The PMT-driven p -coumaroylation of poplar lignins impacts lignin structure and improves wood saccharification

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Research Article

p-Coumaroylation of poplar lignins impacts lignin structure and improves wood saccharification

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Abstract

The enzymatic hydrolysis of cellulose into glucose, referred to as saccharification, is severely hampered by lignins. Here, we analyzed transgenic poplars (*Populus tremula* × *Populus alba*) expressing the Brachypodium (*Brachypodium distachyon*) *p*-coumaroyl-Coenzyme A monolignol transferase 1 (*BdPMT1*) gene driven by the Arabidopsis (*Arabidopsis thaliana*) Cinnamate 4-Hydroxylase (*AtC4H*) promoter in the wild-type (WT) line and in a line overexpressing the Arabidopsis Ferulate 5-Hydroxylase (*AtF5H*). *BdPMT1* encodes a transferase which catalyzes the acylation of monolignols by *p*-coumaric acid (*p*CA). Several *BdPMT1*-OE/WT and *BdPMT1*-OE/*AtF5H*-OE lines were grown in the greenhouse, and *BdPMT1* expression in xylem was confirmed by RT-PCR. Analyses of poplar stem cell walls (CWs) and of the corresponding purified dioxan lignins (DLs) revealed that *BdPMT1*-OE lignins were as *p*-coumaroylated as lignins from C3 grass straws. For some transformants, *p*CA levels reached 11 $mg{\cdot}g^{-1}$ CW and 66 $mg{\cdot}g^{-1}$ DL, exceeding levels in Brachypodium or wheat (*Triticum aestivum*) samples. This unprecedentedly high lignin *p*-coumaroylation affected neither poplar growth nor stem lignin content. Interestingly, *p*-coumaroylation of poplar lignins was not favored in *BdPMT1*-OE/*AtF5H*-OE transgenic lines despite their high frequency of syringyl units. However, lignins of all *BdPMT1*-OE lines were structurally modified, with an increase of terminal unit with free phenolic groups. Relative to controls, this increase argues for a reduced polymerization degree of *BdPMT1*-OE lignins and makes them more soluble in cold NaOH solution. The *p*-coumaroylation of poplar samples improved the saccharification yield of alkali-pretreated CW, demonstrating that the genetically driven *p*-coumaroylation of lignins is a promising strategy to make wood lignins more susceptible to alkaline treatments used during the industrial processing of lignocellulosics.

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Introduction

Wood appears as a major feedstock for traditional or innovative biorefineries producing pulp, chemicals, or fermentable sugars. However, most industrial fractionations of lignocellulosics are detrimentally affected by lignins. For instance, the enzymatic hydrolysis of cellulose into glucose, referred to as saccharification, is severely hampered by lignins that hinder the accessibility of enzymes to cell wall (CW) polysaccharides. Indeed, the economically effective production of cellulosic ethanol necessitates costly, polluting and energy-intensive pretreatments that most often aim at reducing the lignin shield effect (Yang and Wyman, 2008; Sun et al., 2016). Since the last decades, lignin engineering in trees has been the subject of intensive studies to produce tailor-made wood more amenable to efficient deconstruction by milder processes (Pilate et al., 2012; Chanoca et al., 2019; Mahon and Mansfield, 2019). However, lignins play key roles in wood and sufficient lignin amounts are required to warrant tree growth, development and defense. On this basis, reducing lignin content may result in impaired tree growth and redesigning lignin structure appears as a better strategy to obtain wood biomass more adapted to industrial deconstruction without yield penalty.

Lignins primarily result from the enzymatically driven oxidation of monolignols, mainly coniferyl alcohol and sinapyl alcohol that give rise to guaiacyl (G) and syringyl (S) units, respectively. It is now well established that lignin biosynthesis is very plastic and that, besides the main monolignols, a number of other molecules may participate in the formation of lignin polymers (Mottiar et al., 2016; del Río et al., 2020). For instance, *p*-coumaroylated sinapyl alcohol and, to a lower extent, *p*-coumaroylated coniferyl alcohol, are naturally incorporated into grass lignins (Grabber et al., 1996; Lu and Ralph, 1999; Hatfield et al., 2009; Ralph, 2010). This *p*-coumaroylation of grass monolignols is specifically catalyzed by a *p*-coumaroyl-coenzyme A monolignol transferase (PMT) studied in various grass species (Hatfield et al., 2009; Withers et al., 2012; Marita et al., 2014; Petrik et al., 2014). The *p*-coumaroylation of dicot lignins was recently achieved by introducing the rice (*Oryza sativa*) *PMT* gene into poplar and Arabidopsis plants (Smith et al., 2015), but the *p*-coumaroylation level of transgenic dicot CW reported in this study was modest (varying from 1 to 3.5 $mg{\cdot}g^{-1}$ CW) and much lower than that of lignified grass stems (*p*-coumaric acid [*p*CA] ranging from 6 to 39 $mg{\cdot}g^{-1}$ CW; Hatfield et al., 2009). In contrast, the introduction of two different Brachypodium *PMT* genes (*BdPMT1* or *BdPMT2*) under the control of the *AtC4H* promoter into various Arabidopsis lines boosted the *p*-coumaroylation of mature stem lignins up to the grass lignin level (Sibout et al., 2016). In addition to a high *p*CA content, the Arabidopsis *BdPMT1*-OE lignins displayed other traits specific to grass lignins, i.e. a high frequency of free phenolic units in lignins and an increased solubility in cold alkali.

In this work, we explored the potential of introducing the *proAtC4H::BdPMT1* construct into poplar in order to beneficially tailor lignin structure without biomass penalty. To this end, *BdPMT1* was expressed not only in the poplar wild-type (WT) background, but also in a transgenic poplar line overexpressing the *AtF5H* gene (*AtF5H*-OE). By so doing, we obtained several independent transformants that were grown in the greenhouse together with the corresponding controls during 3 months. In this study, we first evaluated the growth of the *BdPMT1*-OE lines and the *p*-coumaroylation of their stem lignins, as compared to control trees. We then investigated the effect of the *BdPMT1* expression on lignin content and structure before subjecting the transgenic and control poplar stems to alkali-solubilization assays and saccharification tests.

Results and discussion

The expression of heterologous *BdPMT1* gene under the control of the *AtC4H* promoter does not alter poplar growth

The BdPMT1 acyltransferase (referred to as Bradi2g36910) has been shown to be specific to monolignol *p*-coumaroylation (Petrik et al., 2014). *BdPMT1* is a close homolog of the rice *OsPMT* that was introduced by Smith et al. (2015) into poplar and Arabidopsis plants. We used the *AtC4H* promoter to drive the expression of *BdPMT1* as it is highly expressed in Arabidopsis xylem tissues during lignification (Bell-Lelong et al., 1997) and is also efficient to drive transgene expression in poplar wood (Franke et al., 2000). The transformation was performed in two poplar genetic backgrounds, the WT line and a transgenic line overexpressing the *AtF5H* gene. The *AtF5H* expression was driven by a poplar cellulose synthase A4 promoter, known to be highly active in the fibers and vessels of poplar developing xylem (Hai et al., 2016). The *AtF5H*-OE poplar line was chosen to test the hypothesis that the *p*-coumaroylation of poplar lignins may be favored by a high frequency of S units based on the two following published data: (1) the *p*-coumaroylation of grass lignins mostly occurs on S units (reviewed in Ralph, 2010; Karlen et al., 2018) and (2) overexpressing the *AtF5H* gene in poplar substantially increases the frequency of S lignin units (Franke et al., 2000).

The *Agrobacterium tumefaciens*-mediated transformation yielded several independent transformants in the WT background (referred to as *BdPMT1*-OE/WT lines) and in the *AtF5H*-overexpressing background (referred to as *BdPMT1*-OE/*AtF5H*-OE lines). Three *BdPMT1*-OE/WT lines and two *BdPMT1*-OE/*AtF5H*-OE lines were randomly chosen for further analyses: They were acclimatized and grown for 3 months in the greenhouse together with corresponding control plants (Supplemental Figure S1A). Semi-quantitative RT-PCR with *BdPMT1* specific primers revealed a substantial *BdPMT1* transcript abundance in developing xylem of *BdPMT1*-OE lines, with some variations between lines, whereas no *BdPMT1* expression could be detected in the WT or *AtF5H*-OE control trees (Supplemental Figure S1B). Likewise, when using primers directed to *AtF5H*, a strong RT-PCR signal was observed in the *AtF5H*-OE transgenic

lines (Supplemental Figure S1C). Relative to the control trees, the *BdPMT1*-OE did not induce any significant difference in height and diameter (Figure 1) and the transgenic poplar plants did not show any obvious phenotype difference when compared to WT trees.

The *BdPMT1*-OE poplar stems and their corresponding purified dioxane lignin fractions are *p*-coumaroylated to the levels of C3 grass samples

CW samples from the stems of 3-month-old greenhouse-grown poplar trees were subjected to mild alkaline hydrolysis to quantify *p*-hydroxybenzoic acid (Bz), *p*CA, and ferulic acid (FA) ester-linked to CW polymers. Poplar wood is typified by the occurrence of Bz ester-linked to lignins (Smith, 1955; Venverloo, 1969) and preferentially to the γ position of S lignin units (Lu et al., 2004; Morreel et al., 2004). Most *BdPMT1*-OE poplar samples displayed similar *p*-hydroxybenzoylation levels as their corresponding controls (Table 1). In addition to Bz, mild alkaline hydrolysis of poplar samples released small amounts of FA consistently and significantly in slightly smaller quantities in all *BdPMT1*-OE/WT lines compared to the WT (Table 1). In plant CW, FA preferentially acylates noncellulosic polysaccharides (Ishii, 1997). Some of these FA esters can be oxidatively coupled to monolignols and act as lignin nucleation sites (Ralph, 2010). The small differences of FA esters between poplar lines might reflect some variations in the feruloylation degree of CWs polymers and/or in their ferulate mediated cross-linking.

While expressing the *BdPMT1* gene both in the WT and the *AtF5H*-OE backgrounds had no effect on Bz units ester-linked to poplar CW, this transformation dramatically increased CW *p*-coumaroylation with up to 1,000-fold higher levels compared to the trace amounts of the controls (Table 1). Remarkably enough, this quantity was boosted up

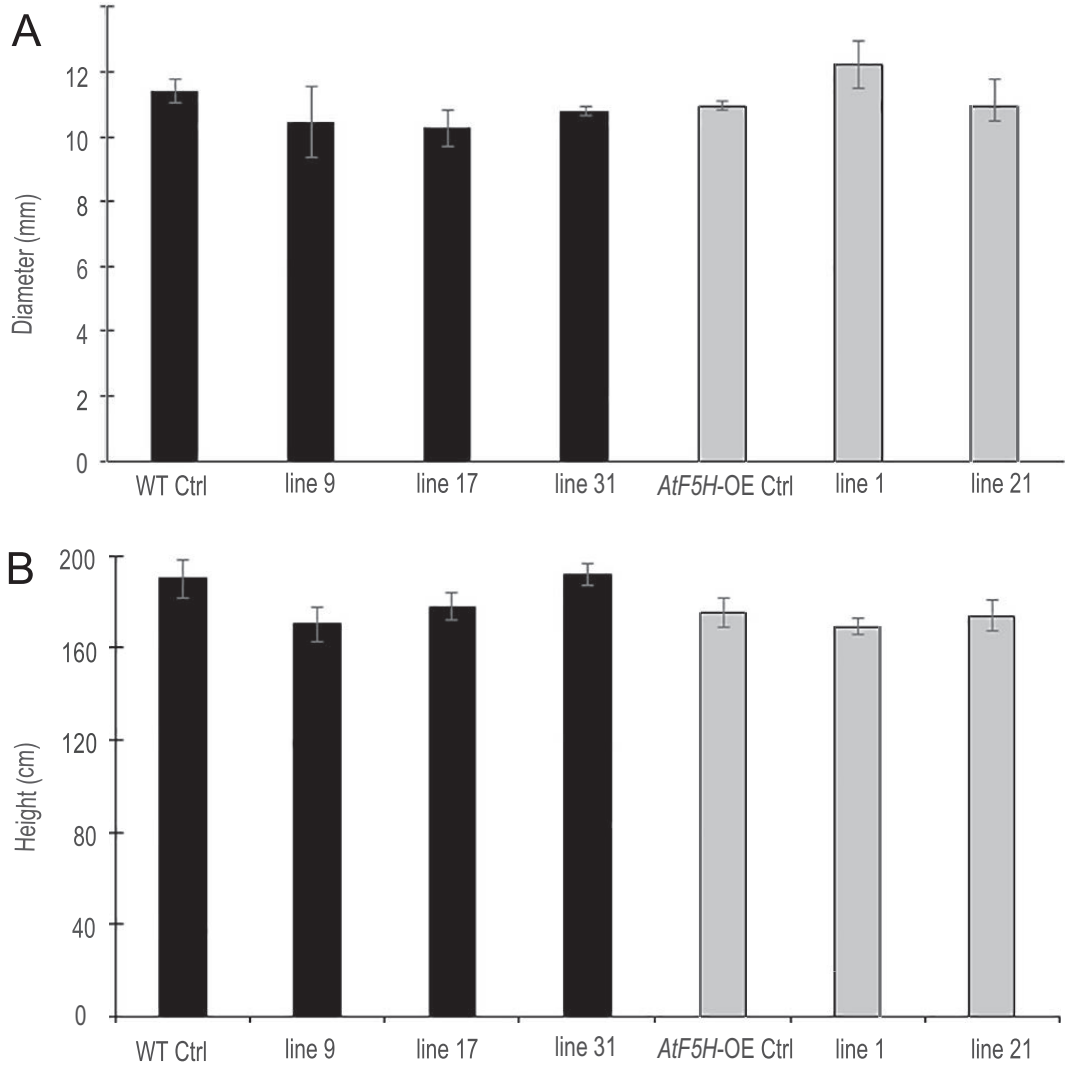

Figure 1 Growth response to the introduction of the *proAtC4H::BdPMT1* construct into the poplar WT background (black bars) and into the *AtF5H*-OE background (gray bars), as compared to control (Ctrl) trees. The basal diameter (A) and the tree height (B) were measured on 3-month-old greenhouse-grown trees. Data are means (SD) values of three or four biological replicates. Duncan tests (at $P < 0.05$) did not reveal any significant differences between poplar lines.

Table 1 Amount of Bz, *p*CA, and FA released by mild alkaline hydrolysis of extract-free poplar stems (referred to as CWs) from *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds, as compared to their respective controls

Line (*n* Replicates)	Bz mg·g^{-1} CW	*p*CA mg·g^{-1} CW	FA mg·g^{-1} CW
WT control (3)	3.86 (0.10)^{a,b}	0.01 (0.00)^{f}	0.22 (0.00)^{a}
BdPMT1-OE/WT line 9 (3)	3.21 (0.51)^{b}	7.12 (0.49)^{b}	0.15 (0.02)^{c}
BdPMT1-OE/WT line 17 (3)	3.66 (0.28)^{a,b}	10.69 (0.49)^{a}	0.18 (0.01)^{b}
BdPMT1-OE/WT line 31 (3)	3.57 (0.11)^{ab}	3.63 (0.42)^{d}	0.10 (0.00)^{d}
AtF5H-OE control (4)	3.29 (0.14)^{b}	0.01 (0.00)^{f}	0.06 (0.00)^{e}
BdPMT1-OE/*AtF5H*-OE line 1 (4)	3.06 (0.56)^{b}	0.76 (0.04)^{e}	0.07 (0.01)^{de}
BdPMT1-OE/*AtF5H*-OE line 21 (3)	4.35 (0.08)^{a}	4.92 (0.19)^{c}	0.13 (0.02)^{c}

The data represent mean (SD) values from n biological replicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$).

to about 11 mg·g^{-1} CW in *BdPMT1*-OE/WT line 17. As compared to grass mature stems, the *p*CA levels of this poplar line exceeded those of most C3 grass CW, but remained lower than those of C4 grass CW (Supplemental Table S1). With the exception of line 1, the obtained *BdPMT1*-OE poplar lines were as *p*-coumaroylated as extract-free *proAtC4H::BdPMT1* Arabidopsis mature stems (*p*CA amounts ranging between 3.5 and 12.6 mg·g^{-1} CW; Sibout et al., 2016). In contrast, these levels were much higher than the values reported for *OsPMT*-OE poplar lines (*p*CA range: 1.2–3.5 mg·g^{-1} CW) or for *OsPMT*-OE Arabidopsis lines (*p*CA range: 1.0–2.0 mg·g^{-1} CW) when *OsPMT* expression was driven by the 35S CAMV promoter or by the CELLULOSE SYNTHASE7 promoter (Smith et al., 2015). In agreement with Smith et al. (2015), the *p*-coumaroylation of poplar CW did not affect their *p*-hydroxybenzoylation (Table 1). The high *p*-coumaroylation of poplar CWs obtained in the present work is very likely related to the efficiency of the *AtC4H* promoter, in agreement with recent data obtained with *BdPMT1*-OE Arabidopsis lines (Sibout et al., 2016).

Isolation of dioxan lignin (DL) fractions followed by their mild alkaline hydrolysis recently proved to be an efficient strategy to demonstrate that *p*CA units introduced in *BdPMT1*-transformed Arabidopsis plants are ester-linked to lignins (Sibout et al., 2016). The isolation method consists in mild acidolysis (refluxing CW samples in dioxane/0.2 M aq. HCl for 30 min under N_2), which provides a rough lignin extract then purified to recover DL fractions. This isolation method relies on the hydrolysis of some ether bonds in lignins to make the insoluble native lignin polymers partially soluble into the reaction medium. The purified DL fractions contain a low amount of sugar contaminants (<10% by weight) and the mild isolation procedure mostly preserves lignin-linked *p*CA esters, if present (Chazal et al., 2014). Purified poplar DL fractions were isolated from a few control and *BdPMT1*-OE poplar lines and then subjected to mid-infrared (IR) spectroscopy. Their mid-IR spectra not only confirmed their low contamination by sugar components,

Table 2 Amount of *p*CA released by mild alkaline hydrolysis of DL fractions isolated from control and *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds

Line	*p*CA mg·g^{-1} DL
WT control	3.21 (0.17)
BdPMT1-OE/WT line 9	50.00 (0.77)
BdPMT1-OE/WT line 17	66.52 (0.47)
BdPMT1-OE/WT line 31	31.36 (0.43)
AtF5H-OE control	0.87 (0.04)
BdPMT1-OE/*AtF5H*-OE line 21	33.98 (0.53)

The data represent mean (SD) values from technical duplicates.

but also suggested that the lignin fractions isolated from *BdPMT1*-OE/WT and *BdPMT1*-OE/*AtF5H*-OE lines were enriched in *p*CA esters (Supplemental Figure S2). Relative to their respective controls, the IR spectra from *BdPMT1*-OE lines displayed increased signals at 1,604, 1,164, and 833 cm^{-1}, which can be assigned to the occurrence of *p*CA units (Chazal et al., 2014). More importantly, high *p*CA amounts (from 31 to 66 mg·g^{-1} DL, Table 2) were released by mild alkaline hydrolysis of the purified DL fractions isolated from *BdPMT1*-OE poplar lines, as confirmed by both high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analyses (Supplemental Figure S3). The upper values were similar to the *p*CA levels of DL fractions isolated from C3 grass CW, but remained lower than those of DL fractions isolated from C4 grass species (Supplemental Table S1). Alkaline hydrolysis of the DL fractions isolated from control samples released very low amounts of *p*CA units (Table 2), which reveals that *p*CA acylates poplar lignins to a weak extent and is in agreement with results obtained for Arabidopsis lignins (Sibout et al., 2016). The *p*CA contents of DL fractions from *BdPMT1*-OE poplar line were found to be 6- to 10-fold higher than those from the corresponding CW (Table 1). Such an outstanding enrichment further establishes that most *p*CA units introduced in the transgenic poplars are ester-linked to lignins.

Analytical pyrolysis further confirms the high *p*-coumaroylation of *BdPMT1*-OE poplar lines

The main advantages of the pyrolysis-GC/MS (Py-GC/MS) method is its high-throughput screening capabilities together with its low sample demand (Ralph and Hatfield, 1991; Lapierre, 1993). When subjected to this method, lignified CW samples provide lignin-derived phenolics originating from G and S lignin units. In addition, during pyrolysis, ester-linked Bz and *p*CA units (if present) are decarboxylated to produce phenol and 4-vinylphenol, respectively. The relative abundances (area %) of the main G and S pyrolysis products and of phenol and 4-vinylphenol generated from the poplar CW samples are listed in Table 3.

The pyrolysis S/G ratio calculated from the relative amount of lignin-derived S and G pyrolysis compounds was not significantly affected in the *BdPMT1*-OE/WT lines (Table 3). This result suggests that the proportion of G and S lignin unit is not affected by the introduction of *BdPMT1*

Table 3 Relative percentage values of the peaks assigned to the main phenolics released by Py-GC/MS of poplar CWs from *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds, as compared to their respective controls

Line (*n* Replicates)	Phenol	4-Vinylphenol	G Compounds[a]	S Compounds[b]	S/G Ratio
WT control (4)	5.33 (0.46)^{a,b}	0.21 (0.07)^{e}	24.76 (1.31)^{a}	69.71 (1.57)^{b}	2.82 (0.21)^{b}
BdPMT1-OE/WT line 9 (4)	4.60 (0.94)^{a,b}	10.73 (0.35)^{b}	21.84 (0.80)^{b}	62.83 (1.11)^{c}	2.88 (0.13)^{b}
BdPMT1-OE/WT line 17 (4)	5.43 (0.43)^{a,b}	15.44 (0.71)^{a}	20.69 (0.78)^{bc}	58.44 (0.43)^{d}	2.83 (0.12)^{b}
BdPMT1-OE/WT line 31 (4)	5.06 (0.56)^{a,b}	5.40 (1.46)^{d}	24.10 (0.89)^{a}	65.44 (2.40)^{bc}	2.72 (0.19)^{b}
AtF5H-OE control (4)	4.99 (0.70)^{a,b}	0.10 (0.04)^{e}	18.64 (0.94)^{cd}	76.38 (1.53)^{a}	4.11 (0.29)^{a}
BdPMT1-OE/*AtF5H*-OE line 1 (4)	4.40 (1.17)^{b}	1.15 (0.010)^{e}	17.86 (1.69)^{d}	76.59 (2.74)^{a}	4.32 (0.52)^{a}
BdPMT1-OE/*AtF5H*-OE line 21 (3)	6.21 (0.42)^{a}	7.43 (0.25)^{c}	16.40 (0.85)^{d}	69.95 (0.56)^{b}	4.27 (0.24)^{a}

These area values are expressed as percentage of the total area per sample (set to 100). The data represent mean (SD) values from n biological replicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$)

aG compounds include: guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, 4-allylguaiacol (two isomers), vanillin, acetoguaiacone, guaiacylacetone.

bS compounds include: syringol, 4-methylsyringol, 4-ethylsyringol, 4-vinylsyringol, 4-allylsyringol (two isomers), syringaldehyde, acetosyringone, syringylacetone.

in poplar. In agreement with literature data (Franke et al., 2000; Stewart et al., 2009), this ratio was substantially increased in the *AtF5H*-OE control line as well as in the *BdPMT1*-OE/*AtF5H*-OE lines 1 and 21.

The relative percentage of pyrolysis-derived phenol did not discriminate the various transgenic samples from their control. This result is quite consistent with mild alkaline hydrolysis which provided similar Bz amounts from most transgenic lines and their respective controls. In contrast, the relative amount of 4-vinylphenol was dramatically increased in the *BdPMT1*-OE lines as compared to their controls. Such a relative increase concomitantly decreased the relative percentage of the lignin-derived pyrolysis G and/or S compounds (Table 3). Even though the pyrolysis-derived 4-vinylphenol might originate from tyrosine residues of putatively present protein contaminants, it is essentially produced from the decarboxylation of CW-linked *p*CA units (Ralph and Hatfield, 1991). The relative abundance of 4-vinylphenol was found to nicely echo the level of alkali-releasable *p*CA, as revealed by the positive correlation between *p*CA amount and the 4-vinylphenol % ($R^2 = 0.982$; Supplemental Figure S4). In other words, the relative amount of pyrolysis-derived 4-vinylphenol may be viewed as a good signature of the CW *p*-coumaroylation level. To further confirm that 4-vinylphenol prominently originates from *p*CA decarboxylation, a few pyrolysis assays were carried out in the presence of tetramethylammonium hydroxyde (TMAH). The TMAH-Py-GC/MS method yields methyl 4-methoxybenzoate (Bz_{Me}) and methyl 4-methoxy-*p*-coumarate (pCA_{Me}) from Bz and *p*CA units, respectively (Kuroda et al., 2001, 2002). As shown in the pyrograms outlined in Figure 2, the relative intensity of the Bz_{Me} peak was similar in the *BdPMT1*-OE and in their corresponding controls whereas the pCA_{Me} peak was prominent in the *BdPMT*-OE poplar lines.

The expression of *BdPMT1* transformation has no or little effect on the lignin content of poplar stems, but a strong impact on lignin structure

The most *p*-coumaroylated transgenic poplar lines were analyzed for their lignin content, using both the Klason lignin (KL) and the acetyl bromide lignin (ABL) methods. As shown in Table 4, the *BdPMT1* transformation had no impact on the lignin content of the poplar stem CW. This result contrasts with those obtained for *proAtC4H::BdPMT1* Arabidopsis transformants provided with similar *p*-coumaroylation levels as these poplar transgenics, but with 10%–30% lower lignin contents than their controls (Sibout et al., 2016). Introducing the *proAtC4H::BdPMT1* into Arabidopsis plants seemed to affect the metabolic flux to lignins and thereby the stem lignin content whereas such an effect was not observed in the *BdPMT1*-OE poplar lines.

A major structural trait of native lignins is their percentage of free phenolic groups, which has a strong impact on lignin susceptibility towards industrial alkaline or oxidative treatments. When thioacidolysis is performed on CW exhaustively permethylated with diazomethane or trimethylsilyldiazomethane, the percentages of free phenolic groups in β-O-4 linked G or S lignin units, referred to as %GOH or %SOH, can be evaluated. These percentages have been shown to nicely parallel that of the whole polymer (Lapierre, 2010). With the objective to evaluate the impact of the *BdPMT1* transformation on the structure of poplar native lignins, we employed this analytical approach, the principle of which is outlined in Figure 3. Past studies have shown that the thioacidolysis yield is not affected by the mild permethylation procedure (Lapierre et al., 1988; Lapierre, 2010). Whatever the sample, the *p*-hydroxyphenyl (H) thioacidolysis monomers were found to be obtained as trace components (<1% of the monomer yield) and, in consequence, these minor H units were not considered in the following. In agreement with the Py-GC/MS data, the thioacidolysis S/G ratio was not affected by the *BdPMT1* transformation in the WT background (Table 5). Consistently with the pyrolysis data (Table 3) and as compared to the WT, the thioacidolysis S/G ratio was found to be drastically increased in the *AtF5H*-OE samples (Table 5).

At this stage of the study and from the simultaneous examination of both thioacidolysis S/G ratio (Table 5) and *p*CA level (Table 1), our anticipated hypothesis that a high frequency of sinapyl alcohol in *AtF5H*-OE poplar lines might increase the *BdPMT1*-induced acylation of poplar lignins is most likely to be ruled out. This conclusion is consistent with literature data reporting on the impact of *F5H* overexpression in plant species provided with acylated S lignin

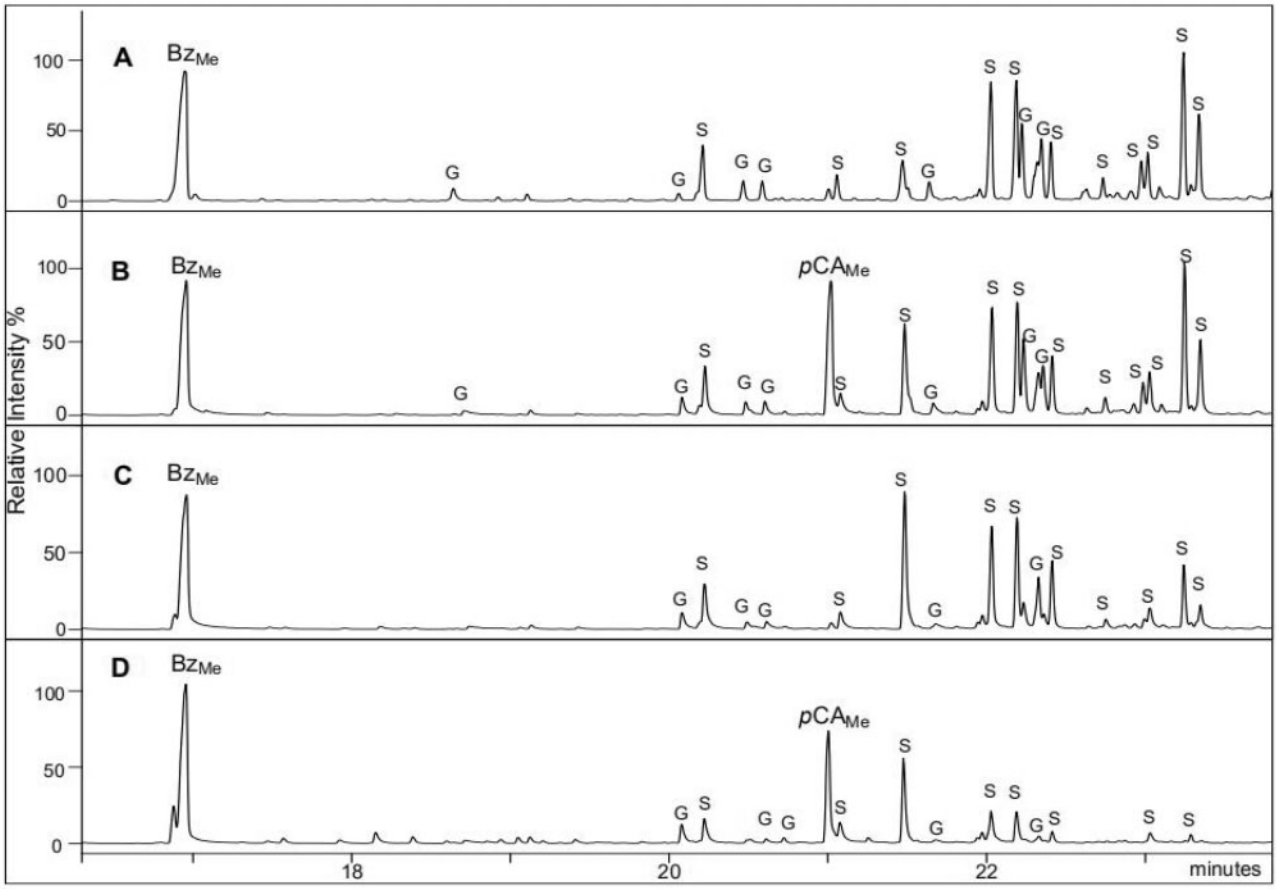

Figure 2 Traces of poplar CWs after Py-GC/MS in the presence of TMAH. (A) WT control, (B) *BdPMT1*-OE/WT line 9, (C) *AtF5H*-OE control, and (D) *BdPMT1*-OE/*AtF5H*-OE line 21. Bz_{Me}, 4-methoxybenzoate; pCA_{Me}, methyl 4-methoxy-*p*-coumarate; peaks quoted G and S correspond to methylated G and S compounds, respectively.

Table 4 Lignin content of extract-free poplar stems from *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds, as compared to their respective controls

Line	KL (%)	ABL (%)
WT control	21.82 (0.21)^{a}	19.18 (0.33)^{ab}
BdPMT1-OE/WT line 9	21.22 (0.09)^{a}	18.77 (0.50)^{b}
BdPMT1-OE/WT line 17	21.60 (0.21)^{a}	19.47 (0.44)^{ab}
BdPMT1-OE/WT line 31	21.09 (0.43)^{a}	19.27 (0.28)^{ab}
AtF5H-OE control	20.86 (0.23)^{a}	19.95 (0.23)^{a}
BdPMT1-OE/*AtF5H*-OE line 21	20.87 (0.69)^{a}	19.86 (0.13)^{a}

The data represent mean (SD) values from biological triplicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$). The lignin content is expressed as weight percentage of the sample and was determined using the KL and the ABL methods

units. For instance, upregulating *F5H* in poplar increased the S frequency up to 97.5%, whereas the incorporation of *p*-hydroxybenzoic acid in lignins was two-fold lower than the control level (Stewart et al., 2009). Upregulating *F5H* in rice also increased the frequency of S units up to 89%, whereas *p*CA levels were similar in the transgenic and control plants (Takeda et al., 2017).

In agreement with literature data (Lapierre, 1993, 2010), the control poplar samples displayed a %GOH and a %SOH close to 20% and 3%, respectively, which confirms that S units essentially are internal units. Even though the impact of *F5H* upregulation on poplar lignins is out of the main scope of this study, the data of Table 5 revealed that, in addition to the expected higher S/G ratio, lignins from *AtF5H*-OE control plants have more terminal units with free phenolic groups than WT lignins (Table 5). This result is consistent with a published paper about lignin structure in *F5H*-upregulated poplars (Stewart et al., 2009). In this study and relative to the WT samples, lignin fractions isolated from *F5H*-upregulated poplars were shown to concomitantly have a twice higher frequency of phenolic OH and a lower degree of polymerization (Stewart et al., 2009). More strikingly and whatever the genetic background, both %GOH and %SOH were significantly increased in the *p*-coumaroylated lignins of the *BdPMT1*-OE poplar lines (Table 5). The increase in %GOH or in %SOH was found to be nicely correlated to the *p*CA level of the *BdPMT1*-OE/WT lines ($R^2 = 0.95$ for %GOH and 0.93 for %SOH; Figure 4). This result means that the incorporation of *p*-coumaroylated monolignols in poplar lignins increases the frequency of free phenolic terminal units relative to internal units. Such a structural change may be accounted for by the occurrence of lignin polymers with lower polymerization degree and/or with a higher content of biphenyl or biphenyl ether branching structures.

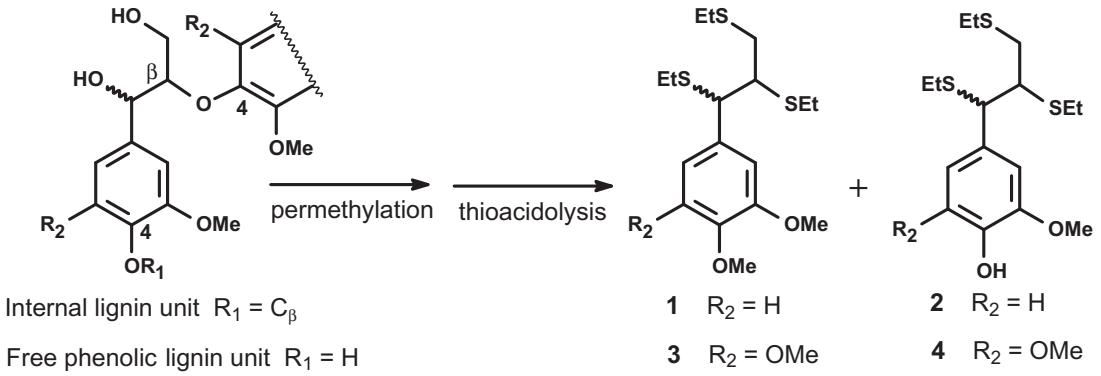

Figure 3 Principle of the evaluation of free phenolic units in lignin by thioacidolysis of permethylated samples. Lignin units only involved in β-O-4 bonds give rise to thioacidolysis guaiacyl ($R_2 = H$) and syringyl ($R_2 = OMe$) monomers. Terminal G and S units with free phenolic group ($R_1 = H$) are first methylated at C4, then degraded to monomers **1 and 3** (erythro/threo mixture), respectively. Internal G and S units ($R_1 = C_\beta$ of another lignin sidechain) are degraded to monomers **2 and 4**, respectively (erythro/threo mixture), EtS = SEt = thio-ethyl.

Table 5 Thioacidolysis of TMSD-methylated poplar CWs from *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds, as compared to their respective controls

Line	S/G Molar Ratio	% Free Phenolic Units in β-O-4 Linked G or S Units	
	(**3** + **4**)/(**1** + **2**)	%GOH 100 × **1**/(**1** + **2**)	%SOH 100 × **3**/(**3** + **4**)
WT control	2.05 (0.03)^{b}	19.45 (0.22)^{d}	2.81 (0.07)^{e}
BdPMT1-OE/WT line 9	2.09 (0.18)^{b}	22.85 (0.12)^{b}	3.65 (0.19)^{bc}
BdPMT1-OE/WT line 17	2.12 (0.19)^{b}	23.65 (0.48)^{a}	4.44 (0.25)^{a}
BdPMT1-OE/WT line 31	2.08 (0.06)^{b}	21.09 (0.26)^{c}	3.44 (0.10)^{cd}
AtF5H-OE control	3.12 (0.13)^{a}	20.85 (0.44)^{c}	3.26 (0.03)^{d}
BdPMT1-OE/*AtF5H*-OE line 21	2.96 (0.15)^{a}	23.10 (0.49)^{b}	4.02 (0.21)^{bc}

The S/G molar ratio corresponds to the ratio of the S monomers (**3** + **4**) to the G monomers (**1** + **2**; monomers shown in Figure 3). The molar % of free phenolic groups in β-O-4 linked G or S units, referred to as %GOH or %SOH, is calculated according to the outlined formula. The data represent mean (SD) values from biological triplicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$).

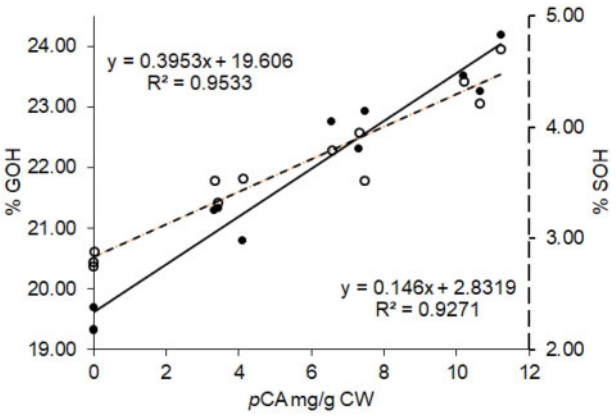

Figure 4 Relationships between the *p*CA amounts in poplar CWs and the percentage of G lignin units with free phenolic groups (%GOH, black circles, full line) or the percentage of S lignin units with free phenolic groups (%SOH, white circles, dotted lines). The lignin structural traits %GOH and %SOH are evaluated by thioacidolysis of permethylated samples for *BdPMT1*-OE poplars and their WT controls

The alkaline hydrolysis of the DL fractions isolated from the *BdPMT1*-OE poplar lines revealed that their *p*CA units were primarily ester-linked to lignins. With the objective to more precisely localize these *p*CA esters on lignin units, we subjected the *BdPMT1*-OE/WT line 17 and the WT samples to 1-h long thioacidolysis experiments, followed by Raney nickel desulfuration in order to identify the syringylpropanol and/or guaiacylpropanol units acylated by *p*-dihydrocoumaric acid (diHCA). This short thioacidolysis time is necessary as *p*CA esters do not survive the standard 4-h long thioacidolysis method (Lapierre, 1993; Sibout et al., 2016). When applied to the *BdPMT1*-OE/WT line 17, the method provided substantial amount of syringylpropanol acylated by diHCA while this dimer could not be observed with a longer thioacidolysis duration (Supplemental Figure S5 and Supplemental Table S2). Interestingly enough and in contrast to the results reported by Smith et al. (2015), its G analogue could not be detected. Taken together and similarly to grass lignins, these results support the hypothesis that the *p*-coumaroylation of lignins in the *BdPMT1*-OE/WT line 17 primarily involves S lignin units.

The analysis of the lignin-derived dimers obtained with the standard thioacidolysis method followed by Raney nickel desulfuration was comparatively performed for the WT line and for the *BdPMT1*-OE/WT line 17. With the caveat that the results were obtained from four technical replicates of WT and line 17 samples, this analysis supported the following conclusions. When expressed as relative

percentage of the total area of the main dimers (set to 100; Supplemental Table S2), the relative amount of dimers with biphenyl or biphenyl ether bonds was not increased by the presence of BdPMT1 and suggests that *BdPMT1*-OE/WT line 17 does not contain lignins more branched than the WT ones. In contrast, the relative percentage of the syringaresinol-derived dimers displayed a 1.4-fold increase in the case of the *BdPMT1*-OE/WT line 17 sample relative to the WT level (Supplemental Table S2). The syringaresinol structures exclusively originate from the dimerization of sinapyl alcohol and are thus starting points for lignin growth (Ralph et al., 2004). Their higher relative recovery from the *BdPMT1*-OE/WT line 17 further argues for the occurrence of lignin polymers with lower polymerization degrees than in the WT sample.

The *BdPMT1*-driven substantial *p*-coumaroylation of poplar samples makes their lignins more easily solubilized in cold alkali

The enrichment in free phenolic G and S units is very likely to improve the lignin susceptibility to alkaline treatments that are employed in chemical pulping or in the cellulose-to-ethanol conversion process. The beneficial impact of lignin terminal units with free phenolic groups on the CW delignification induced by alkaline treatment has been established for a long time for grass samples (Lapierre et al., 1989; Lapierre, 2010) and confirmed for poplar trees deficient in cinnamyl alcohol dehydrogenase (CAD) activity (Lapierre et al., 1999, 2004; Van Acker et al., 2017), for tobacco (*Nicotiana tabacum*) plants deficient in cinnamoyl-coenzyme A reductase (CCR) activity (O'Connell et al., 2002) and for *BdPMT1*-transformed Arabidopsis lines (Sibout et al., 2016). The results of a mild alkaline treatment applied to the poplar samples are shown in Table 6. The residue recovered after this treatment, referred to as the saponified residue (SR), was obtained with similar yields whatever the line. The percentage of the alkali-soluble lignin (%Alk-L) was calculated from the SR recovery yield and the lignin amount of the CW and SR samples. From the data of Table 6, we can see that the lignins from the *AtF5H*-OE control samples are more alkali-soluble than the lignins from the WT samples. This result is consistent with the higher frequency of terminal units with free phenolic groups in the *AtF5H*-OE control samples as compared to the WT samples (Table 5). Whatever the genetic background, the percentage of the alkali-soluble lignin (%Alk-L) revealed that the *BdPMT1*-OE lines are more easily delignified by the employed mild alkaline treatment compared with their control lines. Whereas 15%–20% of the lignin polymers were solubilized by cold alkali for the controls, the %Alk-L was substantially increased in the *BdPMT1*-OE lines (up to 26%–28% in lines 9, 17, and 21, Table 6). As reported for transgenic CAD- or CCR-deficient plants (Lapierre et al., 1999; O'Connell et al., 2002), increasing the percentage of lignin units with free phenolic groups has beneficial effects on the kraft pulping properties of the lignocellulosic biomass, thereby decreasing the energy and environmental costs of this industrial process. The introduction of *BdPMT1* in trees would likely improve the pulping properties of poplar wood.

Table 6 Impact of a mild alkaline treatment (aq. NaOH 1 M, overnight, room temperature) on extract-free poplar stems from control and *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds

Line	%SR	%ABL in SR	%Alk-L
WT control	68.03 (0.88)^{a}	23.69 (0.04)^{a}	15.5 (2.1)^{d}
BdPMT1-OE/WT line 9	67.17 (0.46)^{a}	20.85 (0.47)^{b}	25.5 (1.1)^{a,b}
BdPMT1-OE/WT line 17	65.25 (0.73)^{b}	21.52 (0.24)^{a,b}	28.1 (1.7)^{a}
BdPMT1-OE/WT line 31	68.17 (0.22)^{a}	22.11 (0.75)^{a,b}	21.7 (1.6)^{b,c}
AtF5H-OE control	69.02 (0.65)^{a}	22.96 (0.28)^{a,b}	20.5 (1.3)^{c}
BdPMT1-OE/*AtF5H*-OE line 21	67.65 (1.28)^{a}	21.52 (0.13)^{a,b}	26.4 (1.7)^{a}

The percentage of the recovered saponified residue (%SR) is expressed relative to the initial sample. The lignin content of the SR sample is measured as acetyl bromide lignin (%ABL). The percentage of alkali-soluble lignins (%Alk-L) is calculated from the ABL content of the CW and from the %SR recovery yield. The data represent mean (SD) values from biological triplicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$).

The relationship of the free phenolic groups in poplar lignins to their susceptibility towards cold alkaline treatment is further illustrated in Figure 5. On this scheme, we have gathered the data from 17 different poplar lines, comprising the current *BdPMT1*-OE/WT lines and CAD-deficient ones (Lapierre et al., 2004), together with their respective controls. The effect of the %GOH structural property onto the solubility of poplar lignins in cold alkali is supported by the positive correlation between %GOH and %Alk-L ($R^2 = 0.9513$; Figure 5).

The BdPMT1-driven *p*-coumaroylation of poplar samples results in improved saccharification after cold alkaline pretreatment

It is well established that the detrimental role of lignins on the cost-effective enzymatic conversion of lignocellulosic polysaccharides into fermentable sugars makes necessary the use of pretreatments (Yang and Wyman, 2008; Wang et al., 2015; Sun et al., 2016). Among these pretreatments, alkaline

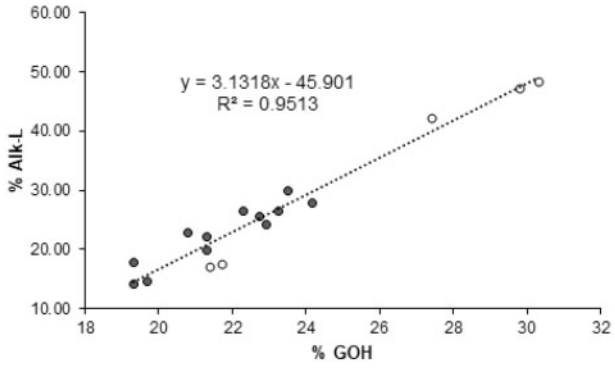

Figure 5 Relationship between the percentage of G lignin units with free phenolic groups (%GOH) and the solubility of poplar lignins in cold alkali (%Alk-L). The data correspond to *BdPMT1*-OE trees and their WT controls (black circles) as well as to CAD-deficient trees and their corresponding controls (white circles).

technologies with sodium hydroxide or with lime have emerged as major procedures for the conversion of lignocellulosic biomass (Kim et al., 2016). Most alkaline pretreatments are carried out under mild conditions (temperature below 60°C) and with moderate alkaline charge (0.5%–10% NaOH w/v) and long reaction time (several hours to several days; Carvalho et al., 2016; Kim et al., 2016; Moreno and Olsson, 2017; Rezania et al., 2020). These simple processes improve saccharification by partially removing lignins and hemicelluloses and by cellulose swelling (Carvalho et al., 2016). Sodium hydroxide pretreatments are more effective with grass feedstocks than on woody ones, which is related to the higher solubility of grass lignins in alkali (Beckmann et al., 1923; Scalbert et al., 1986; Lapierre et al., 1989). From these various literature data about NaOH pretreatments and from the analytical results that we obtained so far on the *BdPMT1*-OE poplar lines, we could anticipate that an alkaline pretreatment would be well suited to reduce the lignin-related recalcitrance of poplar wood to saccharification. Accordingly, the saccharification experiments run on the poplar samples were preceded by a cold alkaline pretreatment (aq. NaOH 1M, overnight, room temperature). Even though the optimization of this pretreatment was out of the scope of this study, we selected reaction duration and temperature as well as NaOH charge that were similar to literature data about alkaline pretreatment technologies (Carvalho et al., 2016; Kim et al., 2016). The saccharification efficiency was evaluated both by the weight loss (%WL) and by the amount of released glucose (Glc; Table 7). In agreement with their higher level of alkali-soluble lignins, the alkali-pretreated *AtF5H*-OE control samples displayed a higher saccharification efficiency than the alkali-pretreated WT samples (Table 7). More importantly, the saccharification efficiency was higher in the alkali-pretreated *BdPMT1*-OE lines, compared with their corresponding control (Table 7). In contrast, when the assays were carried out without any alkali pretreatment, low saccharification yields were observed and the transgenic samples were not significantly different from their controls (Supplemental Table S3). Not unexpectedly, in the WT background, the best saccharification results from alkali-pretreated samples were obtained for the lines provided with the concomitant highest *p*CA level, %GOH and %Alk-L. The enrichment of poplar lignins in free and readily ionizable phenolic groups favored lignin solubilization in alkali, which consequently improved the saccharification of alkali-pretreated samples. Taken together, these results reveal that the lignins from the current *BdPMT1*-OE poplar plants share common features with grass lignins. As compared to nongrass lignins from WT plants, these common features are (1) a substantial *p*-coumaroylation of S lignin units, (2) a higher level of free phenolic units, and (3) a higher solubility in cold alkali. At this point, we may hypothesize that, similar to grass lignins, lignins from the *BdPMT1*-OE poplar lines obtained herein are distributed in the CWs as small lignin domains which are both rich in free phenolic groups and more easily extracted by cold alkali treatment (Lapierre, 2010).

Table 7 Saccharification of the poplar SR obtained after a mild alkaline treatment (aq. NaOH 1 M, overnight, room temperature) and corresponding to *BdPMT1*-OE lines obtained in the WT and *AtF5H*-OE backgrounds, as compared to their respective controls

SR from Line	%WL	Glc $mg \cdot g^{-1}$ SR	Glc $mg \cdot g^{-1}$ CW
WT control	39.8 (1.3)^{d}	307.7 (16.0)^{e}	210.1 (11.3)^{e}
BdPMT1-OE/WT line 9	52.1 (1.6)^{a,b}	417.5 (23.2)^{b,c}	280.1 (17.4)^{b,c}
BdPMT1-OE/WT line 17	55.1 (2.0)^{a}	452.4 (17.7)^{a,b}	294.2 (8.3)^{a,b}
BdPMT1-OE/WT line 31	45.8 (0.3)^{c,d}	369.3 (17.9)^{d}	251.8 (11.9)^{d}
AtF5H-OE control	44.2 (2.2)^{c,d}	401.4 (6.7)^{c,d}	277.1 (6.6)^{c,d}
BdPMT1-OE/*AtF5H*-OE line 21	49.4 (1.5)^{b,c}	461.7 (22.4)^{a}	312.4 (16.5)^{a}

The saccharification efficiency is evaluated both by the weight loss (%WL) and by the released glucose (Glc). Glc yields are expressed either relative to the SR samples or to the initial CW samples. The data represent mean (SD) values from biological triplicates. Different letters in columns indicate significant differences (Duncan test, $P < 0.01$).

Conclusion

In this study, we have shown that *p*-coumaroylating poplar lignins up to the level of grass lignins has consequences that go far beyond a simple lignin decoration and that deeply change not only lignin structural traits, but also important industrial potentialities of lignified CW. Remarkably enough the expression of *BdPMT1* under the control of the *AtC4H* promoter introduced neither any growth penalty, nor reduced lignin content in the various transgenic greenhouse-grown poplar lines that were obtained in two genetic backgrounds. In agreement with a recent study (Sibout et al., 2016), choosing the lignin-specific *AtC4H* promoter to drive the heterologous expression of *BdPMT1* in dicot CW had very likely a key role in changing wood properties.

Since the last decades and with the objective to facilitate the industrial conversion of lignocellulosics into pulp or into bioethanol, many approaches have been used to genetically modify lignin content and/or structure (reviewed in Boerjan and Ralph, 2019; Halpin, 2019; Mahon and Mansfield, 2019; Ralph et al., 2019). Among the lignin structural traits that can be affected by the genetic transformation of angiosperm species, the S/G ratio is probably the most systematically scrutinized one (Chanoca et al., 2019). In contrast, the relative frequency of free phenolic units in native lignins is a key structural trait, which is surprisingly overlooked despite its biological significance and its major effect on the susceptibility of lignins to alkaline or oxidative treatments. In past studies, redesigning native lignins with more free phenolic groups (and therefore with increased alkali-solubility) could be obtained with other genetic transformations, such as CCR or CAD downregulation (O'Connell et al., 2002; Lapierre et al., 2004). In this work, we provide another compelling evidence that the genetically driven increase of free phenolic units in lignins is an efficient strategy for the rational design of lignocellulosics more adapted to industrial biorefineries.

Materials and methods

Production of plant materials

The *BdPMT1* expression in poplar was conducted using the same molecular construct as described in Sibout et al. (2016), with the *BdPMT1* sequence inserted into the pCC0996 vector under the control of the *AtC4H* promoter (Weng et al., 2008). This construct was introduced using *A. tumefaciens* cocultivation into the hybrid poplar clone INRA 717-1B4 as well as in a 717-1B4 transgenic line named *AtF5H*-OE, according to the method described in Leplé et al. (1992). The *AtF5H*-OE line was previously transformed with an *AtF5H* gene inserted into the pH7m24GW vector (Karimi et al., 2007) under the control of the promoter 1.3-kb upstream of the hybrid poplar *CesA4* gene (Potri.002G257900). Several transgenic lines from both genetic backgrounds were selected for further analyses. Three to five ramets of each line were acclimatized and grown in a S2 greenhouse for 3 months, from April until July following a random design plantation. Height and stem diameter were measured before plant sampling for molecular and biochemical analyses.

Differentiating xylem samples were collected by a light scraping at the surface of the debarked stem. Samples were immediately frozen in liquid nitrogen and stored at −80°C until use. DNA was prepared using Nucleospin DNA Plant II kit (Macherey-Nagel, Hoerdt, France) and the integration of *BdPMT1 and F5H* genes was verified by PCR using the following primers pairs: PMT 5′-CCTCATCATGCAGGT GACAG-3′ and 5′-GAAGCAGTTGCCGTAGAACC-3′; F5H 5′-ACGGCTCTTGTCATCGTTGT-3′; and 5′-GTTATGTTGCGGG TCAGTGC-3′. Likewise, RNA was extracted from differentiating xylem using a Nucleospin RNA Plant kit (Macherey-Nagel, Hoerdt, France). The expression level of the *BdPMT1 and AtF5H* gene in each tree was evaluated by semi-quantitative RT-PCR performed in standard conditions on 1-µg total RNA using the same primers as above.

Analyses of CW phenolics

Preparation of CW samples and dioxane lignins

Most analyses of CW phenolics were carried out from biological replicates (three or four per line) harvested from 3-month-old poplar trees. For each tree, the 20-cm-long basal part of the stem was collected, manually debarked, air–dried and ground to 0.5 mm. Extract-free samples were prepared by exhaustive water and ethanol extraction in an accelerated solvent extractor (ASE350, Dionex). The dried and extract-free samples are referred to as CW samples.

The isolation of DL fractions was performed from 1 to 2 g of CW as previously described (Sibout et al., 2016). Fourier transform infrared (FTIR) spectra of DL fractions were run on a Thermo Scientific Nicolet IS5 spectrophotometer and in KBr pellets.

Analytical pyrolysis

Py-GC/MS was done using a CDS model 5250 pyroprobe autosampler interfaced to an Agilent 6890/5973 GC/MS. The CW samples (about 300 µg) were pyrolyzed in a quartz tube at 500°C for 15 s. The pyrolysis products were separated on a capillary column (5% phenyl methyl siloxane, 30 m, 250 µm i.d., and 0.25-µm film thickness) using helium as the carrier gas with a flow rate of 1 mL·min^{-1}. The pyrolysis and GC/MS interfaces were kept at 290°C and the GC was programmed from 40°C (1 min) to 130°C at +6°C min^{-1}, then from 130 to 250°C at +12°C min^{-1} and finally from 250°C to 300°C at +30°C min^{-1} (3 min at 300°C). The various phenolic pyrolysis compounds were identified by comparison to published spectra (Ralph and Hatfield, 1991). Py-GC/MS in the presence of TMAH was similarly performed but with addition of 3 µL of a 25% (w/v) TMAH methanolic solution (Aldrich) onto the CW sample. The methylated pyrolysis products were identified by comparison of their mass spectra with those of the NIST MS library or with published TMAH-pyrograms (Kuroda et al., 2001, 2002).

Determination of lignin content

The determination of KL content was perfomed from about 300 mg of CW (weighted to the nearest 0.1 mg) and as previously described (Méchin et al., 2014). The quantitation of ABL was done from about 5 mg of CW (weighted to the nearest 0.01 mg) according to a recently published procedure (Sibout et al., 2016).

Determination of ester-linked p-hydroxybenzoic and p-hydroxycinnamic acids by mild alkaline hydrolysis

About 5–10 mg of poplar CW or DL samples were put into 2-mL Eppendorf tube together with 1 mL of 1 M NaOH and 0.1 mL of *o*-coumaric internal standard (IS) methanolic solution. The IS amount was 0.05 mg for CW samples and 0.25 mg for DL ones. Mild alkaline hydrolysis was proceeded on a carousel overnight and at room temperature. After acidification (0.2 mL of 6 M HCl) and centrifugation (1,500*g*, 10 min), the supernatant was subjected to solid phase extraction as previously described (Ho-Yue-Kuang et al., 2016). The recovered methanolic samples were analyzed by HPLC combined with diode array detection (HPLC–DAD). For HPLC separation, 1 µL of sample was injected onto an RP18 column (4 × 50 mm, 2.7-µm particle size, Nucleoshell, Macherey-Nagel) with a flow rate of 0.25 mL min^{-1}. The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the gradient was as follows: 0 min 5% B; 12 min, 20% B; 14 min, 80% B; 16 min, 5% B. The quantitative determination of alkali-released Bz, *p*CA, and FA was performed from the 250–400 nm DAD chromatograms and after calibration with authentic compounds.

Analysis of lignin structure by thioacidolysis

Thioacidolysis (4-h long) followed by GC/MS of the trimethylsilylated lignin-derived compounds was carried out from about 10 mg of CW samples using the simplified procedure previously published (Méchin et al., 2014), with some adaptations to the CW type concerning the IS amount and the reagent to sample ratio. In brief, 5–10 mg (weighed to

the nearest 0.1 mg) were put together with 2 mL of freshly prepared thioacidolysis reagent and 0.1 mL of IS solution (heinecosane C21, 5 mg·mL^{-1} in CH_2Cl_2) in a glass tube (Teflon-lined screwcap). The closed tubes were then heated at 100°C (oil bath) and for 4 h with occasional gentle shaking. After tube cooling, 2 mL of 0.2 M $NaHCO_3$ were added to destroy the excess of BF_3 etherate. Then, 0.025 mL of 6 M HCl was added to ensure that the pH was less than 3, before the addition of 2-mL CH_2Cl_2 and tube mixing. A small amount (about 0.5 mL) of the lower organic phase was withdrawn with a glass Pasteur pipette, dried over anhydrous Na_2SO_4 and then directly subjected to trimethylsilylation. This silylation was performed with 10 µL of the solution together with 100-µL BSTFA (Sigma-Aldrich) and 10 µL of GC-grade pyridine (1 h at room temperature). The GC/MS analyses were carried out as previously described (Méchin et al., 2014). Some short thioacidolysis assays (1-h long) were also carried out and were followed by desulfuration experiments according to a published method (Lapierre et al., 1995). In addition, thioacidolysis from exhaustively permethylated CW samples was run according to Sibout et al. (2016) and using the same thioacidolysis and GC/MS conditions.

Investigation of some CW properties

Alkali solubilization assays

About 300 mg of poplar CW were subjected to mild alkaline hydrolysis in 10 mL of 1 M NaOH, into a 25-mL plastic tube agitated overnight on a carousel and at room temperature. The alkali-treated residue, referred to as the SR, was recovered by centrifugation (2,000*g*, 20 min), washed with 1 M HCl before centrifugation and then with water (three times with centrifugation following each washing step). The final residue was freeze-dried, weighted to calculate its recovery yield and subjected to KL or ABL determination. The weight percentage of alkali-soluble lignin (%Alk-L) was calculated from the weight percentages of ABL in CW (%ABL_{CW}) and in SR (%ABL_{SR}) samples and from the SR recovery yield (%SR), as follows:

$$\%\text{Alk} - \text{L} = \left(100 \times \%\text{ABL}_{\text{CW}} - (\%\text{SR} \times \%\text{ABL}_{\text{SR}})\right) / \%\text{ABL}_{\text{CW}}$$

Saccharification assays

Saccharification experiments were performed from about 30 mg of samples (weighed to the nearest 0.1 mg) under the conditions previously described (Sibout et al., 2016). Saccharification efficency was calculated both from the weight loss and from the glucose yield.

Statistical analyses

Statistical analyses were performed with R software (version 3.2.3). Duncan's multiple range tests were performed with the package agricolae (https://cran.r-project.org/web/packages/agricolae/).

Accession numbers

BdPMT1 = Bradi2g36910, accession number NM_00128785 in the Genbank database.

AtF5H = accession number U38416 in the GenBank database.*pCesA4* = Potri.002G257900, accession number AC21305 in the GenBank database.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Molecular analyses on greenhouse-grown plant material.

Supplemental Figure S2. IR spectra (KBr pellet) of DL fractions isolated from *BdPMT1*-OE/WT and *BdPMT1*-OE/*AtF5H*-OE lines as compared to their controls.

Supplemental Figure S3. HPLC and GC/MS analyses of low-molecular weight phenolics released by alkaline hydrolysis of DL fractions isolated from WT and *BdPMT1*-OE/WT lines.

Supplemental Figure S4. Correlation between the amount of ester-linked *p*CA and the relative % of 4-vinylphenol released by analytical pyrolysis of *BdPMT1*-OE poplar trees.

Supplemental Figure S5. Partial GC/MS chromatograms of the main dimers obtained after 1- or 4-h-long thioacidolysis followed by Raney nickel desulfuration from WT or *BdPMT1*-OE/WT lines.

Supplemental Table S1. Amount of *p*CA ester-linked to grass CWs and to the corresponding purified DL fractions.

Supplemental Table S2. Relative amount (% area) of the main dimers obtained after thioacidolysis and Raney nickel desulfuration of extract-free poplar stems.

Supplemental Table S3. Saccharification of extract-free poplar stems with no pretreatment.

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Conflict of interest statement. The authors declare they have no conflict of interest.

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