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1 **Running head**: Making Arabidopsis lignins alkali-soluble

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Title: Structural redesigning Arabidopsis lignins into alkali-soluble lignins through the expression of *p*-coumaroyl-CoA:monolignol transferase (PMT)

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Author Contributions

CL and RS performed the research and some experiments, analyzed the data and wrote the paper. HR contributed to the research and complemented the writing. PL performed the plant transformation and production. FL and LC provided technical assistance to CL.

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One sentence summary

Arabidopsis lignins that are genetically *p*-coumaroylated up to the grass lignin level display dramatic structural changes which make them more amenable to solubilization in alkali at room temperature.

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Abstract (250 words max)

Grass lignins can contain up to 10-15% by weight of *p*-coumaric esters. This acylation is performed on monolignols under the catalysis of *p*-coumaroyl-CoA monolignol transferase (PMT). To study the impact of *p*-coumaroylation on lignification, we first introduced the *Brachypodium Bradi2g36910* (*BdPMT1*) gene into Arabidopsis under the control of the constitutive *ZmUBI* promoter. The resulting *p*-coumaroylation was far lower than that of lignins from grass mature 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 *AtC4H* promoter boosted *p*-coumaroylation of mature stems up to the grass lignin level (up to 8-9% 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 CoA 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 to lignification in a grass similar manner.

Introduction

The cost-effective conversion of lignocellulosic feedstocks to 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 carried out to decipher the relationships 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 (Lapierre et al., 1989). Indeed, up to 50% of guaiacyl (G) grass lignin units are free phenolic, versus 25% or less in non-grass lignins (Lapierre, 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 γ-OH sidechain 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-CoA monolignol transferase (PMT) (Whiters et al., 2012). Thanks to this PMT activity, lignins of grass mature stems can contain up to 10-15% (by weight) of CA esters, as evidenced for purified maize lignin fractions (Chazal et al., 2014). The highest lignin *p*-coumaroylation levels have been reported for the grass species belonging to the C4 physiological type (Hatfield et al., 2009; Hatfield and Marita, 2010). It is now well established that CA esters 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 which extent the substantial participation

of CA conjugates to grass lignification can affect grass lignin structural traits other than the simple *p*-coumaroylation at Cγ. 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 to introduce CA esters into dicot lignins has been recently demonstrated by expressing the *Oryza sativa OsPMT* gene into two eudicots, Arabidopsis and poplar (Smith et al., 2015). The resulting trangenic PMT-Arabidopsis or PMT-poplar lines were found to display lignin *p*-coumaroylation, with CA levels ranging between 0.8 and 2% (by weight) of acetyl bromide lignin (Smith et al., 2015). In this study, we aimed to incorporate further more *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 on their solubility in alkali at room temperature. This investigation was conducted on Arabidopsis transgenic lines expressing either the *Brachypodium Bradi2g36910* (hereafter referred to as *BdPMT1*) or the *Brachypodium Bradi1g36980* (hereafter referred to as *BdPMT2*) gene into the WT Col0 genetic background and under the control of the Arabidopsis cinnamate-4-hydroxylase (*At C4H*) promoter that has been shown to efficiently target gene expression to vascular tissues (Bell-Lelong et al., 1997; Weng et al., 2008). In addition, the *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 (F5H) (Chapple et al., 1992) as well as in the *ccr1g* mutant deficient for cinnamoyl-CoA reductase 1 (CCR1) which accumulate unusual levels of soluble ferulate derivatives and incorporate ferulic acid into lignins (Mir Derikvand et al., 2008; Ralph et al., 2008). The impact of these transformations on the *p*-coumaroylation and structure of Arabidopsis lignins as well as on their solubility in alkali at room temperature is evaluated in the present work.

Results and discussion

The expression of either *BdPMT1* or *BdPMT2* gene into Arabidopsis under the control of the *AtC4H* promoter boosts lignin *p*-coumaroylation up to the grass lignin level

The *BdPMT1* gene was recently shown to encode for the monolignol-specific PMT1 enzyme, the activity of which results in the *p*-coumaroylation of *Brachypodium* lignins (Petrik et al., 2014). Lignin *p*-coumaroylation was severely decreased in the *Bdpmt1* 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* (referred to as *BdPMT2*) as the best candidate to fulfill this role because of its substantial transcript level in *Brachypodium* lignified culm (PlaNET, http://aranet.mpimp-golm.mpg.de/). The full-length cDNA of *BdMPT1* or *BdPMT2* was cloned and introduced into WT and mutant Arabidopsis lines under the control of the *AtC4H* promoter. It is noteworthy that, all along the production of transgenic lines (from T1 to T3 generation), the transgenic and the corresponding control plants displayed similar phenotype (Supplemental Fig. S2).

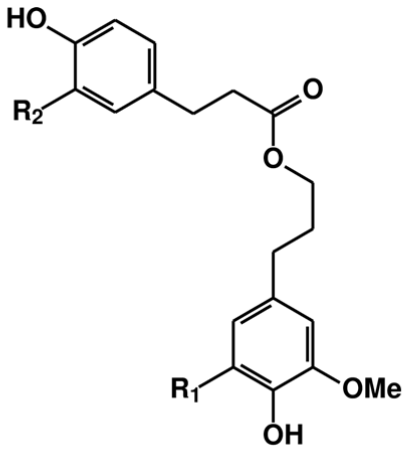
The *AtC4H* promoter-driven expression of the *BdPMT1* or *BdPMT2* gene in Arabidopsis induced an impressive increase of alkali-releasable CA from the cell wall (CW) of WT mature stems (Table 1). While minute, but measurable, amounts of CA were systematically released from WT samples, introducing one 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 CW, Table 1). 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, the CW *p*-coumaroylation was much lower, with values ranging between 0.8 and 1.6 mg/g 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 synthase 7 promoter (1 to 2 mg/g 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 lines 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 in refluxing lignified CWs in dioxane/water mixture (9/1, v/v) containing 0.2 M HCl (30 min under N_2) to recover a rough lignin extract, then purified to get a DL lignin fraction. When applied to grass CWs, this rapid and mild procedure provides purified DL lignins with a high recovery yield (50 to 70% of the whole lignin amount), a low sugar contamination (in the 3 to 10 weight % range) and, more importantly enough, in which most lignin-linked CA esters are preserved (Chazal et al., 2014). Purified DL fractions were obtained from the transgenic and WT samples. When expressed as percentages of the sample Klason lignin (KL) content (discussed later on), their recovery yield was higher for the transgenic lines (mean value ± SD = 52 ± 4 % for the 4 DL fractions isolated from *BdPMT1* and *BdPMT2* expressing lines) than for the control lines (mean value ± SD = 25 ± 5 for the 2 DL fractions isolated from WT samples), which suggests that CA introduction in lignins has made these polymers more amenable to mild acidolysis extraction. Relative to the trace amounts observed for WT CWs (less than 0.02 mg/g CW, Table 1), the corresponding DL fractions were found to be enriched in alkali-releasable CA (about 1 mg/g DL, Table 1), which confirms that WT Arabidopsis lignins are *p*-coumaroylated, albeit to a very low extent. By contrast, outstandingly higher CA levels were released by mild alkaline hydrolysis of DL fractions isolated from *BdPMT1/wt* and *BdPMT2/wt* stems (Table 1). These DL fractions contained 5- to 6-fold more alkali-releasable

157 CA than the corresponding CWs, which conclusively establishes that CA esters are associated
158 with lignins in the corresponding transgenic lines. The highest CA level reached up to 76
159 mg/g DL, a substantial value intermediate between values from C3 grass species (wheat and
160 *Brachypodium* DL in Table 1) and from the highly *p*-coumaroylated DL isolated from maize
161 stems (Table1 and Chazal et al., 2014). To evaluate whether some *p*-coumaric acid may have
162 also acylated arabinose substituents that occur in Arabidopsis hemicelluloses, we subjected
163 the *BdPMT1/wt* or *BdPMT2/wt* mature stems to analytical mild acidolysis, a method recently
164 developed to estimate the amount of *p*-coumaroylated or feruloylated arabinose units in grass
165 arabinoxylans (Petrik et al., 2014). Unlike to grass cell walls, no trace of *p*-coumaroylated
166 arabinose could be detected in transgenic Arabidopsis lines.

167 As a further confirmation that lignin units are *p*-coumaroylated in the *BdPMT1/wt* or
168 *BdPMT2/wt* lines, we subjected the purified DL fractions to 1 hour-long thioacidolysis
169 experiments. The thioacidolysis lignin-derived mixtures were then desulphurated over Raney
170 nickel, as previously described (Lapierre et al., 1995), and analyzed by gas chromatography-
171 mass spectrometry (GC-MS) of their trimethylsilylated (TMS) derivatives. By contrast to the
172 derivatization followed by reductive cleavage (DFRC) technique that leaves lignin γ-esters
173 intact (Lu and Ralph, 1999), most *p*-coumaric esters present in grass lignins do not survive the
174 standard 4 hour-long thioacidolysis (Lapierre, 2010). However, some *p*-coumaroylated lignin
175 units can give rise to diagnostic compounds previously identified among the
176 thioacidolysis/Raney nickel products from maize lignins (Grabber et al., 1996). With 1 hour-
177 long thioacidolysis followed by desulphuration of *BdPMT1/wt* DL lignins, the diagnostic
178 compounds **1a** and **1b** (Fig. 1) were easily observed on the GC-MS trace showing the
179 separation of lignin-derived products (Supplemental Fig. S3). By comparing the mass spectra
180 of their TMS derivatives (Supplemental Fig. S4 and Supplemental Table S2) to published
181 mass spectra (Grabber et al., 1996), compounds **1a** and **1b** were identified as dihydroconiferyl



1a : $R_1 = R_2 = H$
1b : $R_1 = OMe$; $R_2 = H$
2a : $R_1 = H$; $R_2 = OMe$
2b : $R_1 = OMe$; $R_2 = OMe$

Fig.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 hour) followed by Raney nickel desulphuration of Arabidopsis lignins isolated from *BdPMT1*-expressing lines. Compounds **2a** and **2b** are released only from the lignins of *BdPMT1/ccr1g* lines.

alcohol acylated by *p*-dihydrocoumaric acid and as dihydrosinapyl alcohol acylated by *p*-dihydrocoumaric acid, respectively. Accordingly, *p*-coumaroylated G and S units only involved in β-O-4 bonds were the lignin parent structures of compounds **1a** and **1b**. With the standard 4 hour-long thioacidolysis, these diagnostic compounds were minor or trace components relative to the main dimers that 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* mutants

When the *AtC4H*-driven *BdPMT1* expression was performed in the *fah1* mutant, which is impaired in the formation of sinapyl alcohol, a substantial CA incorporation was also obtained up to about 7 mg/g CW and for two independent homozygous lines (referred to as *BdPMT1/fah1* lines) whereas alkali-releasable FA remained as a trace component (Table 2). As previously done for the WT 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 2) and by the identification of the diagnostic compound **1a** obtained with 1 hour-long thioacidolysis (Supplemental Fig. S3). This result means that the *Bd*PMT1 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 WT ones, in agreement with the fact that sinapyl alcohol has been reported to be a better substrate of lignin-specific PMT than coniferyl alcohol (Whithers 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 *BdPMT1/wt* or *BdPMT1/fah1* samples (Tables 1 and 2). 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 have 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 to the WT level, the amount of alkali-releasable FA is slightly increased by CCR1 deficiency (0.053 ± 0.001mg/g CW for the *ccr1g* line (Table 2) versus 0.020 ± 0.06

mg/g CW for the two WT 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 WT 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 2) and by the identification of the four diagnostic compounds **1a**, **1b**, **2a** and **2b** (Fig. 1) among the desulphurated products released by 1 hour-long thioacidolysis (Supplemental Fig. S5). The dihydroferuloylated conjugates **2a** and **2b** displayed mass spectra respectively very similar to those of **1a** and **1b**, but with molecular ions at + 30 Da (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 ones provided that some alteration in the monolignol pathway induces a sufficient feruloyl-CoA building up. In a recent study, lignin feruloylation mediated by the introduction of a gene encoding for an *Angelica sinensis* feruloyl-CoA monolignol transferase AsFMT into poplar has been reported as a novel strategy to introduce chemically labile linkages in lignins (Wilkerson et al., 2014). From the present data, lignin feruloylation seems also to be possible by introducing the *BdPMT1* gene into the appropriate mutant or transgenic background.

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 Klason lignin (KL) content of Arabidopsis CWs (Table 3). 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 10-20% 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 recently reported (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 Klason lignin content of mature stems, the lignin *p*-coumaroylation was actually boosted up to 8-9% by weight for the transgenic lines obtained in WT background, provided that the expression of one or the other PMT-encoding gene was driven by the *AtC4H* promoter, whereas the lignin CA level was only increased to 0.7-0.8% by weight 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, the more pronounced reduction, the higher acylation. 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 similar lignin *p*-coumaroylation extent as in grass lignins.

The susceptibility of extract-free mature cell walls to saccharification by a commercial cellulase preparation and without any pretreatment was evaluated by measuring the released glucose. As expected, the saccharification of Arabidopsis samples displaying a lower KL content yielded more glucose (Table 3). However, the correlation between lignin content and glucose yield was low, which confirms that other factors than lignin content govern the cell wall 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 (by about 10%) induced a large saccharification improvement (glucose 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 exclusively involved in labile β-O-4 interunit bonds. As previously discussed, compounds **1a**, **1b**, **2a** and **2b** were obtained as minor or trace components with the standard 4 hour-long thioacidolysis (Supplemental Fig. S3 and Supplemental Fig. 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 Cγ 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.

As shown in Table 4, the molar frequencies of lignin-derived H, G or S monomers released from mature extractive-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 Klason lignin basis (in μmol/g KL), the total yield of thioacidolysis monomers was noticeably reduced 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 were achieved by the *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 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 WT, *fah1* and *ccr1g* lines), this percentage was weak for S units (less than 1.5% of β-O-4 linked S units were free phenolic, Table 5) and low for G units (14 to 16%, Table 5), 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, 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 5). The percentage of free phenolic groups in β-O-4 linked G units was increased from 14-16% in controls up to 20-55% in the transgenic lines. A higher frequency of terminal lignin units relative to internal ones may originate from a higher branching degree and/or from a lower average molecular weight 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 free phenolic group in lignins may have an outstanding consequence on the solubilization of lignins in alkaline medium and at room temperature, as exemplified by the case study of grass lignins (Lapierre, 2010). On this rationale and to evaluate to which extent the lignins of *BdPMT1/wt* or *BdPMT2/wt* lines mirrored the solubility properties of *p*-coumaroylated grass lignins, we performed simple solubilization tests. The extractive-free stems samples from WT and transgenic lines were

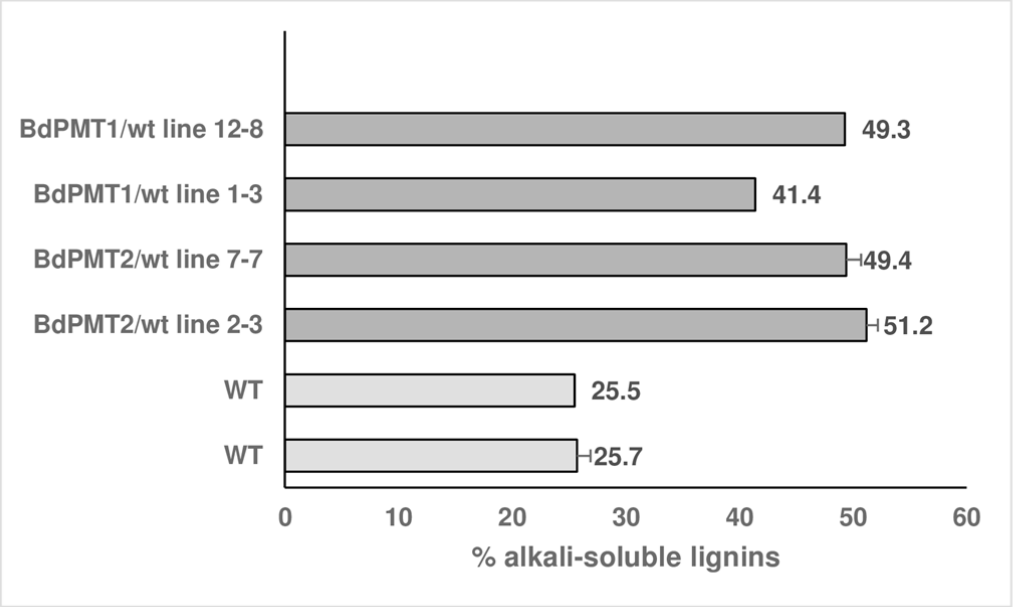


Fig.2. The *p*-coumaroylation of Arabidopsis lignins up to the grass lignin level doubles their solubility in alkali at room temperature. Percentage of Arabidopsis lignins solubilized in NaOH 1M at 20°C (overnight) from extractive-free mature stems of WT and of *BdPMT1*- or *BdPMT2*-expressing lines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

subjected to lignin solubilization assays in NaOH 1 M, at 20°C and overnight. The resulting saponified CWs were carefully recovered, washed and weighted before the determination of their Klason lignin content. The percentage of alkali-solubilized lignins could thus 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 WT lines to about 50% in the transgenic lines displaying lignins as *p*-coumaroylated as 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% by weight of ABL lignins, without any impact on lignin content (Smith et al., 2015). As compared to this recent study, and most likely thanks to the use of the *AtC4H* promoter to drive the *BdPMT* expression, we obtained 5- to 10- fold higher *p*-coumaroylation of lignins from Arabidopsis stems (up to 8-9% by weight of Klason lignin, Supplemental Fig. S6). Such a high *p*-coumaroylation level of Arabidopsis lignins, actually approaching those of lignins from maize mature stems, was the prerequisite for deciphering herein the tremendous impact of *p*-coumaroylated monolignols to 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 for the *bona fide* lignin-specific PMT enzyme, but also with that of *Bradi1g36980* gene (*BdPMT2)*, another PMT-encoding gene with 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, 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.

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 it could be anticipated from a 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, the present results suggest the genetically-induced *p*-coumaroylation as a new

and efficient strategy to make non-grass lignins as amenable to mild alkali pretreatment as grass lignins.

MATERIAL AND METHODS

Production of plant materials

The full-length cDNA sequence of *BdPMT1* or *BdPMT2* genes was amplified by RT-PCR from Bd21-3 stems using the *BdPMT1* forward primer 5'*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGAGAAGAAGTTCACGGTG3' and the *BdPMT1* reverse primer 5'*GGGGACCACTTTGTACAAGAAAGCTGGGTC*TCACTTCCCGGCGGTGAAGGCG3' or the *BdPMT2* forward primer 5'*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGCGGAGCGCGGGCGCGGTG3' and *BdPMT2* reverse primer 5'*GGGGACCACTTTGTACAAGAAAGCTGGGTC*TCAGTCGAGGCGCATCATGTCC3', 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 *the BdPMT2* gene into the pCC0996 vector allowing expression under the control of the *AtC4H* promoter (Weng et al., 2008) by LR Gateway® (Invitrogen). In addition, the *BdPMT1* gene was transferred into the pIPKb2 vector containing the *ZmUbi* promoter (Himmelbach et al., 2007). These various constructions were introduced into the WT, *fah-1* or *ccr1g* backgrounds. Two lines for each construct and per genetic background were selected and used for chemical analysis. Plants were grown together in greenhouse for 12 weeks at 22°C, in a 16-h-light/8-h-dark photoperiod and with 55% relative humidity.

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

378 **Lignin determination**

379 All the measurements were performed on biological triplicates. The determination of the
380 Klason lignin (KL) content was carried out from approximately 300 mg (weighed to the
381 nearest 0.1 mg) of extractive-free sample and according to a protocol adapted from Dence
382 (1992).

383 The acetyl bromide lignin (ABL) determination was performed according to a
384 procedure adapted from Fukushima and Hatfield (2001) and from 6 to 8 mg of extractive-free
385 samples (weighed to the nearest 0.1 mg) mixed with 1.5 mL of freshly prepared 25% acetyl
386 bromide in acetic acid (v/v) in a 2 mL glass vial provided with a Teflon-lined screw cap. The
387 mixture was then digested at 55°C and at 650 rpm in a mixing block for 2h30. After cooling,
388 0.2 mL of the solution were diluted with 3 mL of acetic acid/NaOH 2M mixture (50/9, v/v)
389 and 0.5 mL of 0.5 M hydroxylamine chlorhydrate. The absorbance of the diluted solution was
390 read at 280 nm against a reagent blank. The ABL lignin content was calculated from this
391 absorbance and using the extinction coefficient 20 g $L^{-1}cm^{-1}$.

392 **Analytical mild acidolysis**

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

396 **Isolation of dioxane lignin fractions**

About 2 g of extractive-free sample were suspended in 50 mL dioxane/water mixture (9/1, v/v) containing 0.2 M HCl. The suspension was refluxed for 30 min and under N_2. The cooled reaction mixture was filtrated over a Büchner funnel and the residues was washed with 3 x 10 mL 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 3-4 (saturated $NaHCO_3$ aq. 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 cold water and under magnetic stirring. The lignin precipitate was recovered by centrifugation (30 min at 2000 g, 10°C), washed with pure water, centrifuged again and freeze-dried to recover a purified DL lignin fraction.

Analysis of *p*-coumaric acid and ferulic acid 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) were put into a 2 mL Eppendorf plastic tube together with 1 mL NaOH 1 M aq. solution and with 0.1 mL of *o*-coumaric acid internal standard (IS) methanolic solution. The IS 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 and at room temperature, on a carousel. After acidification with 0.2 mL HCl 6M, the tubes were centrifuged for 15 min at 2000 g and the supernatant was subjected to solid phase extraction, then HPLC-DAD analysis of the recovered methanolic extracts as previously described in detail (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 molecular weight phenolics were re-extracted with AcOEt before GC-MS analyses of their TMS derivatives.

Lignin analysis by thioacidolysis

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

432 The desulphuration of thioacidolysis mixture over Raney nickel was performed at
433 45°C and in dioxane as previously described (Lapierre et al., 1995).

434 **Saccharification assays**

435 About 30 mg of extractive-free CWs were put into a 5 mL plastic tube together with 4 mL of
436 5mM acetate buffer (pH 4.7) containing 4 mg mL^{-1} of a commercial cellulase preparation
437 (Cellulase Onozuka R10 from *Trichoderma viride*, Serva) and 0.1 mg mL^{-1} sodium azide. The
438 reaction tubes together with a blank tube (containing only the enzyme solution) were placed at
439 45°C for 72 h and on a carousel. After centrifugation (1500 g, 20 min), the supernatant was
440 subjected to glucose determination using the K-Gluc Megazyme kit
441 (http://www.megazyme.com) adapted to a 96-well format. In each well of the microplate, 200
442 µL of the glucose reagent was put together with 10 µL of supernatant. The microplate was
443 then covered and the reaction was let to proceed 20 min at 45°C before reading the
444 absorbance at 510 nm in a Spectramax microplate reader. The glucose amount released from

445 the cell wall samples was calculated based on a glucose standard curve and after substraction
446 of the glucose measured in the blank sample.

447 **Alkali solubilization assays**

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

455 **Supplemental data**

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

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

462 **Supplemental Table S3.** Analysis of extract-free mature stems from Arabidopsis WT or
463 transgenic lines expressing the *BdPMT1* gene under the control of the *ZmUbi* promoter.

464 **Supplemental Figure S1.** Phylogeny of the PMT-related subset of the BAHD superfamily in
465 Angiosperms.

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

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

Supplemental Figure S4. Mass spectra (electronic impact, 70 eV) of the 4 lignin-derived compounds diagnostic for the incorporation of *p*-coumaric acid or ferulic acid into Arabidopsis lignins.

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

Supplemental Figure S6. Calculated weight percentage of *p*-coumaric acid linked to the lignins of Arabidopsis mature stems of *BdPMT1*- or *BdPMT2*- expressing lines.

482 **Acknowledgements**

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487

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Table 1. Amount of *p*-coumaric acid (CA) released by mild alkaline hydrolysis of the mature stems of Arabidopsis WT line, of *BdPMT1* or *BdPMT2*-expressing lines under the control of the *AtC4H* promoter and of the corresponding purified dioxane lignin fractions DL. Data from grass samples are given for comparison purpose.

Plant and genotype	CA from whole cell walls mg/g CW	CA from corresponding dioxane lignin (DL) fractions mg/g DL
Arabidopsis extractive-free mature stems		
WT Col0	0.016 (0.002)	0.93 (0.10)
BdPMT1/wt line1-3	7.84 (0.20)*	48.0 (4.1)
BdPMT1/wt line12-8	12.57 (0.39)*	66.3 (1.4)
WT Col0	0.010 (0.004)	1.34 (0.04)
BdPMT2/wt line2-3	13.36 (0.81)*	71.9 (4.3)
BdPMT2/wt line7-7	12.74 (0.23)*	76.6 (2.1)
Grass extractive-free mature stems		
Maize stem *(Z. mays,* F2 line, silage stage*)*	17.39 (0.66)	119.8 (2.1)
B. distachyon Bd21-3 mature stems	8.92 (0.63)	35.4 (1.0)
Wheat straw (*T. aestivum,* cv Champlein)	4.76 (0.10)	23.8 (1.4)

The data for Arabidopsis CW represent mean values (and SD) from 3 different plant pools (about 20 plants per pool) for each line. Asterisks indicate significant difference (one-way ANOVA) compared to the value of the corresponding control at $P < 0.001$. For grass CWs, data are mean values (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.

500 **Table 2.** Amount of *p*-coumaric acid (CA) and ferulic acid (FA) released by mild alkaline
501 hydrolysis of the mature stems of Arabidopsis *BdPMT1*-expressing lines in the *fah1* and the
502 *ccr1g* backgrounds and of the corresponding purified dioxane lignin fractions DL.

Genotype	Whole cell walls		Corresponding dioxane lignin (DL) fractions	
	CA mg/g CW	FA mg/g CW	CA mg/g DL	FA mg/g DL
fah1	0.021 (0.011)	0.020 (0.002)	-	-
BdPMT1/fah1 line1-2	6.48 (0.17)*	0.027 (0.10)	27.3 (1.4)	0.49 (0.05)
BdPMT1/fah1 line12-7	7.08 (0.09)*	0.021 (0.005)	29.6 (0.1)	0.23 (0.02)
ccr1g	0.025 (0.005)	0.053 (0.001)	-	-
BdPMT1/ccr1g line2-7	3.53 (0.35)*	1.58 (0.15)*	20.0 (2.3)	11.5 (1.3)
BdPMT1/ccr1g line 6-5	5.28 (0.18)*	2.49 (0.11)*	-	-

503 Data for Arabidopsis CW data represent mean values (and SD) from 3 different plant pools
504 (about 20 plants per pool) for each line. Asterisks indicate significant difference (one-way
505 ANOVA) compared to the value of the corresponding control at $P < 0.001$. DL isolation was
506 performed from the pool of biological triplicates for 3 transgenic lines only and each DL
507 fraction was then subjected to analytical replicates (n=2 or 3).
508
509

510 **Table 3.** Impact of expressing the *Brachypodium BdPMT1* or *BdPMT2* genes under the
511 control of the *AtC4H* promoter in Arabidopsis lines on the Klason lignin (KL) content or
512 Acetyl bromide lignin (ABL) content of extract-free mature stems as well as on their
513 saccharification efficiency SE (expressed as mg glucose released per gram of cell wall).

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

514

515 The data represent mean values (and SD) from 3 different plant pools (about 20 plants per
516 pool) per genotype. Asterisks indicate significant difference (one-way ANOVA) compared to
517 the value of the corresponding control at $P < 0.001$.

518

519 **Table 4.** Thioacidolysis of extract-free mature stems from Arabidopsis control lines and
520 transgenic lines expressing the *BdPMT1 or BdPMT2* gene under the control of the *AtC4H*
521 promoter.

Line	Thioacidolysis H, G and S monomers: Total yield (H+G+S) and relative molar frequency			
	Total yield µmol/g KL	%H	%G	%S
WT	1064 (34)	1.0 (0.1)	69.7 (0.2)	29.3 (0.1)
BdPMT1/wt line 1-3	891 (53)	1.0 (0.1)	68.5 (0.2)	30.5 (0.3)
BdPMT1/wt line 12-8	713 (7)*	1.3 (0.2)	68.7 (0.1)	30.0 (0.1)
fah1	962 (40)	0.9 (0.2)	99.1 (0.2)	-
BdPMT1/*fah1* line 1-2	675 (23)*	1.3 (0.1)	98.7 (0.1)	-
BdPMT1/*fah1* line 12-7	673 (5)*	1.1 (0.0)	98.9 (0.0)	-
ccr1g	808 (60)	0.9 (0.1)	63.1 (0.6)	36.1 (0.7)
BdPMT1/*ccr1g* line 2-7	602 (78)	0.7 (0.1)	65.9 (1.0)	33.4 (0.9)
BdPMT1/*ccr1g* line 6-5	523 (18)*	0.7 (0.1)	64.9 (0.2)	34.5 (0.2)
WT	853 (17)	0.9 (0.1)	69.7 (0.2)	29.4 (0.3)
BdPMT2/wt line 2-3	532 (15)*	1.3 (0.2)	70.8 (0.7)	27.9 (0.7)
BdPMT2/wt line 7-7	503 (42)*	1.3 (0.2)	71.5 (0.5)	27.3 (0.5)

522 The data represent mean values (and SD) from 3 different plant pools per genotype; asterisks
523 indicate significant difference (one-way ANOVA) compared to the value of the corresponding
524 control at $P < 0.001$.
525

526 **Table 5.** Relative percentages of free phenolic groups in G or S lignin units only involved in
527 β-O-4 bonds, as revealed by thioacidolysis of permethylated extract-free stems from
528 Arabidopsis control line and from *BdPMT1*- or *BdPMT2*- expressing lines under the control
529 of the *AtC4H* promoter.

Line	% free phenolic groups in G or S lignin units only involved in β-O-4 bonds	
	G	S
WT Col0	13.7 (0.1)	1.3 (0.1)
BdPMT1/wt line 1-3	21.8 (0.3)*	2.2 (0.1)*
BdPMT1/wt line 12-8	26.0 (0.0)*	2.7 (0.2)*
fah1	16.7 (0.1)	-
BdPMT1/*fah1* line 1-2	19.1 (0.1)*	-
BdPMT1/*fah1* line 12-7	20.2 (0.6)*	-
ccr1g	16.0 (0.1)	1.1 (0.1)
BdPMT1/*ccr1g* line 2-7	41.6 (3.9)*	4.3 (0.5)*
BdPMT1/*ccr1g* line 6-5	55.4 (0.3)*	6.0 (0.5)*
WT Col0	14.0 (0.1)	1.3 (0.1)
BdPMT2/wt line 7-7	26.3 (0.7)*	2.6 (0.2)*

530 Calculations are done as previously described (Lapierre, 2010). The data represent mean
531 values (and SD) from duplicate analyses of different plant pools per genotype; asterisks
532 indicate significant difference (one-way ANOVA) compared to the value of the corresponding
533 control at $P < 0.001$.
534

Figure Legends

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 hour) followed by Raney nickel desulphuration of Arabidopsis lignins isolated from *BdPMT1*-expressing lines. Compounds **2a** and **2b** are released only from the lignins of *BdPMT1/ccr1g* lines.

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

547

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