quantitative real-time reverse transcription polymerase chain reaction (figs. S3 and S4), with SYBR green and primers as previously described (table S7) (17, 40, 41). Two reference genes, Ubq5 and Cc55, were used for every sample measured. Gene expression was measured in leaf tissue from vegetatively developing rice plants at approximately the V5 stage. We selected samples from the same leaf and developmental stage, that is, developmentally matched, between controls and mutants for each line. For the OsAT5-D1 line, samples were T₂ mutant and nontransgenic negative segregants approximately 5 weeks after sowing. For the Ubi₃₅::AT5 lines, samples were second-generation transgenic (T₁) and negative segregants lacking the transgene from mutant lines 9, 10, and 17.

Analysis of carbohydrate-associated ferulic acid of OsAT5-D1
We used weak acid fractionation to determine whether the changes in hydroxycinnamate content were associated with matrix polysaccharides or lignin. Destarched AIR was created as previously described (17). For weak acid treatment, 2.5 mg of destarched AIR was mixed with 500 µl of either 0.05 M TFA or water, similar to a previously described method (17, 42). Samples were incubated for shaking at 100°C for varying times. At each time point, the supernatant (containing solubilized cell wall material) was separated from the remaining solid by centrifugation. Thawed samples were treated with 2 M NaOH and neutralized with concentrated HCl, trans-cinnamic acid was added as an internal standard, and the samples were frozen to stop the reaction. Thawed samples were then ethyl acetate–extracted and analyzed by high-performance liquid chromatography as previously described (17).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/10/e1600393/DC1

Supplementary Text

fig. S1. Primers used in this study.
fig. S2. The phylogenetic reconstruction of BAHD acyl-CoA ATs is consistent with the convergent evolution of the two feruloyl-CoA monolignol transferases, OsAT5/FMT and AtFMT.
fig. S3. Genomic position and gene expression data for the AT5-D1 rice activation-tagged line.
fig. S4. OsAT5 expression data and DRFC-released ML-DhFCA conjugates from OsAT5 rice lines. Fig. S5. The cell wall compositional differences in OsAT5-D1 straw are predominantly due to the (50 mM TFA, 100°C insoluble fraction.

REFERENCES AND NOTES


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Monolignol ferulate conjugates are naturally incorporated into plant lignins

Troy M. Runge, Kirankumar S. Mysore, Philip J. Harris, Laura E. Bartley and John Ralph

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