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► **To cite this version:**

Richard Sibout, Philippe Le Bris, Frédéric F. Legée, Laurent L. Cezard, Hugues Renault, et al.. Structural Redesigning Arabidopsis Lignins into Alkali-Soluble Lignins through the Expression of p-Coumaroyl-CoA:Monolignol Transferase PMT. *Plant Physiology*, 2016, 170 (3), pp.1358-1366. 10.1104/pp.15.01877 . hal-02437998

**HAL Id: hal-02437998**

**<https://hal.science/hal-02437998>**

Submitted on 14 Jan 2020

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# Structural Redesigning Arabidopsis Lignins into Alkali-Soluble Lignins through the Expression of *p*-Coumaroyl-CoA:Monolignol Transferase PMT<sup>1</sup>

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Grass lignins can contain up to 10% to 15% by weight of *p*-coumaric esters. This acylation is performed on monolignols under the catalysis of *p*-coumaroyl-coenzyme A monolignol transferase (PMT). To study the impact of *p*-coumaroylation on lignification, we first introduced the *Brachypodium distachyon* *Bradi2g36910* (*BdPMT1*) gene into Arabidopsis (*Arabidopsis thaliana*) under the control of the constitutive maize (*Zea mays*) ubiquitin promoter. The resulting *p*-coumaroylation was far lower than that of lignins from mature grass stems and had no impact on stem lignin content. By contrast, introducing either the *BdPMT1* or the *Bradi1g36980* (*BdPMT2*) gene into Arabidopsis under the control of the Arabidopsis cinnamate-4-hydroxylase promoter boosted the *p*-coumaroylation of mature stems up to the grass lignin level (8% to 9% by weight), without any impact on plant development. The analysis of purified lignin fractions and the identification of diagnostic products confirmed that *p*-coumaric acid was associated with lignins. *BdPMT1*-driven *p*-coumaroylation was also obtained in the *fah1* (deficient for ferulate 5-hydroxylase) and *ccr1g* (deficient for cinnamoyl-coenzyme A reductase) lines, albeit to a lower extent. Lignins from *BdPMT1*-expressing *ccr1g* lines were also found to be feruloylated. In Arabidopsis mature stems, substantial *p*-coumaroylation of lignins was achieved at the expense of lignin content and induced lignin structural alterations, with an unexpected increase of lignin units with free phenolic groups. This higher frequency of free phenolic groups in Arabidopsis lignins doubled their solubility in alkali at room temperature. These findings suggest that the formation of alkali-leachable lignin domains rich in free phenolic groups is favored when *p*-coumaroylated monolignols participate in lignification in a grass in a similar manner.

The cost-effective conversion of lignocellulosic feedstocks to the fuel ethanol is strongly dependent on the pretreatment step that makes cellulose accessible to enzymatic hydrolysis (Yang and Wyman, 2008; Kumar et al., 2009, Alvira et al., 2010; Hu and Ragauskas, 2012). Lignins are major targets of such pretreatments, as these phenolic polymers constitute resistant barriers between glycosidases and polysaccharides. Worldwide, research efforts are being carried out to decipher the relationship of lignin structure to lignin susceptibility to pretreatments. Mild alkali pretreatments are well suited

to grass lignocelluloses, as native grass lignins can be substantially solubilized in alkali at room temperature (Beckman et al., 1923). The structural feature that governs this solubilization is the high frequency of free phenolic groups in grass lignins (Lapiere et al., 1989). Indeed, up to 50% of guaiacyl (G) grass lignin units are free phenolic, versus 25% or less in nongrass lignins (Lapiere, 2010). In addition, grass lignins have two other specific features: they are ether linked to ferulic acid (FA) units, which acylate arabinoxylans (Jacquet et al., 1995; Ralph et al., 1995), and they are acylated by *p*-coumaric acid (CA) on the  $\gamma$ -OH side chain of syringyl (S) and, to a lower extent, of G lignin units (Ralph et al., 1994; Grabber et al., 1996; Ralph, 2010). This *p*-coumaroylation, performed on monolignols prior to their polymerization (Lu and Ralph, 2002), is catalyzed by *p*-coumaroyl-coenzyme A monolignol transferase (PMT; Withers et al., 2012). Thanks to this PMT activity, lignins of grass mature stems can contain up to 10% to 15% (w/w) CA esters, as evidenced for purified maize (*Zea mays*) lignin fractions (Chazal et al., 2014). The highest lignin *p*-coumaroylation levels have been reported for grass species belonging to the C4 physiological type (Hatfield et al., 2009; Hatfield and Marita, 2010). It is now well established that CA esters

<sup>1</sup> This work was supported by LabEx Saclay Plant Sciences-SPS (grant no. ANR-10-LABX-0040-SPS to the Institut Jean-Pierre Bourgin).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Catherine Lapiere (catherine.lapiere@versailles.inra.fr).

C.L. and R.S. performed the research and some experiments, analyzed the data, and wrote the article; H.R. contributed to the research and complemented the writing; P.L.B. performed the plant transformation and production; F.L. and L.C. provided technical assistance to C.L.

[www.plantphysiol.org/cgi/doi/10.1104/pp.15.01877](http://www.plantphysiol.org/cgi/doi/10.1104/pp.15.01877)

linked to grass lignins keep free their phenolic group, by contrast to arabinoxylan-linked FA esters, which can be oxidatively etherified to lignin units (Ralph, 2010). However, it is not yet clearly established to what extent the substantial participation of CA conjugates to grass lignification can affect grass lignin structural traits other than the simple *p*-coumaroylation at C $\gamma$ .

In this study, we addressed the question of the possible relationship between the substantial *p*-coumaroylation level of grass lignins and their high frequency in free phenolic lignin units. The feasibility of introducing CA esters into dicot lignins was demonstrated recently by expressing the rice (*Oryza sativa*) *OsPMT* gene into two eudicots, Arabidopsis (*Arabidopsis thaliana*) and poplar (*Populus* spp.; Smith et al., 2015). The resulting transgenic PMT-Arabidopsis and PMT-poplar lines were found to display lignin *p*-coumaroylation, with CA levels ranging between 0.8% and 2% (w/w) of acetyl bromide lignin (Smith et al., 2015). In this study, we aimed to incorporate additional *p*-coumarate esters into Arabidopsis lignins, up to the level reported for the lignins of mature grass stems, and to investigate the impact of such a high incorporation on the structure of Arabidopsis lignins and their solubility in alkali at room temperature. This investigation was conducted on Arabidopsis transgenic lines expressing either the *Brachypodium distachyon* *Bradi2g36910* (hereafter referred to as *BdPMT1*) or the *B. distachyon* *Bradi1g36980* (hereafter referred to as *BdPMT2*) gene into the wild-type Columbia-0 (Col-0) genetic background under the control of the Arabidopsis cinnamate-4-hydroxylase (*AtC4H*) promoter, which has been shown to efficiently target gene expression to vascular tissues (Bell-Lelong et al., 1997; Weng et al., 2008). In addition, *AtC4H* promoter-driven *BdPMT1* expression was also obtained in the Arabidopsis *fah1* mutant devoid of S lignin units due to a mutation in the gene encoding ferulate 5-hydroxylase (Chapple et al., 1992) as well as in the *ccr1g* mutant deficient for CINNAMOYL-COENZYME A REDUCTASE1 (CCR1), which accumulate unusual levels of soluble ferulate derivatives and incorporate FA into lignins (Mir Derikvand et al., 2008; Ralph et al., 2008). The impacts of these transformations on the *p*-coumaroylation and structure of Arabidopsis lignins as well as on their solubility in alkali at room temperature are evaluated in this work.

## RESULTS AND DISCUSSION

### The Expression of Either *BdPMT1* or *BdPMT2* into Arabidopsis under the Control of the *AtC4H* Promoter Boosts Lignin *p*-Coumaroylation up to the Grass Lignin Level

The *BdPMT1* gene was shown recently to encode for the monolignol-specific PMT1 enzyme, the activity of which results in the *p*-coumaroylation of *B. distachyon* lignins (Petrik et al., 2014). Lignin *p*-coumaroylation was decreased severely in the *Bdpm1* mutant, but not reduced to zero, suggesting that some other BdPMT

proteins could act as BdPMT1 surrogates. Among the *BdPMT* gene family (Supplemental Fig. S1), we selected *Bradi1g36980* (*BdPMT2*) as the best candidate to fulfill this role because of its substantial transcript level in *B. distachyon* lignified culm (PlaNET; <http://aranet.mpimp-golm.mpg.de/>). The full-length complementary DNA of *BdPMT1* or *BdPMT2* was cloned and introduced into wild-type and mutant Arabidopsis lines under the control of the *AtC4H* promoter. It is noteworthy that, throughout the production of transgenic lines (from T1 to T3 generation), transgenic and corresponding control plants displayed a similar phenotype (Supplemental Fig. S2).

The *AtC4H* promoter-driven expression of the *BdPMT1* or *BdPMT2* gene in Arabidopsis induced an impressive increase of alkali-releasable CA from the cell wall (CW) of wild-type mature stems (Table I). While minute, but measurable, amounts of CA were released systematically from wild-type samples, introducing one or the other *BdPMT* gene under the control of the *AtC4H* promoter boosted the amount of CW-linked CA esters up to the levels observed for mature grass stem samples (ranging between 5 and 18 mg g<sup>-1</sup> CW; Table I). Such a high incorporation was obtained for two independent *BdPMT1/wt* or *BdPMT2/wt* homozygous lines (T3 generation plants). By contrast, when the *BdPMT1* gene was expressed under the control of the maize constitutive ubiquitin (*ZmUbi*) promoter, CW *p*-coumaroylation was much lower, with values ranging between 0.8 and 1.6 mg g<sup>-1</sup> CW (Supplemental Table S1). Such a modest *p*-coumaroylation level is similar to that obtained when the *OsPMT* gene was introduced in Arabidopsis under the control of the CELLULOSE SYNTHASE7 promoter (1–2 mg g<sup>-1</sup> CW; Smith et al., 2015).

Even though the specificity of BdPMT1 for monolignols is established (Petrik et al., 2014), it was necessary to ascertain that CW-linked CA units introduced in the *BdPMT1/wt* or *BdPMT2/wt* Arabidopsis line were linked to lignins. To this end, we isolated purified dioxane lignin (DL) fractions by mild acidolysis to check whether CA esters remained associated with lignins. The mild acidolysis procedure consists of refluxing lignified CWs in a dioxane:water mixture (9:1, v/v) containing 0.2 M HCl (30 min under N<sub>2</sub>) to recover a rough lignin extract, which was then purified to get a DL fraction. When applied to grass CWs, this rapid and mild procedure provides purified DLs with a high recovery yield (50%–70% of the whole lignin amount), a low sugar contamination (in the 3–10 weight percentage range), and with most lignin-linked CA esters preserved (Chazal et al., 2014). Purified DL fractions were obtained from transgenic and wild-type samples. When expressed as percentages of the sample Klason lignin (KL) content (discussed below), their recovery yield was higher for the transgenic lines (mean  $\pm$  SD = 52%  $\pm$  4% for the four DL fractions isolated from *BdPMT1*- and *BdPMT2*-expressing lines) than for the control lines (25%  $\pm$  5% for the two DL fractions isolated from wild-type samples), which suggests that CA

**Table I.** Amount of CA released by mild alkaline hydrolysis of the mature stems of *Arabidopsis* wild-type and *BdPMT1*- and *BdPMT2*-expressing lines under the control of the *AtC4H* promoter and of the corresponding purified DL fractions

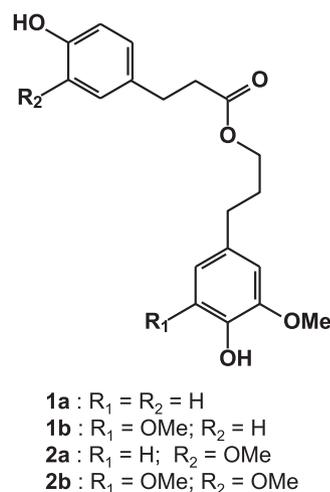
Data from grass samples are given for comparison purposes. The data for *Arabidopsis* CW represent means (and SD) from three different plant pools (about 20 plants per pool) for each line. Asterisks indicate significant differences (one-way ANOVA) compared with the value of the corresponding control at  $P < 0.001$ . For grass CWs, data are means (and SD) from analytical triplicates. For *Arabidopsis* samples, DL isolation was performed from the pool of biological triplicates, and each DL fraction was then subjected to analytical replicates ( $n = 2$  or  $3$ ). For grass DL samples, each DL fraction was analyzed as analytical triplicates.

Plant and Genotype	CA from Whole CWs	CA from Corresponding DL Fractions
	$\text{mg g}^{-1}$	
Arabidopsis extract-free mature stems		
Wild-type Col-0	0.016 (0.002)	0.93 (0.10)
<i>BdPMT1/wt</i> line1-3	7.84 (0.20)*	48.0 (4.1)
<i>BdPMT1/wt</i> line12-8	12.57 (0.39)*	66.3 (1.4)
Wild-type Col-0	0.010 (0.004)	1.34 (0.04)
<i>BdPMT2/wt</i> line2-3	13.36 (0.81)*	71.9 (4.3)
<i>BdPMT2/wt</i> line7-7	12.74 (0.23)*	76.6 (2.1)
Grass extract-free mature stems		
Maize stem (F2 line, silage stage)	17.39 (0.66)	119.8 (2.1)
<i>B. distachyon</i> Bd21-3 mature stems	8.92 (0.63)	35.4 (1.0)
Wheat straw (cv Champlein)	4.76 (0.10)	23.8 (1.4)

introduction in lignins has made these polymers more amenable to mild acidolysis extraction. Relative to the trace amounts observed for wild-type CWs (less than  $0.02 \text{ mg g}^{-1}$  CW; Table I), the corresponding DL fractions were found to be enriched in alkali-releasable CA (about  $1 \text{ mg g}^{-1}$  DL; Table I), which confirms that wild-type *Arabidopsis* lignins are *p*-coumaroylated, albeit to a very low extent. By contrast, significantly higher CA levels were released by mild alkaline hydrolysis of DL fractions isolated from *BdPMT1/wt* and *BdPMT2/wt* stems (Table I). These DL fractions contained 5- to 6-fold more alkali-releasable CA than the corresponding CWs, which conclusively establishes that CA esters are associated with lignins in the corresponding transgenic lines. The highest CA level reached  $76 \text{ mg g}^{-1}$  DL, a substantial value intermediate between the values from C3 grass species (wheat [*Triticum aestivum*] and *B. distachyon* DL in Table I) and the highly *p*-coumaroylated DL isolated from maize stems (Table I; Chazal et al., 2014). To evaluate whether some CA also may have acylated arabinose substituents that occur in *Arabidopsis* hemicelluloses, we subjected the *BdPMT1/wt* or *BdPMT2/wt* mature stems to analytical mild acidolysis, a method recently developed to estimate the amount of *p*-coumaroylated or feruloylated arabinose units in grass arabinoxylans (Petrik et al., 2014). Unlike grass

CWs, no trace of *p*-coumaroylated arabinose could be detected in transgenic *Arabidopsis* lines.

As a further confirmation that lignin units are *p*-coumaroylated in the *BdPMT1/wt* and *BdPMT2/wt* lines, we subjected the purified DL fractions to 1-h-long thioacidolysis experiments. The thioacidolysis lignin-derived mixtures were then desulfurated over Raney nickel, as described previously (Lapierre et al., 1995), and analyzed by gas chromatography-mass spectrometry (GC-MS) of their trimethylsilylated (TMS) derivatives. In contrast to the derivatization followed by reductive cleavage technique that leaves lignin  $\gamma$ -esters intact (Lu and Ralph, 1999), most *p*-coumaric esters present in grass lignins do not survive the standard 4-h-long thioacidolysis (Lapierre, 2010). However, some *p*-coumaroylated lignin units can give rise to diagnostic compounds identified previously among the thioacidolysis/Raney nickel products from maize lignins (Grabber et al., 1996). With 1-h-long thioacidolysis followed by desulfuration of *BdPMT1/wt* DLs, the diagnostic compounds **1a** and **1b** (Fig. 1) were easily observed on the GC-MS trace showing the separation of lignin-derived products (Supplemental Fig. S3). By comparing the mass spectra of their TMS derivatives (Supplemental Fig. S4; Supplemental Table S2) with published mass spectra (Grabber et al., 1996), compounds **1a** and **1b** were identified as dihydroconiferyl alcohol acylated by *p*-dihydrocoumaric acid and dihydrosinapyl alcohol acylated by *p*-dihydrocoumaric acid, respectively. Accordingly, *p*-coumaroylated G and S units only involved in  $\beta$ -O-4 bonds were the lignin parent structures of compounds **1a** and **1b**. With the standard 4-h-long thioacidolysis, these diagnostic compounds were minor or trace components relative to the main dimers, which



**Figure 1.** Acylated compounds diagnostic for *p*-coumaroylation of G and S lignin units (**1a** and **1b**) and for feruloylation of G and S lignin units (**2a** and **2b**). These compounds are recovered after short thioacidolysis (1 h) followed by Raney nickel desulfuration of *Arabidopsis* lignins isolated from *BdPMT1*-expressing lines. Compounds **2a** and **2b** are released only from the lignins of *BdPMT1/ccr1g* lines.

are representatives of lignin-resistant interunit bonds (Supplemental Fig. S3). With short thioacidolysis, they displayed more substantial peaks (Supplemental Fig. S3), with a large predominance of compound **1b** over **1a**, suggesting that the main CA conjugate formed by PMT1 is the one with sinapyl alcohol.

#### Type of Lignin Acylation Induced by Expressing the *BdPMT1* Gene in the Arabidopsis *fah1* or *ccr1g* Mutant

When *AtC4H*-driven *BdPMT1* expression was performed in the *fah1* mutant, which is impaired in the formation of sinapyl alcohol, a substantial CA incorporation also was obtained, up to about 7 mg g<sup>-1</sup> CW, and for two independent homozygous lines (referred to as *BdPMT1/fah1* lines), whereas alkali-releasable FA remained as a trace component (Table II). As done previously for the wild-type background, we could establish that CA was also closely associated with lignins in *BdPMT1/fah1* lines, from the alkaline hydrolysis of the corresponding DL fractions (Table II) and by the identification of the diagnostic compound **1a** obtained with 1-h-long thioacidolysis (Supplemental Fig. S3). This result means that the *BdPMT1* enzyme is able to induce the appearance of *p*-coumaroylated G lignin units by catalyzing the formation of coniferyl *p*-coumarate conjugate. However, this *p*-coumaroylation was achieved to a lower extent in *fah1* lignins than in wild-type ones, in agreement with the fact that sinapyl alcohol has been reported to be a better substrate of lignin-specific PMT than coniferyl alcohol (Withers et al., 2012).

When the *AtC4H*-driven *PMT1* insertion was carried out in the Arabidopsis *ccr1g* background, CA incorporation was also successfully obtained for two independent lines (referred to as *BdPMT1/ccr1g* lines), albeit to a lower extent than in the *BdPMT1/wt* and *BdPMT1/fah1* samples (Tables I and II). Such a lower incorporation might originate from the developmental problems of the CCR1-deficient Arabidopsis lines, echoed by their dwarf

phenotype and lower lignin level (Mir Derikvand et al., 2008). Quite unexpectedly, and in parallel to the accretion of CA esters, a substantial amount of FA was released from the two *BdPMT1/ccr1g* lines. We previously established that CCR1-deficient Arabidopsis mutants accumulate unusual levels of soluble feruloyl malate (Mir Derikvand et al., 2008) and incorporate free FA into lignins by bis 8-O-4 cross coupling (Ralph et al., 2008). In addition, and as compared with the wild-type level, the amount of alkali-releasable FA is increased slightly by CCR1 deficiency (0.053 ± 0.001 mg g<sup>-1</sup> CW for the *ccr1g* line [Table II] versus 0.020 ± 0.006 mg g<sup>-1</sup> CW for the two wild-type samples). The results obtained for the two *BdPMT1/ccr1g* lines suggest that CCR1 deficiency induces the accumulation of feruloyl-CoA that might be used as a substrate by the PMT1 enzyme for the formation of monolignol ferulate conjugates. As done for the wild-type and *fah1* backgrounds, we could ascertain that both CA and FA units introduced in *BdPMT1/ccr1g* CWs were associated with lignins by alkaline hydrolysis of the corresponding DL fraction (Table II) and by identification of the four diagnostic compounds **1a**, **1b**, **2a**, and **2b** (Fig. 1) among the desulfurated products released by 1-h-long thioacidolysis (Supplemental Fig. S5). The dihydroferuloylated conjugates **2a** and **2b** displayed mass spectra very similar to those of **1a** and **1b**, respectively, but with molecular ions at +30 D (Supplemental Fig. S4). In summary, the analyses of *BdPMT1/ccr1g* lines revealed that PMT1 is able to catalyze not only the formation of monolignol *p*-coumarate conjugates but also that of monolignol ferulate conjugates, provided that some alteration in the monolignol pathway induces a sufficient feruloyl-CoA buildup. In a recent study, lignin feruloylation mediated by the introduction of a gene encoding for an *Angelica sinensis* feruloyl-CoA monolignol transferase into poplar was reported as a novel strategy to introduce chemically labile linkages in lignins (Wilkerson et al., 2014). From our data here, lignin feruloylation also seems to be possible by introducing the *BdPMT1* gene into the appropriate mutant or transgenic background.

**Table II.** Amounts of CA and FA released by mild alkaline hydrolysis of the mature stems of Arabidopsis *BdPMT1*-expressing lines in the *fah1* and *ccr1g* backgrounds and of the corresponding purified DL fractions

Data for Arabidopsis CW data represent means (and SD) from three different plant pools (about 20 plants per pool) for each line. Asterisks indicate significant differences (one-way ANOVA) compared with the value of the corresponding control at  $P < 0.001$ . DL isolation was performed from the pool of biological triplicates for three transgenic lines only, and each DL fraction was then subjected to analytical replicates ( $n = 2$  or 3).

Genotype	Whole CWs		Corresponding DL Fractions	
	CA	FA	CA	FA
	<i>mg g<sup>-1</sup></i>			
<i>fah1</i>	0.021 (0.011)	0.020 (0.002)		
<i>BdPMT1/fah1</i> line1-2	6.48 (0.17)*	0.027 (0.10)	27.3 (1.4)	0.49 (0.05)
<i>BdPMT1/fah1</i> line12-7	7.08 (0.09)*	0.021 (0.005)	29.6 (0.1)	0.23 (0.02)
<i>ccr1g</i>	0.025 (0.005)	0.053 (0.001)		
<i>BdPMT1/ccr1g</i> line2-7	3.53 (0.35)*	1.58 (0.15)*	20.0 (2.3)	11.5 (1.3)
<i>BdPMT1/ccr1g</i> line6-5	5.28 (0.18)*	2.49 (0.11)*		

### Lignin *p*-Coumaroylation Induces Lower Lignin Content and Improved Saccharification of Arabidopsis Inflorescence Stems

Increasing the lignin *p*-coumaroylation level up to that of grass lignins induced a noticeable decrease of the KL content of Arabidopsis CWs (Table III). Relative to the corresponding control, the KL amount of *BdPMT1*- or *BdPMT2*- expressing lines was reduced by 10% to 30%. Such an impact was confirmed by acetyl bromide lignin (ABL) determination. All samples displayed an ABL level reduced by 10% to 20% relative to the corresponding control, except the two transgenic lines obtained in the *ccr1g* background. By contrast, no significant KL or ABL reduction could be observed for lower lignin *p*-coumaroylation degree, as reported recently (Smith et al., 2015) or as observed when the *BdPMT1* gene was inserted under the control of the *ZmUbi* promoter (Supplemental Table S3).

When calculated relative to the KL content of mature stems, the lignin *p*-coumaroylation was actually boosted up to 8% to 9% (w/w) for the transgenic lines obtained in the wild-type background, provided that the expression of one or the other PMT-encoding genes was driven by the *AtC4H* promoter, whereas the lignin CA level was only increased to 0.7% to 0.8% (w/w) with the constitutive *ZmUbi* promoter (Supplemental Fig. S6). The relative KL reduction observed in *BdPMT1*- or *BdPMT2*-expressing lines seemed to be somehow related to the lignin *p*-coumaroylation degree. This observation suggests that the lignification process might be indirectly or directly slowed down by the formation of CA-monolignol conjugates, provided that this formation induces a similar lignin *p*-coumaroylation extent to that in grass lignins.

The susceptibility of extract-free mature CWs to saccharification by a commercial cellulase preparation

and without any pretreatment was evaluated by measuring the released Glc. As expected, the saccharification of Arabidopsis samples displaying a lower KL content yielded more Glc (Table III). However, the correlation between lignin content and Glc yield was low, which confirms that other factors than lignin content govern the CW susceptibility to saccharification. The most efficient saccharification was observed in the *ccr1g* background, with an unexpectedly high improvement afforded by introducing both CA and FA esters in lignins. In these samples, and relative to the control, a moderate KL reduction (about 10%) induced a large saccharification improvement (Glc release increased by about 50%).

### The Substantial *p*-Coumaroylation of Arabidopsis Lignins Is Accompanied by Other Severe Structural Changes

The impact of *p*-coumaroylation on lignin structure was evaluated by thioacidolysis. This method specifically provides *p*-hydroxyphenyl (H), G, and S monomers from H, G, and S lignin units that are involved exclusively in labile  $\beta$ -O-4 interunit bonds. As discussed previously, compounds **1a**, **1b**, **2a**, and **2b** were obtained as minor or trace components with the standard 4-h-long thioacidolysis (Supplemental Figs. S3 and S5). This observation rules out the possibility that the yield of lignin-derived S or G monomers might be noticeably reduced by the survival of CA or FA ester linked at the C $\gamma$  group of lignin units. With this consideration in mind, and when expressed relative to the lignin content, thioacidolysis yield closely reflects the frequency of the parent lignin structure. As a corollary, thioacidolysis yield is reduced when the frequency of resistant interunit bonds is increased in lignins.

**Table III.** Impact of expressing the *BdPMT1* and *BdPMT2* genes under the control of the *AtC4H* promoter in Arabidopsis lines on the KL content or ABL content of extract-free mature stems as well as on their saccharification efficiency

Data represent means (and SD) from three different plant pools (about 20 plants per pool) per genotype. Asterisks indicate significant differences (one-way ANOVA) compared with the value of the corresponding control at  $P < 0.001$ .

Genotype	KL	ABL	Saccharification Efficiency of Glc Release
	% by weight		mg g <sup>-1</sup>
Wild type	18.51 (0.06)	14.68 (0.47)	131.0 (3.2)
<i>BdPMT1/wt</i> line1-3	15.84 (0.44)*	13.17 (0.19)*	146.3 (3.8)*
<i>BdPMT1/wt</i> line12-8	15.05 (0.21)*	12.17 (0.52)*	148.0 (2.3)*
<i>fah1</i>	21.12 (0.08)	19.91 (0.59)	115.2 (1.6)
<i>BdPMT1/fah1</i> line1-2	18.39 (0.78)*	16.37 (1.09)*	150.4 (3.8)*
<i>BdPMT1/fah1</i> line12-7	17.63 (0.17)*	15.69 (0.49)*	147.1 (6.5)*
<i>ccr1g</i>	15.58 (0.41)	12.45 (0.22)	165.1 (1.8)
<i>BdPMT1/ccr1g</i> line2-7	13.29 (0.14)*	12.12 (0.18)	234.9 (12.9)*
<i>BdPMT1/ccr1g</i> line6-5	14.10 (1.05)	12.13 (0.36)	254.7 (8.4)*
Wild type	19.84 (0.08)	15.90 (0.22)	122.8 (4.3)
<i>BdPMT2/wt</i> line2-3	15.01 (0.18)*	13.34 (0.21)*	147.6 (3.1)*
<i>BdPMT2/wt</i> line7-7	13.88 (0.21)*	11.95 (0.26)*	155.2 (1.8)*

As shown in Table IV, the molar frequencies of lignin-derived H, G, or S monomers released from mature extract-free stems were found to be similar in the *BdPMT1*- or *BdPMT2*-expressing lines and in the corresponding controls. By contrast, when expressed on a KL basis (in  $\mu\text{mol g}^{-1}$  KL), the total yield of thioacidolysis monomers was reduced noticeably for the Arabidopsis samples displaying substantial lignin *p*-coumaroylation. Such a reduction is very likely diagnostic for a higher frequency of resistant interunit bonds, which counteracts the recovery of thioacidolysis monomers. This impact on lignin structure, reflected by reduced thioacidolysis yield, was not observed when lower lignin *p*-coumaroylation was achieved by *ZmUbi*-driven *BdPMT1* expression (Supplemental Table S3).

The percentage of terminal units with free phenolic groups in native lignins is a major structural feature that governs their solubility in alkaline medium. This feature can be evaluated by the thioacidolysis of permethylated samples (Lapierre, 2010). Whatever the sample and in agreement with past results, about 85% to 90% of H units giving rise to H thioacidolysis monomers were found to be terminal units. In control samples (i.e. in wild-type, *fah1*, and *ccr1g* lines), this percentage was weak for S units (less than 1.5% of  $\beta$ -O-4-linked S units were free phenolic; Table V) and low for G units (14%–16%; Table V), in agreement with previous data reported for Arabidopsis stem lignins (Ralph et al., 2008; Lapierre, 2010). As another major structural change accompanying the *p*-coumaroylation of Arabidopsis lignins, the thioacidolysis of permethylated samples revealed that lignins from *BdPMT1*- or *BdPMT2*-expressing lines were enriched in G or S terminal units with free phenolic groups (Table V). A higher frequency of terminal lignin units relative to internal ones may originate from a higher branching degree and/or a lower average  $M_n$  of native lignins. This structural alteration suggests that the participation of *p*-coumaroylated monolignols to lignification has somehow affected the lignin polymerization mode.

### Arabidopsis *p*-Coumaroylated Lignins Are Twice More Soluble in Alkali and at Room Temperature

A higher frequency of terminal units with a free phenolic group in lignins may have a significant consequence on the solubilization of lignins in alkaline medium and at room temperature, as exemplified by the case study of grass lignins (Lapierre, 2010). Based on this rationale, and to evaluate the extent to which the lignins of *BdPMT1/wt* and *BdPMT2/wt* lines mirrored the solubility properties of *p*-coumaroylated grass lignins, we performed simple solubilization tests. The extract-free stem samples from wild-type and transgenic lines were subjected to lignin solubilization assays in 1 M NaOH at 20°C overnight. The resulting saponified CWs were carefully recovered, washed, and weighed before the determination of their KL content. Thus, the percentage of alkali-solubilized lignins could be evaluated from the alkali-induced weight loss together with the KL levels before and after this mild alkaline treatment (Fig. 2). This simple experiment revealed that the solubility of Arabidopsis lignins was increased from 25% in the wild-type lines to about 50% in the transgenic lines displaying lignins as *p*-coumaroylated as in grass lignins.

### CONCLUSION

It is now well established that grass lignin *p*-coumaroylation results from the PMT-mediated *p*-coumaroylation of monolignols (Hatfield and Marita, 2010; Withers et al., 2012; Petrik et al., 2014). In a recent study, the introduction of a rice *PMT* gene into Arabidopsis and poplar induced the formation of *p*-coumaroylated lignins, with lignin *p*-coumaroylation degree ranging between 0.8% and 2% (w/w) of ABL lignins, without any impact on lignin content (Smith et al., 2015). Compared with this recent study, and most likely thanks to the use of the *AtC4H* promoter to drive *BdPMT* expression, we obtained 5- to 10-fold higher *p*-coumaroylation of

**Table IV.** Thioacidolysis of extract-free mature stems from Arabidopsis control lines and transgenic lines expressing the *BdPMT1* or *BdPMT2* gene under the control of the *AtC4H* promoter

Data represent means (and SD) from three different plant pools per genotype. Asterisks indicate significant differences (one-way ANOVA) compared with the value of the corresponding control at  $P < 0.001$ . ND, Not detected.

Line	Total Yield $\mu\text{mol g}^{-1}$ KL	H %	G	S
Wild type	1,064 (34)	1.0 (0.1)	69.7 (0.2)	29.3 (0.1)
<i>BdPMT1/wt</i> line1-3	891 (53)	1.0 (0.1)	68.5 (0.2)	30.5 (0.3)
<i>BdPMT1/wt</i> line12-8	713 (7)*	1.3 (0.2)	68.7 (0.1)	30.0 (0.1)
<i>fah1</i>	962 (40)	0.9 (0.2)	99.1 (0.2)	ND
<i>BdPMT1/fah1</i> line1-2	675 (23)*	1.3 (0.1)	98.7 (0.1)	ND
<i>BdPMT1/fah1</i> line12-7	673 (5)*	1.1 (0.0)	98.9 (0.0)	ND
<i>ccr1g</i>	808 (60)	0.9 (0.1)	63.1 (0.6)	36.1 (0.7)
<i>BdPMT1/ccr1g</i> line2-7	602 (78)	0.7 (0.1)	65.9 (1.0)	33.4 (0.9)
<i>BdPMT1/ccr1g</i> line6-5	523 (18)*	0.7 (0.1)	64.9 (0.2)	34.5 (0.2)
Wild type	853 (17)	0.9 (0.1)	69.7 (0.2)	29.4 (0.3)
<i>BdPMT2/wt</i> line2-3	532 (15)*	1.3 (0.2)	70.8 (0.7)	27.9 (0.7)
<i>BdPMT2/wt</i> line7-7	503 (42)*	1.3 (0.2)	71.5 (0.5)	27.3 (0.5)

**Table V.** Relative percentages of free phenolic groups in G or S lignin units only involved in  $\beta$ -O-4 bonds, as revealed by thioacidolysis of permethylated extract-free stems from an *Arabidopsis* control line and from *BdPMT1*- or *BdPMT2*-expressing lines under the control of the *AtC4H* promoter

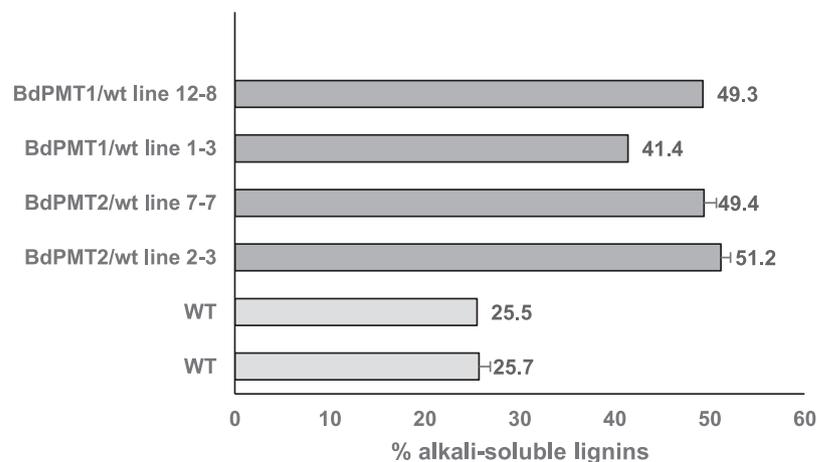
Calculations were done as described previously (Lapierre, 2010). Data represent means (and SD) from duplicate analyses of different plant pools per genotype. Asterisks indicate significant differences (one-way ANOVA) compared with the value of the corresponding control at  $P < 0.001$ .

Line	G	S
Wild-type Col-0	13.7 (0.1)	1.3 (0.1)
<i>BdPMT1</i> /wt line1-3	21.8 (0.3)*	2.2 (0.1)*
<i>BdPMT1</i> /wt line12-8	26.0 (0.0)*	2.7 (0.2)*
<i>fah1</i>	16.7 (0.1)	–
<i>BdPMT1</i> / <i>fah1</i> line1-2	19.1 (0.1)*	–
<i>BdPMT1</i> / <i>fah1</i> line12-7	20.2 (0.6)*	–
<i>ccr1g</i>	16.0 (0.1)	1.1 (0.1)
<i>BdPMT1</i> / <i>ccr1g</i> line2-7	41.6 (3.9)*	4.3 (0.5)*
<i>BdPMT1</i> / <i>ccr1g</i> line6-5	55.4 (0.3)*	6.0 (0.5)*
Wild-type Col-0	14.0 (0.1)	1.3 (0.1)
<i>BdPMT2</i> /wt line7-7	26.3 (0.7)*	2.6 (0.2)*

lignins from *Arabidopsis* stems (up to 8%–9% [w/w] KL; Supplemental Fig. S6). Such a high *p*-coumaroylation level of *Arabidopsis* lignins, actually approaching those of lignins from mature maize stems, was the prerequisite for deciphering herein the tremendous impact of *p*-coumaroylated monolignols on lignification.

In this work, we report the very effective *p*-coumaroylation of *Arabidopsis* lignins, which was achieved not only with the introduction of the *Bradi2g36910* gene (*BdPMT1*) encoding the bona fide lignin-specific PMT enzyme but also with the *Bradi1g36980* gene (*BdPMT2*), another PMT-encoding gene with a still ill-defined role. Results from the introduction of the *BdPMT1* gene into the *fah1* background confirmed that PMT1 is able to efficiently drive the *p*-coumaroylation of G lignin units, which paves the way for the *p*-coumaroylation of gymnosperm lignins. In addition, the introduction of *BdPMT1* into the *ccr1g* background revealed that PMT1 is also able to catalyze in vivo the formation of monolignol-ferulate conjugates if feruloyl-CoA is readily available.

**Figure 2.** The *p*-coumaroylation of *Arabidopsis* lignins up to the grass lignin level doubles their solubility in alkali at room temperature. Values show percentages of *Arabidopsis* lignins solubilized in 1 M NaOH at 20°C (overnight) from extract-free mature stems of wild-type (WT) and *BdPMT1*- and *BdPMT2*-expressing lines under the control of the *AtC4H* promoter. Data were obtained from duplicate solubilization experiments when error bars are given.



When reaching the grass lignin level, the *p*-coumaroylation of *Arabidopsis* lignins was associated with severe structural alterations, namely a higher frequency of resistant interunit bonds and a higher frequency of terminal units with free phenolic groups. As could be anticipated from the higher frequency of G units with free phenolic groups, the solubilization of *Arabidopsis* lignins in alkali and at room temperature was about doubled for the transgenic lines provided with a high lignin *p*-coumaroylation degree. With these structural and solubility modifications, the *p*-coumaroylated *Arabidopsis* lignins are similar to grass lignins. Taken together, these results suggest genetically induced *p*-coumaroylation as a new and efficient strategy to make nongrass lignins as amenable to mild alkali pretreatment as grass lignins.

## MATERIALS AND METHODS

### Production of Plant Materials

The full-length complementary DNA sequences of *Brachypodium distachyon* *BdPMT1* and *BdPMT2* genes were amplified by reverse transcription-PCR from *Bd21-3* stems using the *BdPMT1* forward primer 5'-GGGGACAAGTTTGTA-CAAAAAGCAGGCTTCATGGAGAAGAAGTTCACGGTG-3' and reverse primer 5'-GGGGACCACCTTTGTACAAGAAAGCTGGGTCTCACTCCCGG-CGGTGAAGGCG-3' and the *BdPMT2* forward primer 5'-GGGGACAAG-TTTGTACAAAAAGCAGGCTTCATGGAGCGCGGGCGCGGTG-3' and reverse primer 5'-GGGGACCACCTTTGTACAAGAAAGCTGGGTCTCACTCG-AGCGCATCATGTCC-3', respectively.

The PCR products were ligated into the pDONR207 plasmid by BP Gateway (Invitrogen) reaction. The resulting pEntry clone was used to transfer the *BdPMT1* or *BdPMT2* gene into the pCC0996 vector, allowing expression under the control of the *AtC4H* promoter (Weng et al., 2008) by LR Gateway (Invitrogen) reaction. In addition, the *BdPMT1* gene was transferred into the pIPKb2 vector containing the maize (*Zea mays*) *ZmUbi* promoter (Himmelbach et al., 2007). These various constructs were introduced into the wild-type, *fah1*, or *ccr1g* background. Two lines for each construct and genetic background were selected and used for chemical analysis. Plants were grown together in a greenhouse for 12 weeks at 22°C in a 16-h-light/8-h-dark photoperiod and with 55% relative humidity.

The mature stems of control and transgenic plants were collected, ground to 0.5 mm, and subjected to exhaustive water and ethanol extraction in a Soxhlet apparatus. The extract-free samples were dried at 45°C for 2 d and then used for the analyses of CW-linked phenolics (lignins and CA or FA esters). In this study, the dried extract-free samples are referred to as CW samples.

## Lignin Determination

All measurements were performed on biological triplicates. The determination of KL content was carried out from approximately 300 mg (weighed to the nearest 0.1 mg) of extract-free sample according to a protocol adapted from Dence (1992).

The determination of ABL was performed according to a procedure adapted from Fukushima and Hatfield (2001) using 6 to 8 mg of extract-free samples (weighed to the nearest 0.1 mg) mixed with 1.5 mL of freshly prepared 25% (v/v) acetyl bromide in acetic acid in a 2-mL glass vial provided with a Teflon-lined screw cap. The mixture was then digested at 55°C and at 650 rpm in a mixing block for 2.5 h. After cooling, 0.2 mL of the solution was diluted with 3 mL of acetic acid:2 M NaOH mixture (50:9, v/v) and 0.5 mL of 0.5 M hydroxylamine chlorhydrate. The absorbance of the diluted solution was read at 280 nm against a reagent blank. ABL lignin content was calculated from this absorbance using the extinction coefficient  $20 \text{ g L}^{-1} \text{ cm}^{-1}$ .

## Analytical Mild Acidolysis

We looked for the putative occurrence of *p*-coumaroylated arabinose units in the CWs of wild-type, *BdPMT1/wt*, and *BdPMT2/wt* stems by analytical mild acidolysis according to a recently reported procedure (Petrik et al., 2014).

## Isolation of DL Fractions

About 2 g of extract-free sample was suspended in 50 mL of a dioxane:water mixture (9:1, v/v) containing 0.2 M HCl. The suspension was refluxed for 30 min under  $\text{N}_2$ . The cooled reaction mixture was filtrated over a Büchner funnel, and the residue was washed three times with 10 mL of a dioxane:water mixture (9:1, v/v). All the filtrates containing the dissolved lignins were pooled, and the pH of the resulting solution was adjusted to 3 to 4 (saturated  $\text{NaHCO}_3$  aqueous solution). The solution was concentrated to about 10 mL and by rotoevaporation at 45°C. The concentrated DL solution was then injected into about 100 mL of cold water under magnetic stirring. The lignin precipitate was recovered by centrifugation (30 min at 2,000g and 10°C), washed with pure water, centrifuged again, and freeze-dried to recover a purified DL fraction.

## Analysis of CA and FA Released by Mild Alkaline Hydrolysis of CW or DL Samples

About 5 to 10 mg of CW or DL samples (weighed to the nearest 0.1 mg) was put into a 2-mL Eppendorf plastic tube together with 1 mL of 1 M NaOH aqueous solution with 0.1 mL of *o*-coumaric acid internal standard methanolic solution. The internal standard amount was adapted to the analyzed samples (0.01 mg for control samples and 0.1 mg for transgenic samples). The mild alkaline hydrolysis was carried out overnight at room temperature on a carousel. After acidification with 0.2 mL of 6 M HCl, the tubes were centrifuged for 15 min at 2,000g and the supernatant was subjected to solid-phase extraction, then HPLC-diode-array detection analysis of the recovered methanolic extracts was performed as described previously (Ho-Yue-Kuang et al., 2016). To further confirm the occurrence of alkali-released CA or FA from control or transgenic samples, the methanolic extracts were diluted with water and the low-*M*<sub>w</sub> phenolics were reextracted with ethyl acetate before GC-MS analyses of their TMS derivatives.

## Lignin Analysis by Thioacidolysis

The simplified thioacidolysis was applied to extract-free CW or DL samples as described previously (Méchin et al., 2014). In addition, thioacidolysis assays were also performed after comprehensive permethylation of extract-free sample as follows. About 50 mg of extract-free CW sample was suspended in 1 mL of methanol and 1 mL of 2 M trimethylsilyldiazomethane solution in hexane (Acros Organics). The permethylation was then allowed to proceed for 24 h in a 4-mL glass vial provided with a Teflon-lined screw cap under agitation in a mixing block. After 24 h, the agitation was stopped, the vial was carefully opened (to release the nitrogen formed from the permethylation reagent), and the insoluble sample was allowed to decant before removal of the supernatant and the addition of fresh methylating mixture. This permethylation step was repeated twice with fresh reagent before washing of the permethylated sample with methanol and freeze-drying.

The desulfuration of the thioacidolysis mixture over Raney nickel was performed at 45°C in dioxane as described previously (Lapierre et al., 1995).

## Saccharification Assays

About 30 mg of extract-free CWs was put into a 5-mL plastic tube together with 4 mL of 5 mM acetate buffer (pH 4.7) containing 4 mg  $\text{mL}^{-1}$  commercial cellulase preparation (Cellulase Onozuka R10 from *Trichoderma viride*; Serva) and 0.1 mg  $\text{mL}^{-1}$  sodium azide. The reaction tubes together with a blank tube (containing only the enzyme solution) were placed at 45°C for 72 h on a carousel. After centrifugation (1,500g for 20 min), the supernatant was subjected to Glc determination using the K-Glc Megazyme Kit (<http://www.megazyme.com>) adapted to a 96-well format. In each well of the microplate, 200  $\mu\text{L}$  of the Glc reagent was put together with 10  $\mu\text{L}$  of supernatant. The microplate was then covered and the reaction was allowed to proceed for 20 min at 45°C before reading the  $A_{510}$  in a Spectramax microplate reader. The Glc amount released from the CW samples was calculated based on a Glc standard curve after subtraction of the Glc measured in the blank sample.

## Alkali Solubilization Assays

About 600 mg (weighed to the nearest 0.1 mg) of extract-free samples was put into a 50-mL plastic tube together with 20 mL of 1 M NaOH. The suspension was agitated on a carousel overnight at room temperature, then carefully filtrated over a Büchner porcelain funnel protected with a Whatman paper filter number 4. The residue was then carefully washed with water, then with 1 M HCl, and finally with water again, before freeze-drying. The final dry saponified residue was carefully weighed to calculate its recovery yield and then subjected to KL determination.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Phylogeny of the PMT-related subset of the BAHD superfamily in angiosperms.

**Supplemental Figure S2.** Representative images of Arabidopsis plants expressing the *BdPMT1* or *BdPMT2* gene under *AtC4H* promoter control in the wild-type, *fah1*, and *ccr1g* backgrounds.

**Supplemental Figure S3.** GC-MS separation of the main lignin-derived dimers obtained by thioacidolysis (1 h or 4 h long) and Raney nickel desulfuration of DL fractions purified from Arabidopsis mature stems of *BdPMT1/wt* and *BdPMT1/fah1* lines.

**Supplemental Figure S4.** Mass spectra (electronic impact, 70 eV) of the four lignin-derived compounds diagnostic for the incorporation of CA or FA into Arabidopsis lignins.

**Supplemental Figure S5.** GC-MS separation of the main lignin-derived dimers obtained by thioacidolysis (1 h or 4 h long) and Raney nickel desulfuration of a DL fraction purified from Arabidopsis mature stems of the *BdPMT1/ccr1g* line.

**Supplemental Figure S6.** Calculated weight percentage of CA linked to the lignins of Arabidopsis mature stems of *BdPMT1*- and *BdPMT2*-expressing lines.

**Supplemental Table S1.** Amount of CA released by mild alkaline hydrolysis of extract-free mature stems from Arabidopsis wild-type and transgenic lines expressing *BdPMT1* under *ZmUbi* promoter control.

**Supplemental Table S2.** Abbreviated mass spectra (electronic impact, 70 eV) of the main TMS derivatives of lignin-derived dimers recovered after thioacidolysis and Raney nickel desulfuration of Arabidopsis extract-free stems.

**Supplemental Table S3.** Analysis of extract-free mature stems from Arabidopsis wild-type and transgenic lines expressing the *BdPMT1* gene under the control of the *ZmUbi* promoter.

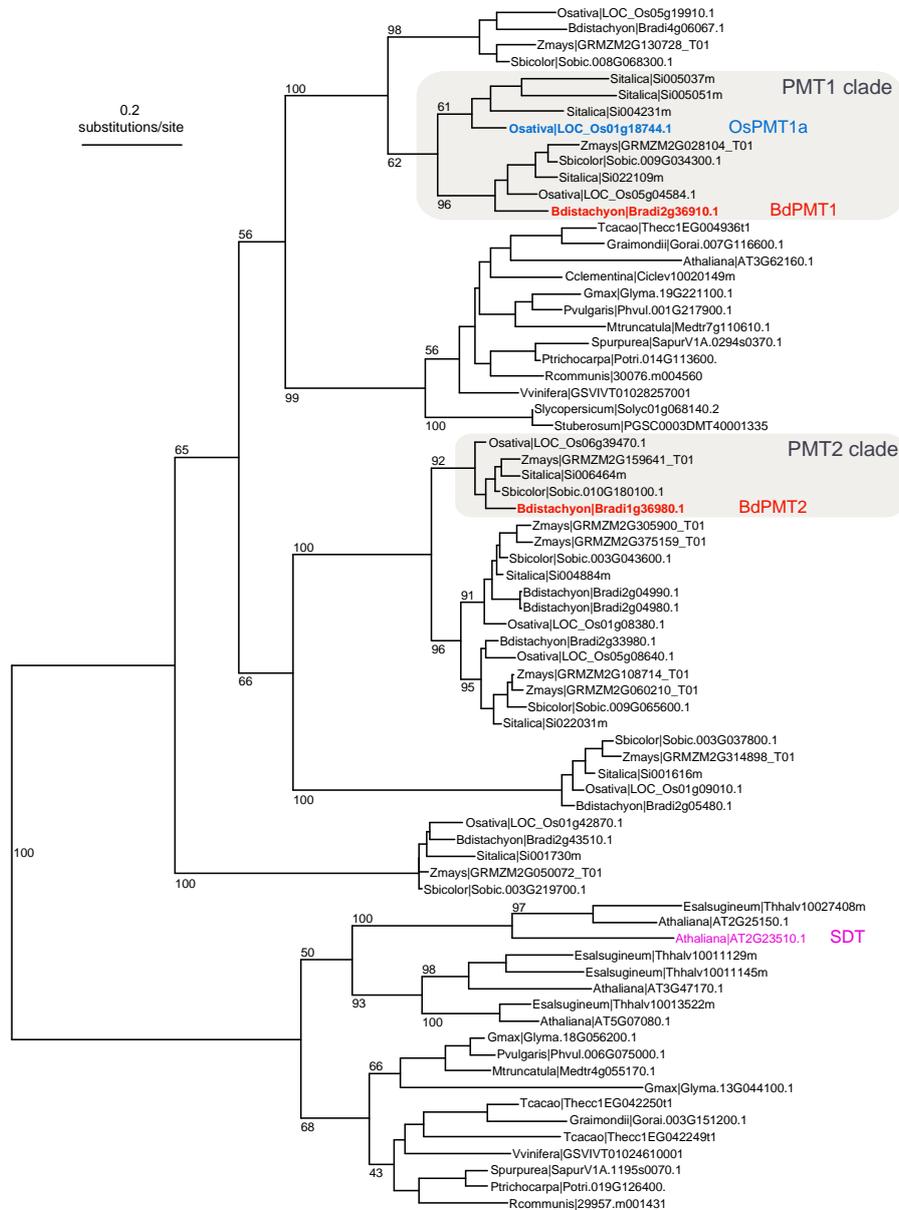
## ACKNOWLEDGMENTS

We thank Clint Chapple for kindly providing the vector pCC0996 allowing the expression of PMT under the control of the *AtC4H* promoter and Sébastien Antelme for growing *B. distachyon* 21-3 plants.

Received December 1, 2015; accepted January 28, 2016; published January 29, 2016.

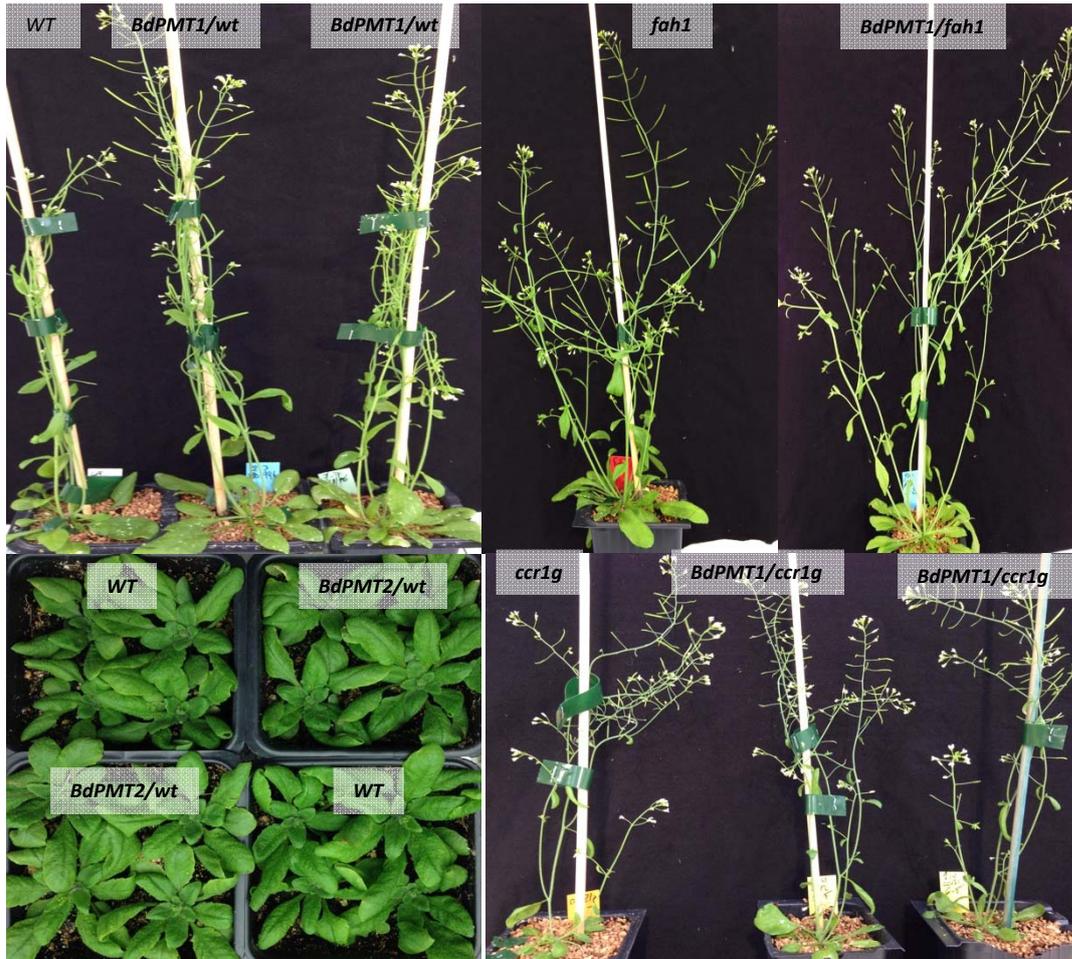
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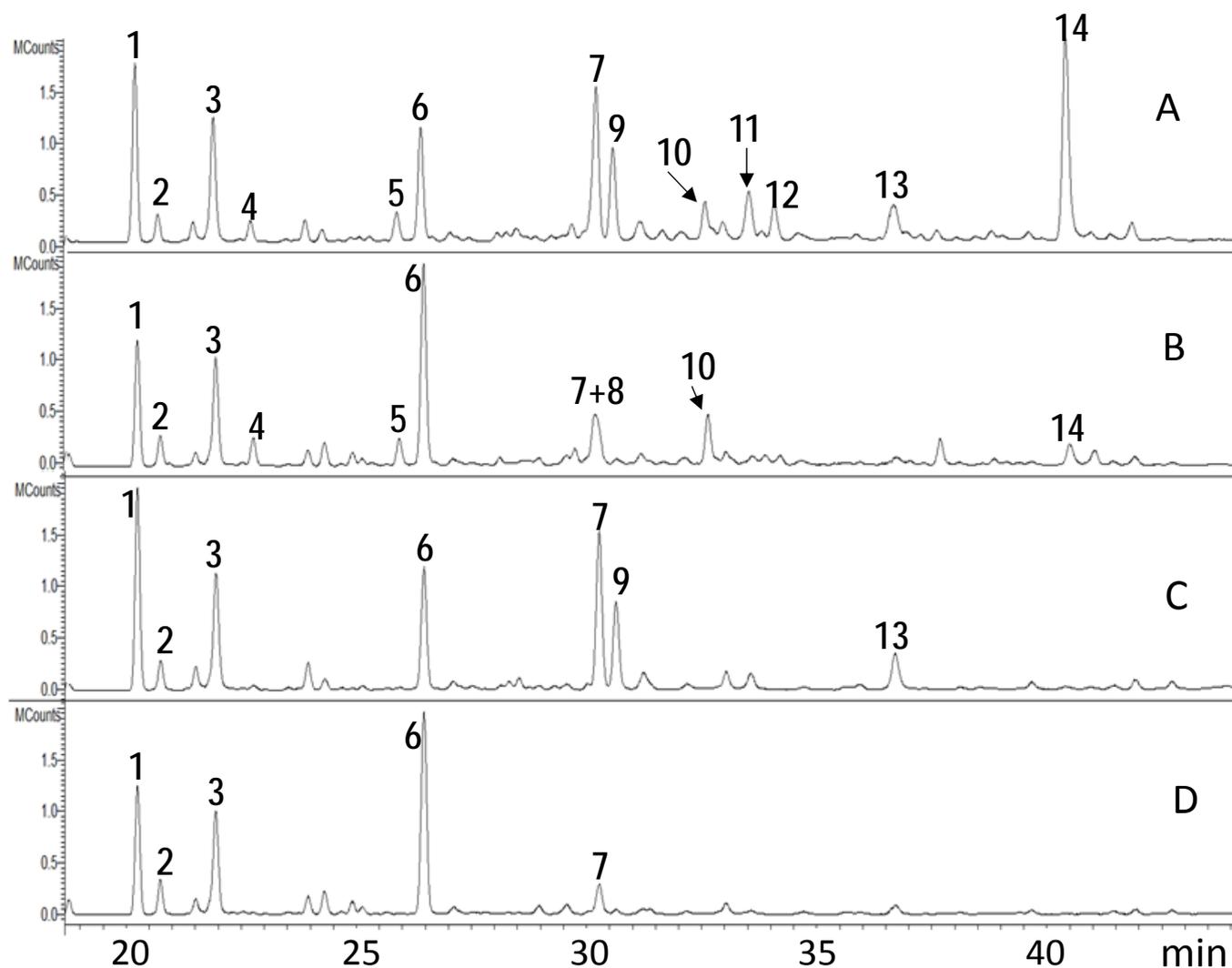


**Supp. Fig. 1.** Phylogeny of the PMT-related subset of the BAHD superfamily in Angiosperms.

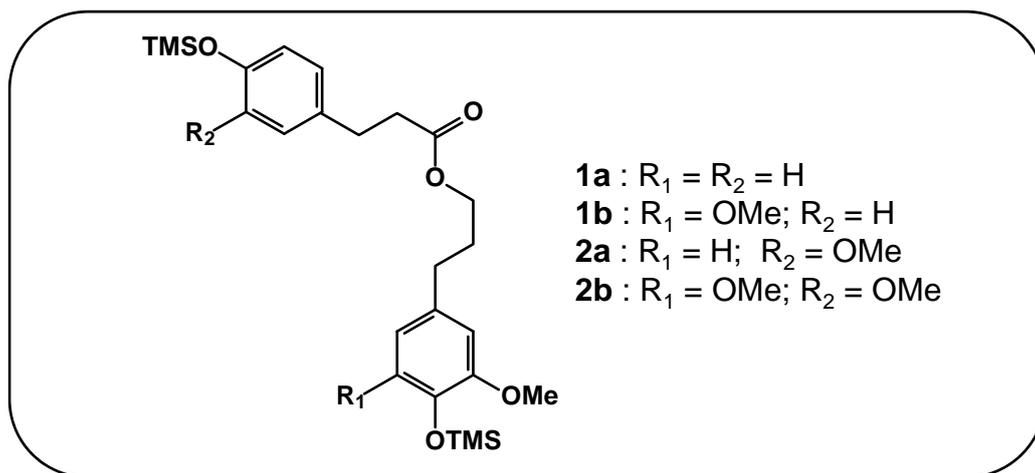
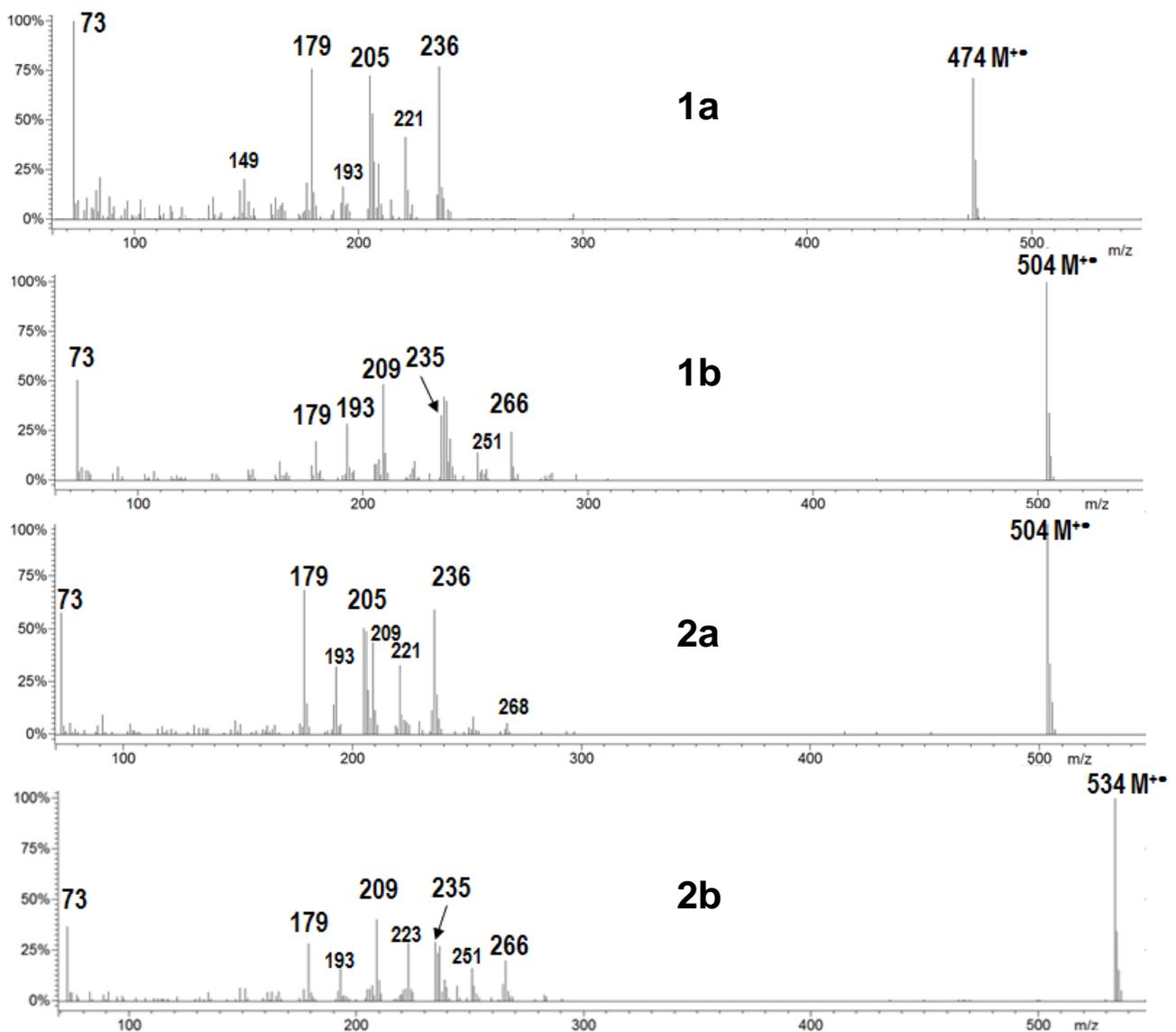
Protein maximum likelihood tree was built using 73 sequences derived from 18 species. Two distinct PMT clades containing proteins characterized either previously or in this study can be defined. Bootstraps values (%) are indicated on main branches. Proteins alignment, ambiguous sites masking and subsequent phylogeny reconstruction were performed with the MUSCLE v3.8, Gblocks and PhyML3.0 softwares, respectively.



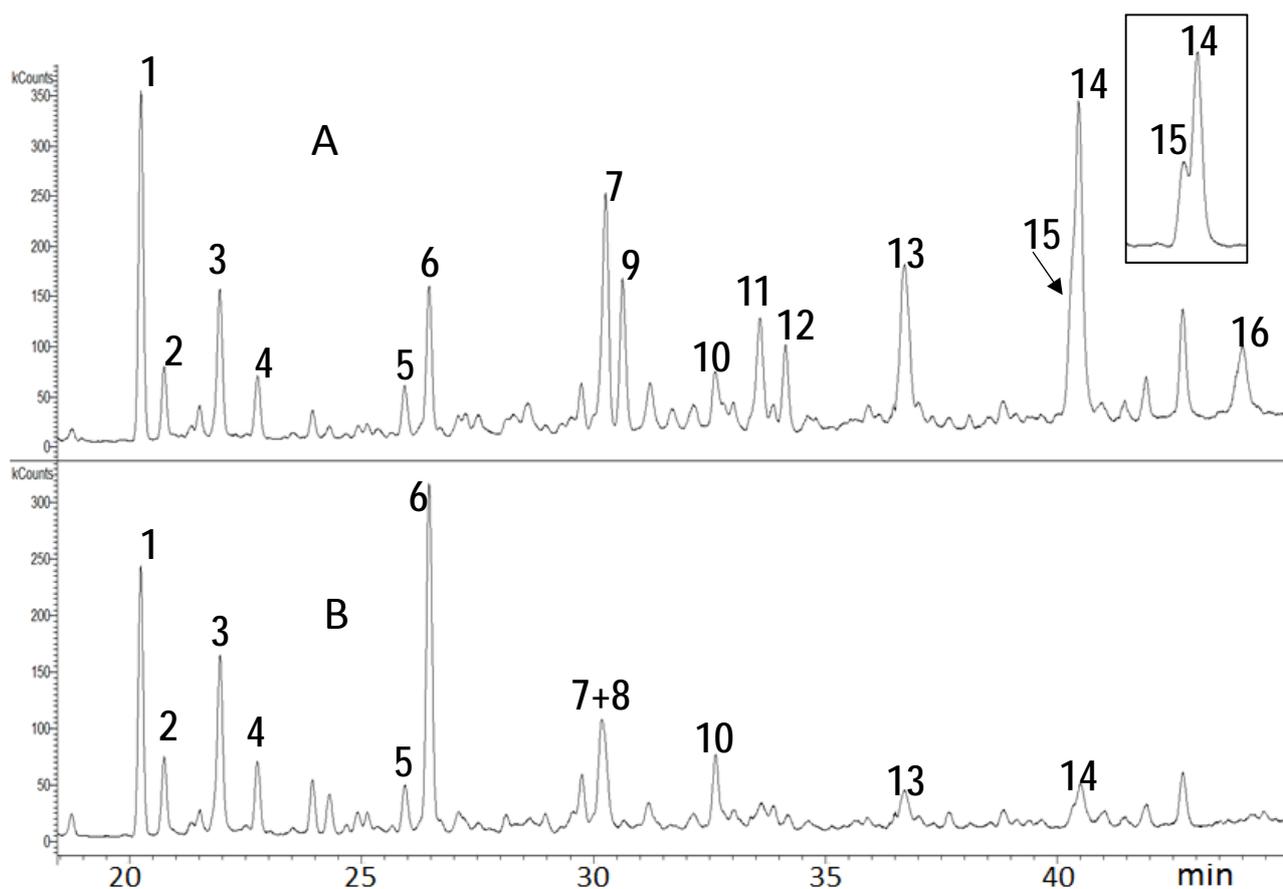
**Supp. Fig. 2.** Representative pictures of Arabidopsis plants expressing the *BdPMT1* or *BdPMT2* gene under the control of the *AtC4H* promoter and in the WT, *fah1* or *ccr1g* background. Relative to the corresponding control, there is no visible impact of the PMT expression.



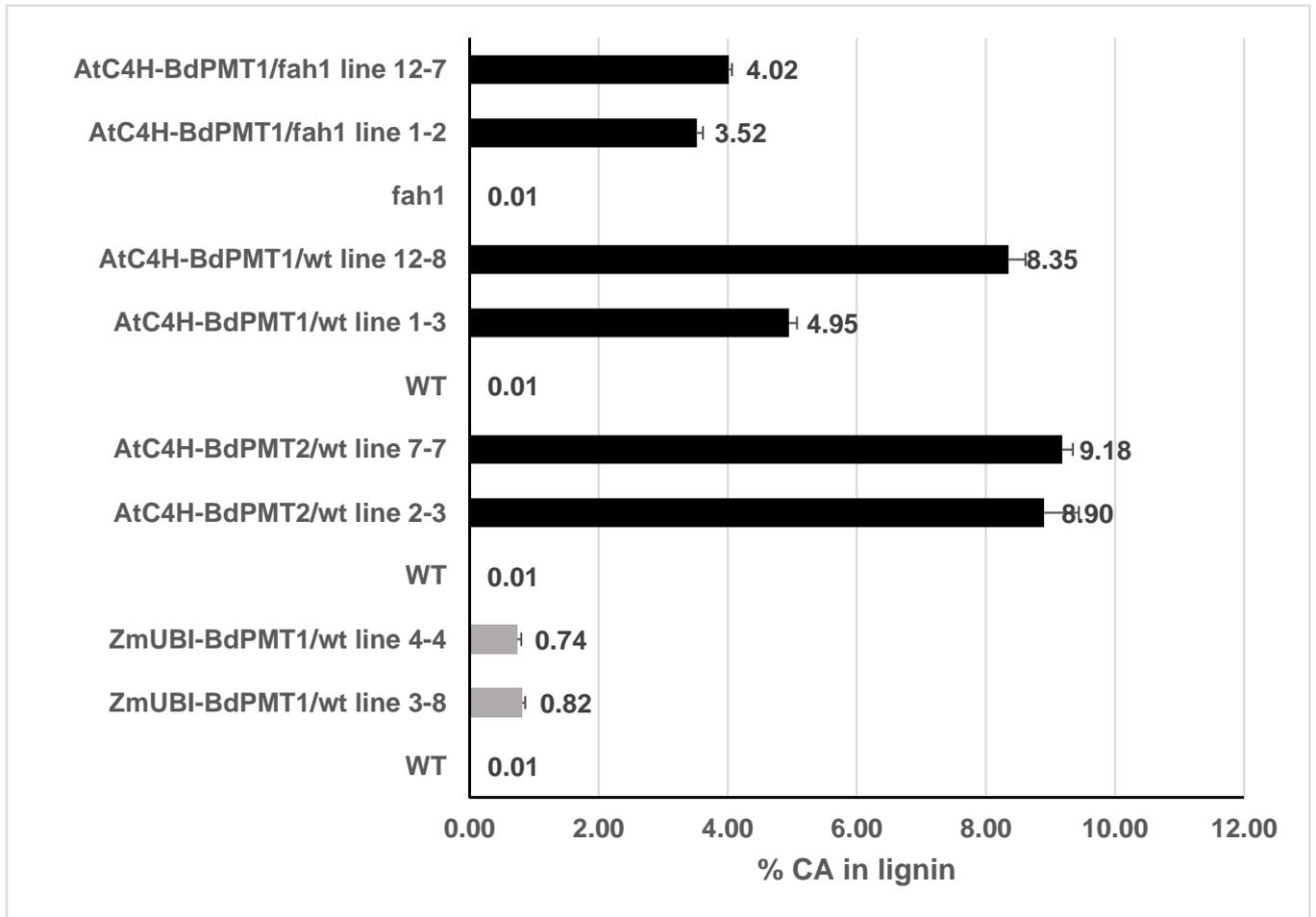
**Supp Fig. 3.** Partial GC-MS traces showing the separation of the main dimers (analyzed as their TMS derivatives) obtained by thioacidolysis and Raney nickel desulphuration of dioxane lignin fractions purified from *Arabidopsis* mature stems of *BdPMT1/wt* (A and B) and of *Bd910/fah1* (C and D) lines. Peak identification is given in Supplemental Table 2. Traces A and C are obtained from 1-hour long thioacidolysis. Traces B and D are obtained with the standard 4 hour-long thioacidolysis. Peaks 13 and 14, which respectively correspond to dihydroconiferyl and sinapyl alcohol acylated by *p*-dihydrocoumaric acid (compounds **1a** and **1b** Fig. 1), are reduced to trace components after 4-hour long thioacidolysis.



**Supp Fig. 4.** Mass spectra (electronic impact, 70 eV) of the 4 compounds diagnostic for the incorporation of *p*-coumaric acid (**1a** and **1b**) or ferulic acid (**2a** and **2b**) into *Arabidopsis* lignins. The compounds display very similar mass spectra with prominent molecular ions together with allylsyringol (at 266 m/z) or allylguaiacol (at 236 m/z) ions respectively diagnostic for S or G units. Compounds **2a** and **2b** are observed only in *BdPMT1/ccr1g* lines.



**Supp Fig. 5.** Partial GC-MS traces showing the separation of the main dimers (analyzed as their TMS derivatives) obtained by thioacidolysis and Raney nickel desulphuration of a dioxane lignin fraction purified from *Arabidopsis* mature stems of *BdPMT1/ccr1g* line with A] 1 hour-long thioacidolysis and B] 4 hour-long thioacidolysis. Peak identification is given in Supplemental Table 2. Peaks 13 and 14 correspond to dihydroconiferyl and sinapyl alcohol acylated by *p*-dihydrocoumaric acid, respectively. Peaks 15 and 16 correspond to dihydroconiferyl and sinapyl alcohol acylated by dihydroferulic acid, respectively. The inset in part A shows a better separation of peaks 14 and 15 obtained with different chromatographic conditions. These acylated compounds disappear almost entirely after 4-hour long thioacidolysis.



**Supp Fig. 6.** Weight percentage of *p*-coumaric acid CA ester-linked to Arabidopsis lignins in the mature stems of control lines (WT or *fah1*) or transgenic lines expressing the *BdPMT1* or *BdPMT2* gene under the control of the *AtC4H* promoter (black bars) or of the *ZmUbi* promoter (grey bars). These percentages are calculated values (% of Klason lignin) obtained with the assumption that all CA units are ester-linked to lignins.

**Supplemental Table S1.** Amount of *p*-coumaric acid (CA) released by mild alkaline hydrolysis of extractive-free mature stems from Col0-derived Arabidopsis WT and transgenic lines expressing *BdPMT1* under the *ZmUbi* promoter control.

Genotype	CA mg/g
WT	0.02 (0.004)
<i>ZmUbi-BdPMT1</i> line 3-2	0.77 (0.01)
<i>ZmUbi-BdPMT1</i> line 4-1	0.89 (0.02)
<i>ZmUbi-BdPMT1</i> line 1-6	1.05 (0.01)
<i>ZmUbi-BdPMT1</i> line 4-4	1.31 (0.12)
<i>ZmUbi-BdPMT1</i> line 1-12	1.42 (0.09)
<i>ZmUbi-BdPMT1</i> line 3-8	1.61 (0.09)

The data represent mean values (and SD) from 3 different plant pools (about 20 plants per pool) per genotype.

**Supplemental Table S2.** Abbreviated mass spectra (electronic impact, 70 eV) of the main compounds corresponding to peaks quoted in Supp. Fig. 3 and in Supp. Fig. 5. These lignin-derived dimers are analysed as TMS derivatives.

Peak number	Dimer type <sup>a</sup>	m/z (relative intensity)
1	GG Biphenyl 5-5'	474 (100), 459 (50), 445 (40), 415 (15), 385 (10), 357 (8), 343 (10), 147 (5), 73 (100)
2	GG Biphenyl ether 4-O-5'	402 (100), 387 (40), 373 (40), 372 (50), 357 (25), 343 (25), 221 (15), 209 (10), 179 (5), 157 (5), 73 (70)
3	GG Diarylpropane $\beta$ -1'	418 (10), 403 (3), 209 (100), 193 (5), 179 (25), 149 (5), 73 (25)
4	SG Biphenyl ether 4-O-5'	432 (100), 417 (40), 403 (25), 387 (8), 373 (8), 239 (5), 172 (8), 73 (50)
5	SG Diarylpropane $\beta$ -1'	448 (25), 433 (5), 239 (100), 223 (10), 209 (50), 193 (5), 179 (15), 149 (5), 73 (30)
6	GG $\beta$ -5' without CH <sub>2</sub> OH at C $\gamma$	460 (25), 445 (5), 251 (20), 236 (7), 221 (5), 209 (100), 207 (20), 193 (7), 179 (5), 73 (40)
7	GG $\beta$ -5' with CH <sub>2</sub> OH at C $\gamma$	562 (25), 472 (15), 457 (5), 352 (10), 322 (5), 263 (15), 209 (40), 191 (50), 179 (15), 103 (5), 73 (100)
8	SG $\beta$ -5' without CH <sub>2</sub> OH at C $\gamma$	490 (30), 251 (10), 239 (100), 236 (5), 209 (25), 207 (5), 73 (30)
9	GG $\beta$ -O-4' <sup>b</sup>	490 (1), 324 (5), 294 (4), 235 (15), 223 (5), 209 (100), 193 (5), 179 (20), 149 (8), 73 (40)
10	SS $\beta$ - $\beta'$ from syringaresinol	532 (70), 517 (5), 501 (15), 445 (10), 384 (8), 306 (60), 291 (8), 275 (15), 239 (8), 187 (20), 73 (100)
11	SG $\beta$ -5' with CH <sub>2</sub> OH at C $\gamma$	592 (30), 502 (5), 471 (5), 352 (5), 263 (10), 239 (50), 209 (10), 191 (60), 179 (5), 103 (5), 73 (100)
12	SG $\beta$ -O-4' <sup>b</sup>	520 (8), 354 (10), 324 (4), 265 (20), 239 (100), 223 (7), 209 (15), 179 (12), 73 (45)
13	Compound <b>1a</b>	474 (75), 236 (75), 221 (45), 209 (25), 207 (25), 206 (55), 205 (70), 193 (22), 179 (80), 149 (20), 73 (100)
14	Compound <b>1b</b>	504 (100), 266 (25), 251 (15), 239 (25), 237 (43), 236 (45), 235 (35), 223 (10), 209 (45), 193 (25), 179 (20), 73 (50)
15	Compound <b>2a</b>	504 (100), 268 (5), 253 (7), 236 (55), 221 (30), 209 (45), 206 (50), 205 (55), 193 (31), 179 (70), 73 (60)
16	Compound <b>2b</b>	534 (100), 266 (25), 251 (20), 239 (10), 237 (23), 236 (20), 235 (25), 223 (25), 209 (40), 193 (20), 179 (30), 73 (40)

<sup>a</sup> G : guaiacyl unit; S : syringyl unit; Compounds **1a** and **1b** are dihydroconiferyl and dihydrosinapyl alcohols acylated by *p*-dihydrocoumaric acid (Fig. 1); Compounds **2a** and **2b** are dihydroconiferyl and dihydrosinapyl alcohols acylated by dihydroferulic acid (Fig. 1).

<sup>b</sup> Peaks 9 and 12 diagnostic for residual  $\beta$ -O-4' dimers are observed with 1 hour-long thioacidolysis.

**Supplemental Table S3.** Analysis of Arabidopsis extract-free mature stems from Col0-derived WT or transgenic lines expressing the *Brachypodium BPMT1* gene under the control of the constitutive *ZmUbi* promoter : weight percentage of Klason lignin (KL) and total yield of (H+G+S) thioacidolysis monomers expressed on a KL basis.

Genotype	KL %	ABL %	(H+G+S) μmol/g KL
WT	19.00 (0.68)	14.65 (0.69)	1125 (18)
<i>BdPMT1/wt</i> line3-8	19.66 (0.31)	16.29 (0.26)	1025 (47)
<i>BdPMT1/wt</i> line4-4	19.16 (0.31)	15.03 (0.35)	994 (23)

The data represent mean values (and SD) from 3 different plant pools per genotype.