Lignin acylation, the decoration of hydroxyls on lignin structural units with acyl groups, is common in many plant species. Monocot lignins are decorated with p-coumarates by the polymerization of monolignol p-coumarate conjugates. The acyltransferase involved in the formation of these conjugates has been identified in a number of model monocot species, but the effect of monolignol p-coumarate conjugates on lignification and plant growth and development has not yet been examined in plants that do not inherently possess p-coumarates on their lignins. The rice (Oryza sativa) p-COUMAROYL-Coenzyme A MONOLIGNOL TRANSFERASE gene was introduced into two eudicots, Arabidopsis (Arabidopsis thaliana) and poplar (Populus alba × grandidentata), and a series of analytical methods was used to show the incorporation of the ensuing monolignol p-coumarate conjugates into the lignin of these plants. In poplar, specifically, the addition of these conjugates did not occur at the expense of the naturally incorporated monolignol p-hydroxybenzoates. Plants expressing the p-COUMAROYL-Coenzyme A MONOLIGNOL TRANSFERASE transgene can therefore produce monolignol p-coumarate conjugates essentially without competing with the formation of other acylated monolignols and without drastically impacting normal monolignol production.

Lignification of plant cell walls prototypically involves the polymerization of the monolignols (MLs), p-coumaryl alcohol, coniferyl alcohol (CA), and sinapyl alcohol (SA), predominantly by stepwise radical coupling of each monomer to the phenolic end of the growing polymer (Sarkanen and Ludwig, 1971; Boerjan et al., 2003; Ralph et al., 2004). The contribution of various MLs to the lignins depends on plant species, cell type, plant tissue, and tissue age. Although the majority of the lignin polymer is derived from these three MLs, the lignification process has a high degree of metabolic plasticity (Boerjan et al., 2003; Ralph et al., 2004; Ralph, 2007; Vanholme et al., 2012). Of particular interest are ML conjugates in which the ester group can be acetate (Ac; Sarkanen et al., 1967; Ralph, 1996; Ralph and Lu, 1998; Del Río et al., 2007; del Río et al., 2008; Martinez et al., 2008), p-hydroxybenzoate (pBz; Ververloo, 1971; Monties and Lapiere, 1981; Landucci et al., 1992; Tomimura, 1992a, 1992b; Hibino et al., 1994; Sun et al., 1999; Kuroda et al., 2001; Lu et al., 2004, 2015; Morreel et al., 2004; Rencoret et al., 2013), p-coumarate (pCA; Monties and Lapiere, 1981; Ralph et al., 1994; Crestini and Argyropoulos, 1997; del Río et al., 2008, 2012a, 2012b; Withers et al., 2012; Rencoret et al., 2013; Petrik et al., 2014), or ferulate (FA; Grabber et al., 2008; Ralph, 2010; Wilkerson et al., 2014). In all cases, the MLs are acylated before polymerization as proven by the presence in the lignins of unique β-β coupling products that only arise when one or both of the MLs are acylated, preventing the formation of the typical resinols from internal trapping of the quinone methide intermediates by the γ-OH (Lu and Ralph, 2002, 2008; Del Río et al., 2007, 2015).

The BAHD acyltransferase, FERULOYL-CoA MONOLIGNOL TRANSFERASE (FMT), was recently identified in Angelica sinensis and transformed into poplar (Populus alba × grandidentata), which naturally incorporates other acylated MLs, namely ML-pBz conjugates, into its lignin (Wilkerson et al., 2014). Plants that incorporate ML-FAs into their lignins have the potential to be particularly important economically, because their lignin backbones are permeated with...
readily cleavable ester bonds, facilitating lignin breakdown and removal under alkaline pretreatment conditions. Determining the extent to which ML-FA s are incorporated into the lignin polymer is, however, extremely difficult because of the diversity of products generated during the polymerization events, which is described in the supplemental information in Wilkerson et al., 2014.

There is currently only one technique, derivatization followed by reductive cleavage (DFRC), that can release diagnostic chemical marker compounds from lignins containing ML-FA s (Lu and Ralph, 2014; Wilkerson et al., 2014). The DFRC method selectively cleaves β-ethers while leaving ester linkages intact. This technique was recently used to show that ML-FA conjugates are fully incorporated into the lignin of the FMT poplar (Wilkerson et al., 2014), but the extent of incorporation, the spatial distribution, the exact mechanism of delivery to the developing cell wall, and the efficiency of incorporation remain largely unknown.

The biological role of pCA in lignin has been highly speculative. It is hypothesized that the pCA moieties may function as a radical sensitizer (Takahama and Oniki, 1996, 1997; Takahama et al., 1996; Ralph et al., 2004; Hatfield et al., 2008; Ralph, 2010). Peroxidases and/or laccases readily oxidize pCA to a radical but are poor oxidizers for SA. Free radicals of pCA readily undergo radical transfer to SA, which in turn, forms a homodimer or couples to the end of a growing polymer chain. Conjugating pCA to an ML, like SA, to form SA-pCA, the most prevalent ML-pCA conjugate in grasses, creates a compound with a built-in radical sensitizer that can participate in the polymerization event. The prevalence of these conjugates in potential biofuel crops and the impact that these ester-linked conjugates have on the lignin polymer during pretreatment and downstream fermentation processes have driven the search to find the genes and their enzymes responsible for acylating MLs in monocots (Withers et al., 2012; Marita et al., 2014). The enzyme preferentially forms a γ-ester through its specificity toward p-coumaryl-CoA and an ML, and has kinetic efficiency with p-coumaryl alcohol > SA > CA. In most grasses, the PMT enzyme predominantly produces SA-pCA conjugates that are then incorporated into the lignin polymer (Petrik et al., 2014).

To test the role of PMT during cell wall lignification, genetic manipulation of PMT genes has been performed in Brachypodium distachyon and maize (Zea mays), two model monocots. The suppression and overexpression of a BdPMT revealed the PMT to be involved only in the acylation of MLs before polymerization and not in the acylation of hemicelluloses (Petrik et al., 2014). RNA interference-mediated suppression of BdPMT resulted in decreased incorporation of ML-pCA conjugates into the cell wall without adversely affecting growth, height, or digestibility of the mature plants. Even deleterious mutations in the BdPMT gene, which resulted in a complete absence of pCA-acylating B. distachyon lignins, did not affect plant growth or development (Petrik et al., 2014). The arabinose-bound FA and pCA levels remained virtually unchanged in the PMT-misregulated plants, illustrating the specificity of the PMT enzyme for the p-coumaryl-CoA substrate and its ML acylation. The PMT enzyme identified in maize (pCAT = ZmPMT) also displayed the highest catalytic efficiency with p-coumaryl-CoA and SA as substrates (Marita et al., 2014). RNAi-mediated suppression of ZmPMT also resulted in decreased production of the ML conjugates. The effect on the lignin polymer when introducing PMT into plants that do not normally express a homologous enzyme is, however, unknown.

pCAs, because they favor radical transfer over radical coupling, are overwhelmingly seen as free-phenolic pendant entities on the lignin polymer (Ralph et al., 1994; Ralph, 2010). As a result, the pCA itself can be completely quantified by simple saponification. The units to which the pCA is attached are, like their normal ML-derived counterparts, not fully releasable from lignin as identifiable monomers (during degradative reactions), but the pCA’s terminal location makes p-coumarylated units more readily releasable and detectable than if they participated in lignification (as FAs do). Examining the effect of PMT and its resulting conjugates on lignification in plants that do not naturally produce such conjugates will contribute to our understanding of the role of PMT in lignification in general.

In this study, we aimed to assess the ability of the model eudicot plants Arabidopsis (Arabidopsis thaliana) and poplar, neither of which naturally produces ML-pCA conjugates, to express a PMT gene and incorporate these novel conjugates into their cell wall lignins. We also investigated the effect that the introduction of PMT has on the native levels of ML-pBz conjugates in poplar lignin. Various analytical techniques were optimized and used to examine the cell walls of the transgenic plants for pCA conjugates and determine whether they were specifically incorporated into the lignin polymer in the cell wall.

RESULTS AND DISCUSSION

Plant Transformations

In an attempt to maximize the number of cells producing and incorporating the ML conjugates into the eudicot lignins, the PMT transgenes were expressed universally in poplar under the control of the CAULIFLOWER MOSAIC VIRUS promoter (pro35S) and specifically, in lignifying cells of Arabidopsis under the control of the CELLULOSE SYNTHASE7 promoter (proCESA7). In poplar stem, the 35S promoter targets
PMT to all cell types (Wilkerson et al., 2014), whereas the Arabidopsis proCESA7 is specific to secondary cell wall cellulose synthesis and therefore, targets PMT to all tracheary elements and fibers in the xylem tissue (Smith et al., 2013). The generation of OsPMT transgenic Arabidopsis and poplar did not manifest in altered growth, architecture, or developmental timing, regardless of promoter used (data not shown).

NMR Examination of Cell Walls for the Presence of pCA

Heteronuclear single-quantum coherence (HSQC) NMR spectroscopy was used to determine the extent of variation in extract-free whole-cell wall (WCW) samples of wild-type and transgenic plants; specifically, we assessed whether there were changes in the relative abundance of syringyl (S) and guaiacyl (G) units derived from the dominant MLs (CA and SA) and if novel cell wall acylation by pCA could be detected. In poplar and Arabidopsis, wild-type plants did not contain any detectable pCA esters, indicating that acylation is not inherent to their lignification (Fig. 1, A and C). As expected, the wild-type poplar samples did show a significant amount of pBz, accounting for 5% of the total peak volume of the total S and G lignin units (Fig. 1A). The WCW samples of the pro35S::PMT transgenic poplar and the proCESA7::PMT transgenic Arabidopsis plants (hereafter referred to as PMT-Poplar and PMT-Arabidopsis) clearly contained pCAs (Fig. 1, B and D). These esters in poplar represent 2% to 8% of the total S and G lignins (by volume integrals), whereas in Arabidopsis, these same conjugates represent about 1% to 3% of the total lignin. (Note that the relative percentage of pCA is overestimated by the HSQC experiment, because it is a mobile pendant component with a longer relaxation time than the polymer backbone [Mansfield et al., 2012].) Interestingly, the biosynthesis of the PMT products, and their incorporation into lignin did not negatively impact the production of the traditional MLs, because the S:G ratio and the acetyl bromide (AcBr) lignin content in both poplar and Arabidopsis and the Klason lignin content in poplar remained unchanged (Table I). Empirically, as determined by wet chemistry (mild base hydrolysis), the pBz level was 1.2% of the cell wall of wild-type poplar trees and ranged from 1.1% to 1.8% of the cell wall in the PMT-poplar trees (Table I). The production of pBz was, therefore, not significantly altered in the PMT-poplar transgensics, suggesting that not only is the biosynthesis of ML-pCA conjugate a unique pathway introduced into poplar and Arabidopsis, but it essentially does not occur at the expense of MLs or naturally occurring acylated MLs.

Because the pCA detected in the WCW could be conjugated to either the polysaccharide or lignin component of the wall, extract-free WCW samples were treated with cellulase to remove the cell wall polysaccharides (Fig. 1, E–H). In the resulting isolated enzyme lignin (EL) samples, the pCA signatures remained in both poplar and Arabidopsis at levels (on a lignin basis) similar to those in the WCW samples (Fig. 1, F and H), suggesting that the ML-pCA conjugates synthesized are being incorporated into the lignin. Additional evidence for this contention is provided below.

Plants with 50% to 80% acylation typically have S to G ratios ranging from 4 to 9 and, consequently, primarily linear, unbranched lignins (del Rio et al., 2008). In contrast, the S to G ratio of poplar and especially, Arabidopsis is lower compared with many plants that naturally produce ML-Ac or ML-pCA conjugates (del Rio et al., 2008). However, lignin acylation is not correlated to the S to G ratio (del Rio et al., 2008), and therefore, the relatively low levels of S lignin do not impact the ability of either Arabidopsis or poplar to produce and incorporate acylated MLs.

Confirming the Presence of pCA by Tetramethyl-Ammonium Hydroxide Pyrolysis-Gas Chromatography-Mass Spectrometry

Pyrolysis of plant biomass converts plant material into a complex mixture of low M, compounds that can be analyzed by gas chromatography (GC)-mass spectrometry (MS). When tetramethyl-ammonium hydroxide (TMAH) is used in the pyrolysis reaction, carboxylic acids/esters are efficiently converted into methyl esters (and in the case of phenols and phenolic ether products, the phenol is also methylated). When pCA or its esters are pyrolyzed with TMAH, they are efficiently converted to methyl 4-methoxycinnamate, which is easily identified among the other pyrolysis products by GC-MS (Fig. 2; Table II).

By examining the pyrolysis GC-MS traces of WCW wild-type Arabidopsis and PMT-Arabidopsis samples and normalizing the signal intensity for the polysaccharide-derived 1,2,4-trimethoxybenzene, very little change was observed in most of the pyrolysis products. The PMT-Arabidopsis sample, however, had a strong signal for the pCA-derived methyl 4-methoxycinnamate (Fig. 2, magenta, peaks 15 and 21 with yellow highlighting). Performing a similar comparison between the wild-type and PMT-poplar samples showed only minor changes in most of the pyrolysis products and again, the presence of methyl 4-methoxycinnamate (pCA) only in the transgenic plants (Fig. 2, cyan, peaks 15 and 21). In the poplar samples, the lignin-bound pBz is converted to methyl 4-methoxybenzoate, and consistent with the wet chemical estimation, there is little change in the amount of pBz (Fig. 2, peak 9 with green highlighting) compared with the other products. This again indicates that the formation and subsequent lignification of ML-pCA conjugates do not affect the production and subsequent incorporation of ML-pBz conjugates into lignin.

Measurement of Total pCA by Saponification

If it is assumed (as is reasonable) that all of the pCA incorporation in the transgenic plants is the result of the introduced PMT, that pCA is only on the lignin (not on...
polysaccharides), and that, as in grasses, pCA is always a terminal free-phenolic unit, then the pCA level on lignin should be easily quantified by simply measuring the pCA released by saponification. As a corollary, this method provides a measure of the total amount of pCA introduced by expression of the PMT enzyme in these transgenic plants, regardless of whether it is on the lignin or the wall polysaccharides. As in the two-dimensional (2D)-HSQC-NMR and pyrolysis-GC-MS analyses, pCA was absent in both wild-type Arabidopsis and poplar samples when analyzed by mild base hydrolysis GC-flame ionization detection analysis (Fig. 3; Table I). Base hydrolysis of PMT-Arabidopsis transgenic plants showed the presence of 9.9 to 21.0 mg pCA g⁻¹ AcBr lignin. Similar levels of pCA, 8.1 to 23.2 mg g⁻¹ AcBr lignin (5.6–16 mg g⁻¹ Klason lignin), were found in the PMT-poplar transgenic plants (Fig. 3). For comparison, the rind from a mature maize stem released 36.9 mg pCA g⁻¹ AcBr lignin. The PMT-poplar plants had no significant difference in pBz production.

Figure 1. pCA is incorporated into transgenic PMT-poplar and PMT-Arabidopsis lignins. 2D-HSQC-NMR spectra of WCW samples (A–D) and the cellulase-digested EL for each sample (E–H). The pCA (magenta) is only present in the PMT-expressing lines and not wild-type control samples. The amount of pBz (orange) in the poplar samples does not seem to change between the wild type and PMT-expressing poplar. Note that the total lignin is the sum of all S, G, and H units (100%); therefore, when the sum of S + G does not add up to 100%, the remaining fraction should be ascribed to H units.
Table 1. Analytical data on the Arabidopsis and poplar samples

The AcBr and Klason lignin values represent the averages of two technical replicates ± SEM. The ranges indicate the ranges of conjugate levels released from three independent lines, representing three biological replicates, each with two technical replicates. N/D, Not detected; —, assay was not performed.

<table>
<thead>
<tr>
<th>Method/Component</th>
<th>Wild-Type Arabidopsis</th>
<th>PMT Arabidopsis</th>
<th>Wild-Type Poplar</th>
<th>PMT Poplar</th>
<th>Maize (Rindl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcBr lignin (wt% WCW)</td>
<td>9.7 ± 0.8</td>
<td>9.7 ± 0.7</td>
<td>15.7 ± 1.7</td>
<td>15.0 ± 0.5</td>
<td>21.4 ± 0.5</td>
</tr>
<tr>
<td>Klason lignin (wt% WCW)</td>
<td>—</td>
<td>—</td>
<td>21.3 ± 0.7</td>
<td>21.5 ± 1.1</td>
<td>—</td>
</tr>
<tr>
<td>Saponification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Coumaric acid (mg g⁻¹ WCW)</td>
<td>N/D</td>
<td>1.0–2.0</td>
<td>N/D</td>
<td>1.2–3.5</td>
<td>39.6*</td>
</tr>
<tr>
<td>AcBr lignin (mg g⁻¹ calculated)</td>
<td>—</td>
<td>9.9–21</td>
<td>—</td>
<td>8.1–23</td>
<td>185</td>
</tr>
<tr>
<td>Klason lignin (mg g⁻¹ calculated)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5.6–16</td>
<td>—</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid (mg g⁻¹ WCW)</td>
<td>N/D</td>
<td>N/D</td>
<td>1.2</td>
<td>1.1–1.8</td>
<td>N/D</td>
</tr>
<tr>
<td>AcBr lignin (mg g⁻¹ calculated)</td>
<td>—</td>
<td>—</td>
<td>7.6</td>
<td>7.5–12</td>
<td>—</td>
</tr>
<tr>
<td>Klason lignin (mg g⁻¹ calculated)</td>
<td>—</td>
<td>—</td>
<td>5.6</td>
<td>5.1–8.4</td>
<td>—</td>
</tr>
<tr>
<td>DFRC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCA (mg g⁻¹ sample)</td>
<td>N/D</td>
<td>1.1–1.7</td>
<td>N/D</td>
<td>0.9–2.0</td>
<td>4.8</td>
</tr>
<tr>
<td>AcBr lignin (mg g⁻¹)</td>
<td>—</td>
<td>11–18</td>
<td>—</td>
<td>6–13</td>
<td>36.9</td>
</tr>
<tr>
<td>Klason lignin (mg g⁻¹ calculated)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4–8</td>
<td>—</td>
</tr>
</tbody>
</table>

*Maize has a large amount of arabinose-bound pCA that is released during saponification.

(7.5–11.9 mg pBz g⁻¹ AcBr lignin and 5.1–8.4 mg g⁻¹ Klason lignin) compared with wild-type plants (7.6 mg g⁻¹ AcBr lignin and 5.6 mg g⁻¹ Klason lignin; Fig. 3), again supporting the hypothesis that there is little to no competition between the natural production of ML-pBz conjugates and the newly introduced ML-pCA conjugates.

DFRC Analysis of Cell Walls for the Presence of pCA Conjugates

Base hydrolysis can provide a quantifiable estimate of the amount of pCA introduced into the transgenic plants, but it gives no indication of which cell wall component is acylated by pCA. The DFRC method is invaluable for showing that the pCA is on lignin but yields no information on pCA bound to the polysaccharides. DFRC specifically cleaves the β-ether linkages between ML-derived units while retaining the ester linkages coupling pCA to the lignin unit (i.e. releasing a diagnostic ML-dihydro-p-coumarate [DHpCA] conjugate from free-phenolic or etherified β-ether units bearing pCA moieties). The pCA yield (calculated from the conjugate yield) from DFRC analysis is expected to be lower than that of base

Figure 2. TMAH-pyrolysis-GC-MS confirms the presence of pCA in the cell walls of the PMT-expressing plants. TMAH-pyrolysis-GC-MS chromatograms of wild-type (WT) Arabidopsis (gray) overlaid with PMT Arabidopsis (magenta) and wild-type poplar (gray) overlaid with PMT poplar (cyan). The peaks arising from pCA (15 and 21) are indicated with yellow highlighting, and that from pBz (9) has green highlighting. The peak labels refer to the compound numbers defined in Table II.
hydrolysis, because the method only detects the ML-DH\textsubscript{pCA} conjugates that are released after the cleavage of \(\beta\)-ether linkages. (Note that we will dispense with the dihydro descriptor here and refer to them simply as \(\text{pCA}\).) Conjugates associated with any of the so-called condensed units (i.e. those in 4-O-5, 5-5, \(\beta\)-\(\beta\), or \(\beta\)-5 units) will not be released/detected by DFRC, whereas such \(\text{pCA}\) units would still be released and detected by base hydrolysis. In \textit{PMT}-\textit{Arabidopsis}, ML-bound \(\text{pCA}\) comprised approximately 11 to 18 mg \(\text{pCA}\) g\textsuperscript{-1} AcBr lignin, whereas transgenic poplar trees had 6 to 13 mg \(\text{pCA}\) g\textsuperscript{-1} AcBr lignin (4–8 mg \(\text{pCA}\) g\textsuperscript{-1} Klason lignin; Table I). Again, in the rind from mature maize stem, 36.9 mg g\textsuperscript{-1} AcBr lignin was measured. Various lines of our transgenic dicots, which inherently do not incorporate \(\text{pCA}\) in wild-type plants, therefore have up to one-half the amount of \(\text{pCA}\) found in a monocot naturally permeated with \(\text{p}-\text{coumarylated lignins}.

The relative ratios of ML-\(\text{pCA}\) conjugates released from our \textit{PMT}-Arabidopsis and \textit{PMT}-poplar transgenics differed from those previously observed in \textit{B. distachyon} and other grasses. In the grasses, an approximately 90:10 ratio of SA-\(\text{pCA}\):CA-\(\text{pCA}\) is consistently observed (Grabber et al., 1996; Lu and Ralph, 1998a; Withers et al., 2012). In both the \textit{PMT} Arabidopsis and the \textit{PMT} poplar, CA-\(\text{pCA}\) is the most prevalent form, despite the biochemical report that the activity of PMT was lower with CA as a substrate, instead preferring either SA or \(\text{p}-\text{coumarylated alcohol} \) (Withers et al., 2012). Based on the cell wall analysis, it seems that the enzyme uses CA, even in poplar, with its relatively high levels of S units in its lignins. What drives the production of SA-\(\text{pCA}\) versus CA-\(\text{pCA}\) in model eudicots is unknown. It may be the...
greater availability of CA as a substrate at the time that p-coumaroyl-CoA is available for the PMT.

The amount of ML-pCA conjugates released by DFRC from PMT poplar (0.4%–0.8% Klason lignin) is higher than the amount of ML-FA conjugates released from FMT-poplar trees (0.15%–0.52% Klason lignin; Wilkerson et al., 2014). This is fully anticipated, even at the same level of activity, because the pCAs are entirely free-phenolic (and therefore, DFRC releases the conjugates in the same way as it releases the MLs themselves), whereas the FA moiety also undergoes particularly diverse radical coupling of its own, releasing the conjugate from only a fraction of those products (see supplemental figure S2 in Wilkerson et al., 2014).

Overall, all of the methods clearly illustrate the successful incorporation of pCA esters into plant cell walls using this transgenic approach in Arabidopsis and poplar. The NMR results on the isolated lignins but particularly, the DFRC results prove that this pCA is on lignin and that, as in grasses, it acylates the γ-OH on lignin side chains (Fig. 4).

**Figure 4.** MS evidence for the incorporation of ML-CA conjugates into transgenic PMT Arabidopsis and PMT poplar. A, The DFRC assay releases a fraction of the pCA as diagnostic ML-pCA-derived structural signatures as indicated with cyan shading; both CA-DHPCA (generally just denoted as CA-pCA here) and SA-DHPCA (SA-pCA) are evidenced. The shaded ovals highlight the structural changes caused by electron ionization (blue) and MRM fragmentation (purple). The orange lines indicate bonds that cleave during the MRM transitions to the detected fragment. B, The mass spectra for synthetic model compounds CA-pCA and SA-pCA from a Q3 scan on a GC-triple-quadrupole MS: molecular ion (mass-to-charge ratio \([m/z]=412\) and 442) loss of ketene \([m/z]=42\) to yield the base peak \([m/z]=370\) and 400). C, A stacked plot of the triple-quadrupole MRM chromatograms of DFRC-derived parent ions \([m/z]=370\) and 400) to one of the diagnostic product ions \([m/z]=131\) from WCW of PMT Arabidopsis (magenta), wild-type (WT) Arabidopsis (gray), PMT poplar (cyan), and wild-type poplar (gray).
CONCLUSION
Various chemical and analytical techniques conclusively showed that the introduction of PMT from rice into Arabidopsis and poplar results in the production of ML-pCA conjugates at levels approaching those in monocots. Results from DFRC and 2D-HSQC-NMR of isolated lignins showed that the pCA specifically acylates the lignin polymer and is therefore logically derived from the ML-pCAs biosynthesized only in the presence of the introduced PMT enzyme. In poplar, which naturally produces acylated MLs in the form of pBz, the production of pCA conjugates was essentially independent of the pBz biosynthesis. Together, these data show the ability of Arabidopsis and poplar to incorporate unique ML conjugates into their cell walls and also further highlight the plasticity of the lignification process in plants. However, this apparent plasticity raises some questions that require further examination. In vitro, the preferred substrate for the PMT enzyme is SA, but in the eudicots examined in this study, CA-pCA was the predominant product, despite the importance of SA in Arabidopsis and especially poplar. The reason behind this discrepancy remains unclear. Furthermore, the incorporation of a diversity of monomers into lignin, beyond the three traditional monomers, becomes the question of how the monomers are exported into the cell wall. If the monomers are actively transported out of the cells, which has been hypothesized (Miao and Liu, 2010; Alejandro et al., 2012), the ML transporter(s) would have to be particularly accommodating, or there may be many more transporters involved than previously considered.

MATERIALS AND METHODS
Generating the PMT Constructs and Transgenic Plants

PMT Construct Development
The PMT gene (LOC_Os01g19740) was identified from a list of candidate BAH domains, acyltransferases (Withers et al., 2012). The open reading frame of the gene was cloned into the pDONR221 vector. Cloning to generate the poplar PMT Construct Development was achieved using the Gateway cloning (underlined). The downstream of the transcriptional start codon for PMT was amplified by PCR using the primers 5'-ATCCGCATGCGTCCTCAGTGTTTCAAGTTT-3' (forward) and 5'-gggagggcagctggagattggtgagagcatgtctttgccc-3' (reverse), which adapt probe sites for Gateway cloning (underlined). The promoter region, represented by a 1,127-bp sequence spanning from the transcriptional start codon for CESA7, was amplified by PCR using the primer 5'-AAAAACCGGGCGGTCCTCAAGGATTTCAGTTT-3' (forward) and 5'-gagaggagctcggggtgtttggggtagctgaaagcagca-3' (reverse), which adapt probe sites for Gateway cloning (underlined). The CESA7 promoter was cloned into pDONR221 and subsequently transferred into the pKGW vector using LR Gateway cloning. The PMT gene was amplified by PCR using the primers 5'-ATCCGCATGCGTCCTCAGTGTTTCAAGTTT-3' (forward) and 5'-gggagggcagctggagattggtgagagcatgtctttgccc-3' (reverse) and placed into the pKGW vector by blunt end cloning following the promoter.

Plant Transformations and Growth Conditions
For generation of poplar transgenics, Agrobacterium tumefaciens strain EHA105 containing the pmuSS::YFP::PMT construct was used to transform a poplar hybrid (P99; Willkerson et al., 2014). The Arabidopsis PMT expression construct was introduced into A. tumefaciens strain GV3101 and transformed into Arabidopsis Columbia-0 using the floral dip method (Clough and Bent, 1998). Seeds from the dippled plants were plated on one-half-strength Murashige and Skoog media (Sigma) supplemented with 50 mg mL−1 kanamycin to select for transformants. Two-week-old seedlings were transferred to soil (Sunshine Mix 4; Sungrow Horticulture) and grown in a growth chamber at 21°C and in 16-h light/8-h-dark cycles until mature (approximately 2 months old). Rosette leaves from mature plants were subjected to genotyping using the PMT primers listed above to confirm that the transformation was effective. First and second generation transformants were used for the chemical analyses.

Analyses

2D-HSQC-NMR Analysis of Cell Wall Components
The preparation of the extract-free WCs and the EL was performed as described previously (Lu and Ralph, 2003; Wagner et al., 2007; Kim and Ralph, 2010). The ball-milled extract-free Arabidopsis and poplar samples were prepared in 5-mm NMR tubes and suspended/suspended in dimethyl sulfoxide (DMSO)·d6-pyridine-d5 (4:1; 500 μL) for NMR (Kim and Ralph, 2010; Mansfield et al., 2012).

EL samples were prepared following the procedure described previously (Chang et al., 1975; Wagner et al., 2007). Briefly, the material (200 mg) was digested at room temperature with 20 mg of crude cellulases (Celluclast; Sigma) in 40 mL of Ac buffer (pH 5.0) on a shaker at 3.6 rpm for 3 days. The samples were then centrifuged (Sorval Biofuge Primo; 8,500 rpm or 10,000g for 10 min) and deacetylated. The cellulase digestion was then repeated. After deacetylating the second hydrolysate, the sample was rinsed with reverse osmosis water (3 × 40 mL) and hydrolyzed. From the resulting brown powder (40 mg), 10 to 15 mg were transferred to a 5-mm NMR tube and dissolved/suspended in DMSO·d6-pyridine-d5 (4:1; 500 μL).

The gel was characterized by HSQC spectroscopy under the conditions previously reported (Kim and Ralph, 2010; Mansfield et al., 2012). The spectroscopic data were acquired on a Bruker Biospin Avance 700-MHz NMR spectrometer equipped with an inverse gradient 5-mm TXI 1H-13C-15N Cryo-probe. The central DMSO solvent peak was used as an internal reference (δH = 39.5 ppm and δC1 = 24.9 ppm). Peak assignments for S, G, and H-hydroxyphenyl (H) lignin units as well as previously assigned spectra (Lu and Ralph, 2003; Marita et al., 2003; Kim et al., 2008; Kim and Ralph, 2010; Ralph and Landucci, 2010; del Río et al., 2012a, 2012b; Mansfield et al., 2012; Rencoret et al., 2013).

Analytical Pyrolysis
To confirm the formation of pCA by the PMT transgene, the pyrolysis GC-MS spectra of the transgenic plant lines were compared with those of the corresponding wild-type plants. The sample (approximately 300 μg) was treated with 50 μL of TMAH (25%, w/w in methanol) and air dried to remove excess methanol. The pyrolysis GC-MS was performed on a Py-2020ID Microfurnace Pyrolyzer (Frontier Laboratories Ltd.) connected to a Shimadzu 2010 GC equipped with an Agilent DB-1701 Fused-Silica Capillary Column (30 m × 0.25 mm i.d. × 0.25-μm film thickness) and a Shimadzu QP2010 Plus Mass Spectrometer (electron impact at 70 eV). The helium carrier gas was held at a constant linear velocity (36.3 cm s−1). The pyrolysis was performed in helium atmosphere by lowering the sample into the 500°C furnace, where the injection port was operating with a split ratio of 10:1. The GC oven program was a 50-min temperature gradient as follows: 50°C hold for 2 min, ramp at 20°C per minute to 100°C, ramp at 6°C per minute to 300°C, and hold at 300°C for 12 min. Compound identification was performed by comparison of the detected mass spectra with those in the National Institute of Standards and Technology library and comparison with spectra reported in the literature from pyrolysis of plant tissue in the presence of TMAH (Cliftord et al., 1995; Kuroda, 2002; del Río et al., 2012a, 2012b).

AcBr Lignin
To estimate the amount of lignin in each Arabidopsis and poplar sample to express the amount of pCA as a proportion of lignin in the sample, AcBr lignin analysis was performed as previously described (Hattie et al., 1999; Fukushima and Hatfield, 2004; Chang et al., 2008) with the absorbance at λ = 280 nm and extinction coefficients of ε280 = 20.0 for poplar and ε280 = 23.3 for Arabidopsis. Briefly, the samples (2–3 mg) were treated with 0.5 mL of 20% (v/v) AcBr in acetic acid for 2 h at 50°C. The solution was then quantitatively transferred to a 10-mL volumetric flask containing 2 mL of NaOH (2 mL) and 0.5 M HCl until the absorbance at 280 nm was constant. The solution was then made to volume (10 mL) with 0.5 M HCl, and the absorbance was measured at 280 nm. The amount of pCA was calculated from the absorbance and extinction coefficients.
hydrolsine (0.35 mL) and adjusted to a final volume of 10 mL using acetic acid. Samples were then transferred to quartz cuvettes, and the absorbance was measured at 280 nm. The average weight of lignin (percentage) from two technical replicates was used to estimate the amount of lignin in the samples, and this was used to express the amount of pCA in terms of the amount of lignin for base hydrolysis and DFRC experiments.

**Mild Alkaline Hydrolysis to Determine Releasable pCA and pBz**

The determination of ester-linked pCA and pBz was by mild alkaline hydrolysis following previously published procedures (Ralph et al., 1994).

**DFRC Method for Lignin-Bound pCA**

The quantitation of lignin-bound pCA was performed using the DFRC method that cleaves lignin b-ethers while retaining esters (Lu and Ralph, 1997, 1998b, 1998c, 1999; Petrini et al., 2014). The same dry extract-free WCs were used for pyrolysis and base hydrolysis (20–40 mg) were treated with a solution of AcBr: acetic acid (1:4, v/v) at 50°C for 3 h. The acetylated and benzyl-brominated lignin solution was quantitatively transferred to a 50-mL round-bottomed flask using glacial acetic acid, and the solvent was evaporated to dryness on a rotary evaporator (water bath heated to 50°C). The dry film was treated with absolute ethanol (2 mL), which was then removed on a rotary evaporator. The dry sample was then immediately dissolved in a mixture of 1,4-dioxaneacetic acid: water (5:4, v/v/v; 5 mL), and zinc nanopowder (150 mg) was added to the flask in four portions at 15-min intervals. The reaction was stirred for 1 h (16 h for Arabidopsis samples) at room temperature and then quenched with saturated ammonium chloride. The quenched reaction was spiked with an internal standard (diethyl 5,5'-dithio-2,2'-diphenyl disulfide) and extracted with 1,4-dioxane:ethyl acetate (1:1; 8 mL) for Arabidopsis and ethyl acetate:hexane (1:1; 8 mL) for poplar and ethyl Ac:hexane (1:1; 8 mL) for sweetgum and spruce. Holzforschung


