Manipulation of Lignin Monomer Composition Combined with the Introduction of Monolignol Conjugate Biosynthesis Leads to Synergistic Changes in Lignin Structure

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The complexity of lignin structure impedes efficient cell wall digestibility. Native lignin is composed of a mixture of three dominant monomers, coupled together through a variety of linkages. Work over the past few decades has demonstrated that lignin composition can be altered through a variety of mutational and transgenic approaches such that the polymer is derived almost entirely from a single monomer. In this study, we investigated changes to lignin structure and digestibility in Arabidopsis thaliana in near-single-monolignol transgenics and mutants and determined whether novel monolignol conjugates, produced by a FERULOYL-CoA MONOLIGNOL TRANSFERASE (FMT) or a p-COUMAROYL-CoA MONOLIGNOL TRANSFERASE (PMT), could be integrated into these novel polymers to further improve saccharification efficiency. Monolignol conjugates, including a new conjugate of interest, p-coumaryl p-coumarate, were successfully integrated into high-H, high-G and high-S lignins in A. thaliana. Regardless of lignin composition, FMT- and PMT-expressing plants produced monolignol ferulates and monolignol p-coumarates, respectively, and incorporated them into their lignins. Through the production and incorporation of monolignol conjugates into near-single-monolignol lignins, we demonstrated that substrate availability, rather than monolignol transferase substrate preference, is the most important determining factor in the production of monolignol conjugates, and lignin composition helps dictate cell wall digestibility.

Keywords: 2D-HSQC-NMR • Arabidopsis • DFRC • Lignin mutants • Monolignol biosynthesis • Saccharification

Introduction

Native lignin, although integral to the growth and development of the plant body, is the source of many problems in industry due to its recalcitrant nature and the difficulties associated with its degradation (Himmel et al. 2007, Rinaldi et al. 2016). Lignin polymers are the product of combinatorial coupling reactions between multiple subunits through various categories of covalent cross links (Freudenberg and Neish 1968, Ralph et al. 2004b). Both the number of subunits and the complexity of linkages between the subunits are major factors in the recalcitrance of lignin toward degradation. The majority of the units found in lignin are characterized by their alkyl aryl ether (β-O-4), C–C (β–β, β–S, β–T, S–T) or diaryl ether (4–O–5) bonds that are either refractory to cleavage or require strong acids or bases and high temperatures to break (Rinaldi et al. 2016). Adding to lignin’s resistance to degradation, there is also potential for condensed and non-linear structures to form during processing (Shuai et al. 2016, Lan and Luterbacher 2019, Ralph et al. 2019). In short, most native lignin polymers present a range of traits that are undesirable for industrial processing. Recently, the introduction of ester bonds into the lignin backbone by the incorporation of monolignol conjugates has garnered attention as a method by which to improve the digestibility of the lignin, and therefore the cell wall, without negatively impacting the growth or development of the plant (Marita et al. 2014, Petrik et al. 2014, Wilkerson et al. 2014, Smith et al. 2015, Karlen et al. 2016, Mottiar et al. 2016, Sibout et al. 2016, Zhou et al. 2017, Kim et al. 2017b, Bhalla et al. 2018). Native monolignol conjugates can be found in a wide range of plants, from maize to poplar (Ralph 2010, Wilkerson et al. 2014, Lu et al. 2015, Karlen et al. 2018), but they may also be introduced into model species such as Arabidopsis thaliana and other plants that do not naturally produce them (Wilkerson et al. 2014, Smith et al. 2015). Although such engineered plants represent a major milestone in improving the processing of lignin, they may not yet represent the best lignins from an industrial perspective.
It has been suggested that an optimal lignin for biorefinery operations is one derived from a single type of monomer in which the resulting lignin contains primarily C–O bonds (Li et al. 2018). Although native lignins do not fit this principle, there are a number of plants with mutations in lignin biosynthetic genes that produce altered lignins that approximate this scenario, as exemplified by lignins from O-Methyltransferase (OMT)-deficient plants producing essentially homopolymers of either caffeoyl alcohol or 5-hydroxyconiferyl alcohol (Ralph et al. 2001, Weng et al. 2010, Tobimatsu et al. 2013, Li et al. 2018). Other lignin mutants have been well-described in the model plant A. thaliana. The c3′h null mutant (ref8-1) produces lignin that is predominantly derived from p-coumaryl alcohol and therefore has only p-hydroxyphenyl (H) units (Franke et al. 2002). These plants are dwarfed, but the growth deficiency can be overcome by mutating the mediator5a and mediator5b subunits in the c3′h background (Bonawitz et al. 2014). The triple mutants (medi5a/medi5b/ref8-1, hereafter denoted c3′h/med) therefore have high H-lignin but are not substantially retarded in development. The flux from coniferyl alcohol (G-lignin) formation to sinapyl alcohol (S-lignin) formation requires the enzyme FERULATE 5-HYDROXYLASE (F5H). Null mutations in the FSH gene (fah1 mutant) result in plants that deposit lignin primarily derived from coniferyl alcohol (G-lignin), similar to native gymnosperm lignins (Meyer et al. 1996, 1998). More strikingly, overexpression of the FSH gene under the control of the C4H promoter (pC4H::FSH) results in the production of lignin that is primarily derived from sinapyl alcohol (S-lignin) (Meyer et al. 1998, Marita et al. 1999, Stewart et al. 2009). The latter three mutant backgrounds all represent potential optimal lignins for lignin processing because they are dominated by one type of monomer, with the high-S lignin being the best in terms of having a high β-ether content (Stewart et al. 2009). However, the processability of the lignin might be further improved by adding more labile bonds into the polymer as well, in a so-called ‘zip-lignin’ approach (Wilkerson et al. 2014, Zhou et al. 2017).

*p-COUMAROYL-CoA MONOLIGNOL TRANSFERASE (PMT)* from Oryza sativa (OsPMT = OsAT4) (Mitchell et al. 2007, Withers et al. 2012) and *FERULOYL-CoA MONOLIGNOL TRANSFERASE (FMT)* enzymes are capable of producing chemically labile ester bonds. Two of the best-defined monolignol transferase enzymes are from Oryza sativa (OsPMT = OsAT4) (Mitchell et al. 2007, Withers et al. 2012) and Angelica sinensis (AsFMT) (Wilkerson et al. 2014). OsPMT and AsFMT have previously been successfully transformed into Arabidopsis, resulting in the production of monolignol p-coumarolates or monolignol ferulates and their integration into the lignin polymer (Smith et al. 2013, 2015, 2017).

Crucial to the determination of lignin structure and proof that monolignol conjugates are introduced into the polymer is the derivatization followed by reductive cleavage (DFRC) and two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy (2D-HSQC-NMR) methods. DFRC is a degradative lignin analytical technique releasing the lignin-derived monomers in a reaction that specifically cleaves lignin units bound to the lignin polymer by β–O–4 linkages (Lu and Ralph 1997a, b). Lignin end-units are cleaved to release monomer units, in addition to units bound at their β- and 4-O-positions in β-ether units. DFRC specifically cleaves ether linkages but leaves ester linkages intact (Lu and Ralph 1999, Regner et al. 2018). As a result, zip-lignin units/monolignol conjugates are released as intact units and create a unique chemical fingerprint that is distinct from that of the prototypical H-, G- or S-monomer units. 2D-HSQC-NMR is a valuable complement to DFRC because it profiles the lignin in the intact, non-degraded cell wall without requiring lignin isolation (Kim and Ralph 2010, Mansfield et al. 2012, Tobimatsu et al. 2019). NMR provides information regarding the total lignin units, regardless of the interunit linkage that binds them to the lignin polymer. When the results of the chemical analysis are taken together and if conjugates can be produced in the mutant lines, it is possible to ascertain the proportion of monolignols that are diverted to the production of monolignol conjugates.

Although the integration of conjugates into mutant lignin backgrounds would seem like a straightforward proposal, it is unclear whether plants are capable of efficiently integrating monolignol conjugates into the lignin when there is primarily one type of monomer present. The types of monolignol conjugates that would be produced, i.e. which monolignol(s) and which hydroxycinnaminate(s) are used for conjugate production, are also unknown. The aim of this study was therefore to investigate the production of monolignol conjugates in the different mutant backgrounds to delineate the effects of lignin-pathway manipulations on the nature of the conjugating elements (hydroxycinnamates) and to determine their effects on lignin structure and, ultimately, cell wall digestibility.

**Results and Discussion**

To determine whether monolignol conjugates can be integrated into lignins dominated by single monomers and to elucidate the effect the resulting integration had on lignin structure, high-H, high-G and high-S lignin Arabidopsis mutants and transgenics were generated and grown in parallel with wild-type Arabidopsis. OsPMT or AsFMT were overexpressed in the various mutant and wild-type backgrounds. The wild-type, mutant and transgenic plants generated and analyzed in this study were as follows: Col-0 wild-type Arabidopsis, Col-0 996 (empty vector control), Col PMT, Col FMT, c3′h/med, c3′h/med PMT, c3′h/med FMT, fah1, fah1 PMT, fah1 FMT, pC4H::FSH, pC4H::FSH PMT and pC4H::FSH FMT.

The 6-week-old wild-type, mutant and transgenic Arabidopsis stems were analyzed for plant height and stem biomass yield. No differences in plant height were observed between Col-0 wild-type and Col PMT or Col PMT lines (Supplementary Fig. S1). The mutant and transgenic fah1 plants also displayed wild-type plant height, with the exception of one fah1 FMT line that was slightly taller. The mutant and transgenic pC4H::FSH
and C3′/med lines were somewhat shorter than wild type. The biomass yield of the primary inflorescence stems follows the same trend as the plant height results (Supplementary Fig. S2). Both pC4H::F5H PMT lines had higher biomass yields than their corresponding mutant, and line 215-2 also achieved a greater plant height, but the reasons for this are unclear. The lignin content of the different lines was also examined to determine if differences could be observed between wild-type, mutant, and transgenic lines. The wild-type and mutant lines had similar cysteine-associated sulfuric acid (CASA)-lignin content to the transgenic lines in the same mutant or wild-type background, with the exception of the pC4H:F5H PMT lines, which had a slightly lower lignin content (Supplementary Table S1).

Wild-type Arabidopsis had a high percentage of G-lignin (~80%), followed by S-lignin (~20%), and a trace level of H-lignin, as has been previously reported (Fig. 1, Fig. 2A) (Mansfield et al. 2012, Smith et al. 2013). The AsFMT and OsPMT genes were successfully transformed into the wild-type background under the control of the Arabidopsis C4H promoter. The Col PMT lines produced monolignol p-coumarate conjugates, including both coniferyl p-coumarate and sinapyl p-coumarate (Fig. 1, Fig. 2B). The expression of OsPMT in these lines correlates with the amount of p-coumarates detected through chemical analysis (Fig. 3A). Prior investigation of the enzyme kinetics of the OsPMT enzyme determined that the enzyme had the highest substrate affinity and catalytic efficiency with p-coumaroyl alcohol as a substrate, followed by sinapyl alcohol (Withers et al. 2012). Coniferyl alcohol was the least preferred substrate in vitro. In contrast, there is a slight preference for coniferyl alcohol, the most abundant monolignol, to be used over sinapyl alcohol or p-coumaryl alcohol in the production of monolignol p-coumarates in planta, suggesting that enzyme kinetic parameters are a less important factor than substrate availability in vivo. Similarly, the Col FMT lines produced monolignol ferulates and integrated them into the lignin polymer, and coniferyl ferulate was the primary monolignol conjugate form generated. As with the Col PMT lines, there was a correlation between AsFMT expression and the product detected (Fig. 2C, Fig. 3B). The amounts of monolignol p-coumarates detected by 2D-NMR (Fig. 1) and released by DFRC (Fig. 2C) were substantially higher than the levels of monolignol ferulates. This is most likely due to the prevalence of monolignol p-coumarates as lignin end-units, facilitating their release from the polymer and subsequent detection (Ralph et al. 1994, Ralph 2010, Withers et al. 2012, Rinaldi et al. 2016). By contrast, monolignol ferulates can be integrated into the backbone of the polymer after which only a small percentage of the conjugates can be released by DFRC (Wilkerson et al. 2014). As a result, the amount of monolignol ferulates reported underrepresents the conjugates present in the lignin polymer. Similarly, ferulates are difficult to detect by NMR because they can couple with each other and cross-couple into the lignin in a variety of ways (Wilkerson et al. 2014); as such, each structure is present only at low levels, making their detectability in NMR difficult (Ralph et al. 1995). These results, together with previous reports, confirm that PMT and FMT can successfully be transformed into Arabidopsis and that these transferase enzymes use whichever monolignol that is most prevalent regardless of enzyme kinetic preferences.

One of the types of homogeneous lignin investigated was high-G lignin. High-G lignin was achieved in Arabidopsis through a null mutation in the FSH gene (Humphreys et al. 1999). FSH catalyzes hydroxylation of the 5-position of the aromatic ring of coniferaldehyde and coniferyl alcohol to yield 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, respectively. Without this enzyme, sinapyl alcohol and syringyl lignin synthesis is blocked, and, as a result, the lignin of these ferulic acid hydroxylase 1 (fah1) mutants is 99.3% G-lignin, with only trace levels of H-lignin and S-lignin (Fig. 1). The fah1 mutants were also transformed with OsPMT and AsFMT to determine if high G-lignins can produce monolignol conjugates and to determine what kinds of conjugates are produced. The plants were examined for gene expression of AsFMT or OsPMT, and all but one of the fah1 FMT lines had detectable levels of the transgene (Fig. 3). The fah1 PMT lines also had homogeneous G-lignin, with only trace amounts of H- and S-lignin (Fig. 1, Fig. 2A); the plants produced monolignol p-coumarates, specifically coniferyl p-coumarate, and incorporated it into the lignin polymer (Fig. 2B). These results are in line with a previous study in which BdpMT1 was expressed in the fah1 genetic background (Sibout et al. 2016). Like OsPMT, BdpMT facilitated the production of coniferyl p-coumarate in fah1 plants, but the fah1 BdpMT1 lines were observed to have less p-coumarates than wild-type BdpMT lines (Sibout et al. 2016). This was attributed to the preference of PMT enzymes for sinapyl alcohol as an acceptor substrate. In contrast, fah1 OsPMT lines in this study showed higher levels of p-coumarates than detected in wild-type OsPMT plants. This may be due to differences in expression patterns of the PMTs between these two studies, leading to differences in substrate availability or differences in enzyme kinetics between OsPMT and BdpMT related to coniferyl alcohol. As in the fah1 PMT lines, the fah1 FMT transformants produced coniferyl ferulate and integrated it into the lignin polymer, as evidenced by its release through DFRC and in the whole cell wall (WCW) by 2D-NMR (Fig. 1, Fig. 2C).

High-S lignin is another type of homogeneous lignin available in Arabidopsis. High-S lignin is achieved by overexpressing the FSH gene using a strong lignin-specific C4H promoter (pC4H::FSH) (Meyer et al. 1998, Marita et al. 1999, Stewart et al. 2009). Overexpressing the FSH gene results in a more complete conversion of coniferaldehyde to 5-hydroxyconiferaldehyde and ultimately to sinapyl alcohol and syringyl lignin (S-lignin) production. The pC4H::FSH lines therefore have ~97% S-lignin in contrast to 20% S-lignin in wild-type Arabidopsis plants (Fig. 1). As with the other lignins enriched in one monolignol, the high-S lines were transformed with OsPMT and AsFMT. Introducing PMT and, especially, FMT into high-S lines was aimed at producing primarily sinapyl alcohol conjugates but was also
Fig. 1 2D-HSQC-NMR of Arabidopsis wild-type, mutant and transgenic lines. The aromatic region of the spectra is shown for Col wild-type (A), and the corresponding FMT and PMT transgenics (B, C), the high G-lignin mutant (fah1) (D), and the corresponding transgenics (E, F), the wild-type empty vector control (G), the high-S lignin (pC4H::F5H) transgenic (H), and the corresponding FMT- and PMT-expressing lines (I, J), and the high H-lignin (c3′h/med) mutant (K) and corresponding FMT-expressing transgenic (L).

aimed at elucidating whether other intermediates in the pathway, such as the various hydroxycinnamates, could be satisfactorily used as substrates. Also of interest was whether the overexpression of F5H would result in the production of sinapate, an S-lignin intermediate outside the core monolignol pathway, possibly resulting in sinapyl sinapate conjugates that are of considerable interest in producing more readily degradable zipper lignins. Sinapyl sinapates would make a linear, predictable lignin structure if present in high quantities during lignification.

The pC4H::F5H PMT plants had similar levels of S-lignin to the pC4H::F5H transgenic alone, with 96% S-lignin (Fig. 1). Of the total lignin in these lines, ~6% was p-coumarate, the majority of it was sinapyl p-coumarate, with small amounts of coniferyl p-coumarate and p-coumaryl p-coumarate. The pC4H::F5H PMT lines also had 97% S-lignin and produced significant amounts of sinapyl ferulate (Fig. 1, Fig. 2A, C). There was, again, a correlation between the expression of the PMT and FMT genes and the amount of monolignol conjugate detected on the lignin (Fig. 3). We hypothesized that high S-lignin pC4H::F5H mutants expressing an FMT enzyme would accumulate higher levels of sinapate, as a byproduct of the S-unit branch of the pathway and therefore perhaps produce sinapyl sinapate conjugates. These lines, however, produced only sinapyl ferulate and did not produce detectable levels of sinapyl sinapate. From these data, we can hypothesize that the FMT enzyme is not promiscuous enough to utilize sinapoyl-CoA as a substrate and/or that there are insufficient levels of sinapoyl-CoA to produce the conjugate. In Arabidopsis, only one ligase enzyme has been reported to allow sinapate to generate sinapoyl-CoA (At4CL4) (Li et al. 2015). The gene encoding At4CL4 is expressed in the roots and cotyledon veins of Arabidopsis, neither of which tissues were examined for the presence of sinapyl sinapate in the pC4H::F5H PMT lines, and At4CL4 is not expressed in the stems, which holds the majority of the lignifying tissue (Li et al. 2015). It is therefore possible that sinapyl sinapates can only be produced in Arabidopsis or other plant species if the ligase that produces sinapoyl-CoA is co-expressed with a monolignol transferase enzyme that can similarly use the sinapate substrate.

High-H lignin has been produced in plants by knocking down or knocking out the p-COUMAROYL SHIKIMATE C3′H HYDROXYLASE (c3′h) gene that encodes the C3′H enzyme (Franke et al. 2002). This enzyme is responsible for the 3′-hydroxylation of p-coumaroyl shikimate to generate caffeoyl shikimate and, without this enzyme, plants deposit a lignin dominated by p-hydroxyphenyl (H) units. In Arabidopsis, null mutations in the c3′h gene also result in severe dwarfism (Franke et al. 2002).
Fig. 2 DFRC analysis of Arabidopsis wild-type, mutant and transgenic lines to determine the release of monolignols (A), monolignol p-coumarates (B) and monolignol ferulates (C) from the lignin polymer. HA, p-coumaryl alcohol; CA, coniferyl alcohol; SA, sinapyl alcohol; GpCA, coniferyl p-coumarate; SpCA, sinapyl p-coumarate; GFA, coniferyl ferulate; SFA, sinapyl ferulate. The average areas of p-coumaryl p-coumarates released from the lignin (D) reflect the amount of p-coumaryl p-coumarate that was detected as pendant moieties (cis- or trans-Ac-HA-Ac-pCA) or integrated into the lignin on both ends via β-ether linkages (d3Ac-HA-d3Ac-pCA).

Fig. 3 Expression of the OsPMT (A) or AsFMT (B) transgene in primary inflorescence stem of 4-week-old plants. Transgene expression was normalized to the reference gene ACTIN2 (At1g18780). Error bars represent standard deviation among biological replicates (n = 3).
This phenotype is not the direct effect of the high H-lignin deposition, however, as mutations in two components of the mediator complex, med5a and med5b, suppress the dwarf phenotype of the refβ mutant. The resulting c3′h/med plants still have lignin dominated by H-units (89%) but grow almost normally (Fig. 1, Supplementary Fig. S1) (Bonawitz et al. 2014). To determine whether these high-H lines can incorporate monolignol conjugates into the lignin, the triple mutant plants were transformed with OsPMT or AsFMT genes. No c3′h/med PMT transformants were obtained for analysis, suggesting that the plants were seedling-lethal. It is possible that the high H-lignin content results in the production of p-coumaryl p-coumarate units, which may be toxic to the plant because they cannot be efficiently integrated into the cell wall or stored in the vacuole. FMT expression in the c3′h/med background was higher than in the other lines, presumably due to the upregulation of phenylpropanoid gene expression previously observed in the med5a med5b background (Bonawitz et al. 2014). Despite high expression of FMT, the c3′h/med FMT lines had a high H-lignin content, as seen in the mutant plants, but did not produce detectable levels of monolignol ferulates (Fig. 1, Fig. 2C, Fig. 3B). The lack of ferulates in these high-H lines was expected, as a disruption in the C3′H gene would negatively impact the production of downstream lignin biosynthetic pathway intermediates and products, including feruloyl-CoA, which is the acyl donor for AsFMT.

A pattern that consistently appeared for all the FMT and PMT transgenic lines is that the acyltransferases were able to use multiple acceptor substrates. Both enzymes predominantly used whichever monolignol substrate was most prevalent. The kinetics of the OsPMT enzyme have been characterized in depth (Withers et al. 2012). This study determined that OsPMT has the highest affinity and the most efficient activity with p-coumaryl alcohol and sinapyl alcohol as substrates with p-coumaroyl-CoA. Very little activity was observed with coniferyl alcohol and other alcohol or acid substrates tested. These kinetic assay results suggest that, regardless of monolignol level, the plants should preferentially produce p-coumaryl p-coumarates and sinapyl p-coumarates. However, this was not the case. The most prevalent monolignol, especially in homogeneous lignin lines, is almost exclusively used for the production of monolignol p-coumarates. This means that coniferyl alcohol is the primary substrate for OsPMT in the high G-lignin lines, but also in the Col PMT lines, which have 81% G-lignin (Fig. 1). Sinapyl p-coumarates are the primary monolignol conjugate products in all the high S-lignin lines. p-Coumaryl p-coumarates were produced at very low levels in all PMT-expressing lines (Fig. 2D), which reflects the low levels of H-lignin in all the control and transgenic lines and further supports the hypothesis that the abundance of monolignols is more important than the substrate preferences observed in the in vitro assays.

These mutant lines presented an opportunity to test whether plants can produce certain types of monolignol conjugates and, more importantly, whether those conjugates are integrated throughout the lignin polymer or exist only as pendant groups. One such conjugate of interest was p-coumaryl p-coumarate.

As has been pointed out (Ralph et al. 1994) and reviewed (Ralph 2010, Ralph and Landucci 2010, Vanholme et al. 2019), p-coumarate units in lignins are almost entirely free-phenolic pendant units on the lignin side chain. p-Coumarate radicals preferentially undergo radical transfer to produce more stable radical species and therefore tend not to enter into radical coupling reactions with guaiacyl or syringyl phenolics (Ralph et al. 2004a, Hatfield et al. 2008, Ralph 2010, Vanholme et al. 2019). In that sense, they are not compatible with the radical coupling reactions occurring during lignification and do not integrate into the backbone of the polymer. We have long surmised that p-coumarates might be compatible with H-lignin formation, however, with radical coupling between H-units and p-coumarates being more likely to occur. To assess whether p-coumaryl p-coumarate can be integrated into an H-lignin polymer, p-coumaryl p-coumarate was synthesized and a biomimetic dehydrogenation polymer (DHP) was prepared using this conjugate and the H-monolignol, p-coumaryl alcohol. 2D-HSQC-NMR of the resulting DHP revealed cross-coupled structures from both moieties of the conjugate, indicating that p-coumaryl p-coumarate integrated into the H-lignin polymer (Fig. 4). The most prevalent units, characterized by their diagnostic interunit linkages, in the DHP were the β-O–4 A, β–5 B and β–β C units typical of DHP lignins. p-Coumaryl p-coumarates were found bound into the DHP in both A′ and B′ units and particularly diagnostic β–β–coupled units C′ and C″ were evident (see Fig. 4 caption that explains how the primed units are diagnostic for the incorporation of the conjugates into lignin). These results indicate that, in vitro, the conjugates and, importantly, the p-coumarate moiety can be integrated into the lignin polymer backbone, as revealed by units C″ (Fig. 4). As a result, the lignin backbone contains ester units and can therefore be classified as a zip-lignin, similarly to those produced using monolignol ferulates in normal G/S-lignins. In other words, p-coumarates can be used as zip-components when the lignification is made to be compatible with their radical coupling. It might be noted that this compatibilization of the lignin is the opposite of the original zip-lignin strategy of making ferulate conjugates that were compatible with the coupling of G and S monomers and oligomers in ‘normal’ lignification (Grabber et al. 2008, Ralph 2010, Wilkerson et al. 2014). The alternative approach here to creating zip-lignins in grasses, by utilizing the existing p-coumarate conjugates and altering the lignins to be p-coumarate-compatible H-lignins, may be thwarted by the same agronomic issues noted with high-H lignin plants in general. Indeed, in this study, correcting for high-H lignin growth defects in Arabidopsis with the med5a/med5b double mutant may have resulted in seedling lethality when the triple mutants were coupled with PMT expression.

The mutant lines expressing OsPMT all produced small amounts of p-coumaryl p-coumarates, but the DFRC and NMR analyses do not indicate whether the conjugates were pendent or integrated into the lignin polymer in planta. A modified
Fig. 4 HSQC-NMR of the synthetic lignin (DHP) prepared from \( p \)-coumaryl \( p \)-coumarate and \( p \)-coumaryl alcohol. Contour peaks are color-coded to match the assigned structures that are coded with conventional descriptors \([A\) for \( \beta \)-ether, \( B\) for phenylcoumaran and \( C\) for resinol\]) for units that may be linked to other \( H\) or \( p\)CA units; the primed variants \([A', B', C'\) and \(X'\)] are \( H\)-units in the polymer that have \( p\)CA units attached and are therefore derived from the \( p\)-coumaryl \( p\)-coumarate conjugates in the polymerization; the double-primed units \([A''\), \(B''\), \(C''\)] are those from the \( p\)-coumaryl \( p\)-coumarate conjugates in which the \( p\)-coumarate moiety itself has coupled into the lignin, providing evidence for cross-coupling of those \( p\)-coumarate moieties to also integrate them fully into the backbone of the polymer. From this spectrum, we can tell little about the etherified and/or cross-linked nature of the \( \beta \)-ether units \([A\), \(A''\) (and \(A'''\), not shown\]), but their etherification status (to demonstrate that both the \( H\)-unit and the \( p\)CA moiety are integrated into the polymer) may be derived from the modified DFRC experiments (see text).

DFRC approach has already been designed to directly answer this question (Martone et al. 2009, Lu and Ralph 2014), i.e. whether the \( p\)-coumarates are incorporated into the polymer only as end-groups or also within the core of the polymer, as is the case for ferulates. The full solubilization achieved by the acetyl bromide reagent suggests that complete acetylation should occur in the first step (see the ‘Materials and Methods’ section for further explanation). In reality, there is some potential to over-estimate etherification as the first acetylation step may not be able to target a proportion of hydroxy end-groups, depending on the access of reagents to the whole lignin polymer.

The results from the modified DFRC provide an approximation for how much conjugate is found as end-groups versus within the polymer, if integration into the polymer does occur. The \( p\)-coumaryl \( p\)-coumarate detected was found only in two forms: as unlabeled conjugates or as doubly labeled conjugates. There was no evidence that singly labeled conjugates were released from the lignin. The Col PMT and \( fah1\) PMT lines released six times and four times as much doubly labeled \( p\)-coumaryl \( p\)-coumarate as unlabeled product by gas chromatography (GC) peak area, respectively. Even allowing for the possibility that some of the doubly labeled product might be end-groups (because of incomplete acetylation in the first acetylation step of DFRC), these data indicate that \( p\)-coumaryl \( p\)-coumarate is being incorporated into the lignin, as we noted for the in vitro DHP study above. The \( p\)C4H::F5H PMT lines released as much unlabeled conjugate as doubly labeled conjugate, but the data still suggest that some conjugate is being integrated into the lignin polymer in these plants. These data are supported by the results of a recent study in a rice \( c3h\) mutant (Takeda et al. 2018).

Previous studies have demonstrated that the addition of significant levels of monolignol ferulates, and occasionally monolignol \( p\)-coumarates, into the lignin polymer can improve the digestibility of the plant cell wall (Wilkerson et al. 2014, Sibout et al. 2016, Zhou et al. 2017, Kim et al. 2017b, Bhalla et al. 2018). The ester linkages that are generated within the conjugates by the monolignol transferase enzymes are more labile bonds than the ether (or other) linkages that are prevalent within the native lignin polymer. Studies in other plant species have shown that engineering plants to have high-\( H\) or high-\( S\) lignin significantly improves cell wall digestibility (refs) (Huntley et al. 2003, Bonawitz et al. 2014). For high-\( S\) lignin, this is due to the regularity of the lignin structure and the high proportion of ether linkages, but the reason(s) for enhanced saccharification in \( H\)-lignin-producing plants is unclear. High-\( H\) lignins have elevated condensation, but may be lower molecular weight and therefore less recalcitrant. Lignin with a high proportion of G-lignin, in contrast, is much more recalcitrant because it has a higher propensity to form condensed linkages that are difficult to cleave. In theory, therefore, the combination
of high-H or high-S lignin and monolignol conjugates in the lignin could lead to further improved digestibility. Partial saccharification analysis was performed to determine which milled WCW plant lines have a higher glucose and pentose release when pretreated with a weak base (6.25 mM NaOH) at room temperature. In all lines, there was no significant difference between the control wild-type or mutant line and the lines also expressing AsFMT or OsPMT genes (Fig. 5). The c3’h/med (high H) lines had a higher glucose and pentose release than the wild-type controls, as did the pC4H:F5H (high S) lines (Fig. 5). The fah1 (high G) lines had levels of glucose and pentose release that did not significantly differ compared to wild-type lines (Fig. 5). Although these results indicate that the monomer composition of the lignin appears to be more important than the presence of monolignol conjugates with respect to the digestibility of the samples, we suspect that engineering monolignol conjugates to higher than the trace levels observed in this study would significantly improve the digestibility of the different lines. A recent study reported levels of monolignol p-coumarates in wild-type and pC4H::F5H poplar that rivaled those seen in grasses, in contrast to the trace levels observed in this study, and the transgenic poplar lines had an improved saccharification efficiency under mild alkaline treatments (Lapierre et al. 2021). This study employed the gene encoding the BdPMT enzyme, a PMT from Brachypodium distachyon, which may reflect the importance of enzyme choice and activity level when engineering monolignol conjugates into the lignin backbone. Beyond the current observations, engineering plants in ways that integrate conjugates more readily into the backbone rather than remaining pendent remains a worthwhile goal that is proving elusive.

**Materials and Methods**

**Plant material and biomass measurements:**

*Arabidopsis thaliana* ecotype (Columbia-0) was used as the wild type. Plants were cultivated at a light intensity of 100 µE m⁻² sec⁻¹ at 22°C under a photoperiod of 16 h light/8 h dark in Redi-Earth Plug and Seedling Mixture (Sun Gro Horticulture, Bellevue, WA, USA) supplemented with Scotts Osmocote Plus controlled-release fertilizer (Hummer International, Earth City, MO, USA). Codon-optimized PMT from *Oryza sativa* (LOC_Os01g18744) and native FMT from *Angelica sinensis* (GenBank JA758320.1) were cloned into the plant binary vector pCC0996 that contains the *A. thaliana* C4H promoter (Bonawitz et al. 2012, Withers et al. 2012, Wilkerson et al. 2014). Constructs were transformed into wild-type, fah1, pC4H:F5H and c3’h/med plants (Chapple et al. 1992, Meyer et al. 1998, Bonawitz et al. 2014). The empty vector control Col 996 was generated by transforming wild type with pCC0996. Plant height measurements were taken from 6-week-old stems, with 8–13 stems analyzed per line. For primary inflorescence stem biomass measurements, 6-week-old stems were stripped from flowers, siliques, cauline leaves and axillary stems and weighed. Statistical differences were identified by one-way analysis of variance and Tukey’s post hoc test.

**RNA extraction and quantitative real-time polymerase chain reaction**

Four primary inflorescence stems from 4-week-old plants were harvested in triplicate, and flowers, siliques, cauline leaves and axillary stems were removed. RNA was extracted using the QiAGEN RNeasy® Plant Kit and DNase-treated using the Invitrogen™ TURBO DNA-free™ kit following the manufacturer’s instructions. Complementary DNA was synthesized using 1 µg of RNA and the Invitrogen™ MultiScribe™ Reverse Transcriptase. Quantitative real-time polymerase chain reaction was performed with primers (Supplementary Table S2) specific for AsFMT or OsPMT, and the Applied Biosystems™ Fast SYBR™ Green PCR Master Mix. ACTIN2 (At1g18780) was used as the reference gene to normalize gene expression.
DFRC of WCW samples

The DFRC analysis was performed essentially as previously reported (Regner et al. 2018), as follows. Each WCW sample (50 mg) was stirred in a 2-dram vial, with polytetrafluoroethylene (PTFE) pressure release cap, in a solution of acetyl bromide/acetic acid (1/4 v/v, 3 ml) at 50°C for 2.5 h. The solvent was removed on a SpeedVac (Thermo Scientific SPD111D, 50°C, 40 min, 1.0 torr, 35 torr/min). The crude film was suspended in absolute ethanol (1 ml), and the ethanol was then removed on the SpeedVac (50°C, 15 min, 6.0 torr, 35 torr/min). The residue was suspended in dioxane:acetic acidwater (5/4/1 v/v, 5 ml), and nano-powdered zinc (250 mg) was added. The reaction was then sealed, sonicated to ensure that the solids were suspended and stirred in the dark at room temperature for 16–20 h. The reaction was quantitatively transferred with dichloromethane (DCM, 3 × 2 ml) to a separatory funnel containing saturated ammonium chloride (10 ml) and the deuterated internal standards [300–600 µg d8-monolignols, 350 µg d10-monolignol-p-coumarates and 200–250 µg d12-monolignol-furanulates]. The organics were extracted with DCM (3 × 10 ml), combined, dried over anhydrous sodium sulfate, filtered and the solvent removed on a rotary evaporator (water bath at <50°C). The free hydroxy groups were then acetylated for 16 h in the dark using a solution of pyridine and acetic anhydride (1/1 v/v, 5 ml), after which the solvent was removed to give a crude oily film.

To remove most of the polysaccharide-derived products, the crude DFRC product was dissolved in 1:1 ethyl acetate:hexane (1.0 ml) and loaded onto a solid phase extraction (SPE) cartridge (Supelco Supelclean LC-Si SPE tube, 3 mL, Sigma-Aldrich, St. Louis, Missouri, USA, P/N: 505048). The products were eluted to give a crude oily film.

Lignin content analysis

The CASA-lignin contents of biomass samples were determined according to the published procedure (Lu et al. 2021) with some modifications. Each biomass sample (2–5 mg) was weighed to the nearest 0.01 mg and suspended in 0.5 ml of 67.25% sodium hydroxide (2.3 g of 30% solution, 2.0 ml) at 25°C for 2.5 h. The resultant solution was called solution A. To another flask containing 450 ml of 20 mM phosphate buffer (pH 6.5), 2.5 mg of 30% solution was added. The resultant precipitate (DHP product) was recovered by centrifugation (10,000 g, 15 min), washed with water (100 ml × 2) and lyophilized (2.82 g, 76.2% wt). The DHP product was dissolved in DMSO-δ6/pyridine-δ4 (1:1 v/v, 0.5 ml) in a 5-mm NMR tube. A 1H–13C NMR correlation experiment (HSQC, hsqcetgpsisp2.2, using adiabatic pulses and decoupling) was recorded on a Bruker BioSpin (Rheinstetten, Germany) Neo 700-MHz NMR spectrometer equipped with a 5-mm QCI 1H/13C/C5N cryograde probe with inverse geometry (proton coils closest to the sample) and previously described (Kim and Ralph 2010). The central DMSO solvent peak was used as the internal reference (δC 39.5, δH 2.49 ppm).

Synthetic lignin from p-coumaryl alcohol and p-coumaryl p-coumarate

Synthesis. p-Coumaryl alcohol and p-coumaryl p-coumarate were synthesized according to published methods with modification (Quideau and Ralph 1992, Lu and Ralph 1998). p-Coumaryl alcohol (2.5 g, 16.67 mmol) and p-coumaryl p-coumarate (12 g, 4.05 mmol) were dissolved in 250 ml acetone. To the solution was added 200 ml of 20 mM phosphate buffer (pH 6.5). The resultant solution was called solution B. About 150 mg of p-coumaryl alcohol was dissolved in 20% acetone aqueous buffer (200 ml) in a 1000-ml flask and then 5 mg horseradish peroxidase (Sigma-Aldrich, St. Louis, Missouri, USA; type VI) was added. To this solution, while stirring at 25°C, two addition of solution A and solution B, simultaneously and slowly (0.5 ml per min) via a peristaltic pump over a 15-h period at 25°C. During the addition, additional peroxidase (5 mg in every 5 h) in 1 ml buffer was added. The resultant precipitate (DHP product) was recovered by centrifugation (10,000 g, 15 min), washed with water (100 ml × 2) and lyophilized (2.82 g, 76.2% wt).

Digestibility analysis

Partial saccharification of glucose and pentose yield was performed as previously described (Santoro et al. 2010). Statistical significance of saccharification results was determined by Student’s t-test with P < 0.05.

Supplementary Data

Supplementary data are available at PCP online.
Data Availability

The AsFMT gene sequence used in this article is available in the Genbank Nucleotide Database at https://www.ncbi.nlm.nih.gov/genbank and can be accessed with the accession number JAT58320.1. The OsPMT gene sequence used in this article is available in the Phytozome database at https://phytozome-next.jgi.doe.gov and can be accessed with the accession number LOC_Os01g18744.

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Disclosures

The authors have no conflicts of interest to declare.

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