Journal of Analytical and Applied Pyrolysis 121 (2016) 155–164

Contents lists available at ScienceDirect

Journal of Analytical and Applied Pyrolysis

journal homepage: www.elsevier.com/locate/jaap

Rapid Py-GC/MS assessment of the structural alterations of lignins in genetically modified plants

Jorge Rencoret[a,*], José Carlos del Río[a], Klaas G.J. Nierop[b], Ana Gutiérrez[a], John Ralph[c]

[a] Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, 41012, Seville, Spain
[b] Department of Earth Sciences – Organic Geochemistry, Faculty of Geosciences, Utrecht University, 3584 CS, Utrecht, The Netherlands
[c] Departments of Biochemistry and Biological Systems Engineering, and the DOE Great Lakes Bioenergy Research Center, the Wisconsin Energy Institute, University of Wisconsin, Madison, WI 53726, USA

ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 11 July 2016
Accepted 16 July 2016
Available online 18 July 2016

Keywords:
Pyrolysis
Lignin
Whole cell-walls
Transgenic plants
Woody
Non-woody

ABSTRACT

Genetic modifications for perturbing the lignin pathway in three different species of angiosperm plants, including non-woody (Arabidopsis and alfalfa) and woody (poplar) plants, were readily evaluated by analytical pyrolysis coupled to gas chromatography-mass spectrometry (Py-GC/MS). Pyrolysis showed that the composition of Arabidopsis plants was severely altered when the expression of the gene encoding the enzyme caffeic acid *O*-methyltransferase (COMT) was downregulated, resulting in a lignin largely enriched in guaiacyl (G) units (88%). Alfalfa plants in which lignin biosynthesis was modified by down-regulation of the *p*-coumarate 3-hydroxylase (C3H) gene, showed extremely high proportions of *p*-hydroxyphenyl (H) units (71%) relative to the naturally prevailing guaiacyl (G) and syringyl (S) units. Finally, Py-GC/MS analyses indicated that overexpression in poplar of the gene that encodes the enzyme ferulate 5-hydroxylase (F5H) resulted in a lignin with a higher content of syringyl lignin units (88%) compared to the wild-type control (71%). In conclusion, Py-GC/MS is a useful and convenient tool for the rapid evaluation of compositional changes in lignin from genetically modified plants.

1. Introduction

Lignin is a complex aromatic biopolymer characteristic of vascular plants, where it provides mechanical support, waterproofs the cell-wall, and protects plants against microbial attack, limiting polysaccharide hydrolysis. Lignin acts as a biological cement between cellulose and hemicelluloses in the secondary cell wall and is the main cause of lignocellulosic biomass recalcitrance to efficient utilization of lignocellulosic substrates. Due to the negative processing effects of the presence of lignin in cell walls, there have been numerous efforts towards engineering lignin biosynthesis to produce plants with lower lignin content or with a lignin structure more amenable to chemical degradation [1]. In this context, a promising approach is to develop genetically modified plants by up- and down-regulating the expression of key genes involved in the biosynthesis of the lignin precursors (Fig. 1). Lignin derives mainly from the oxidative coupling of three *p*-hydroxycinnamyl alcohol monomers, *p*-coumaryl, coniferyl and sinapyl alcohols that produce respectively the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units when incorporated into the lignin polymer [2]. The lignin content as well as the monomer composition and inter-unit linkage distribution are widely variable between species and tissues. In general terms, gymnosperm lignins are composed of G- units with minor amounts of H-units, woody and non-woody angiosperm lignins are G-S lignins with variable S:G ratios, whereas grasses contain all the three units, although the levels of H-units have often been over-attributed [3]. Substantial compositional changes in angiosperm lignins can be obtained from misregulation of single genes in the lignin biosynthetic pathway, with the aim of altering the ratio of the three principal lignin monomers or monolignols [2,4,5]. The lignin of any plant can, in principle, be engineered and altered to produce more desirable lignin polymers thanks to the flexibility of the lignification mechanism that, unlike that of other biopolymers such as cellulose and hemicelluloses, does not follow any structural pattern [6]. In this sense, plants can use any and all of the synthesized monolignols, regardless of the proportion in which they are produced, as long as they are transported to the lignification zone. Among the most impressive genetically modified plants are transgenic hybrid poplar trees with elevated levels of the enzyme ferulate 5-hydroxylase (F5H) that produce lignins highly enriched in syringyl units, enhance biomass conversion [7,8] and are more resistant to

* Corresponding author.
E-mail address: jrencoret@irnase.csic.es (J. Rencoret).

http://dx.doi.org/10.1016/j.jaap.2016.07.016
0165-2370/

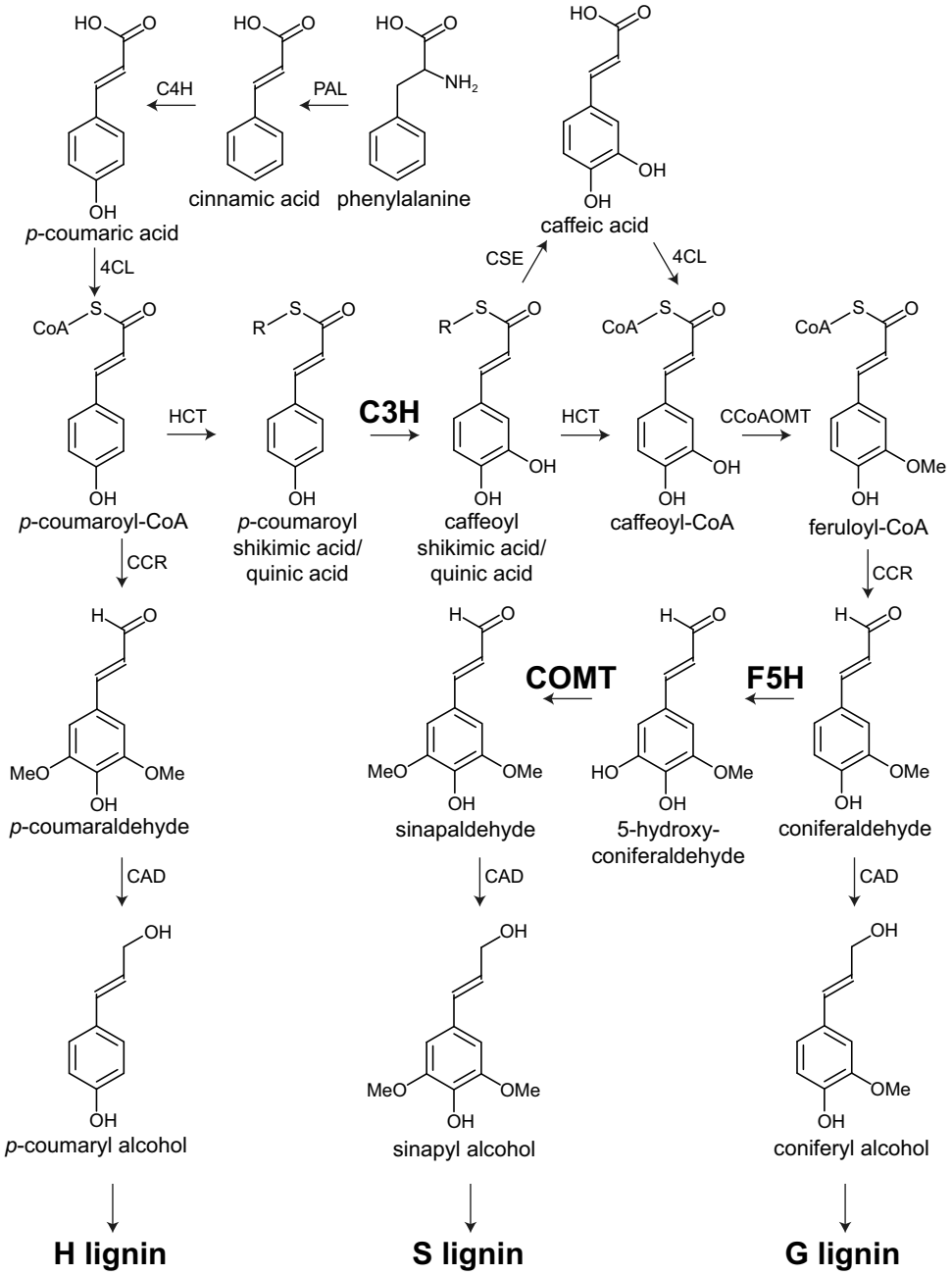

Fig. 1. The main monolignol biosynthetic pathway including the enzymes and metabolites involved (adapted from Vanholme et al.) [5]. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-Hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase. Only the steps that involve the enzymes C3H, COMT and F5H, involved in this study, are highlighted.

fungal attack [9]; alfalfa plants with improved digestibility derived from increasing the amounts of H lignin units by downregulation of the gene that encodes the enzyme *p*-coumarate 3-hydroxylase (C3H) [10]; and Arabidopsis plants that do not produce S lignin, but generate 5-hydroxy-guaiacyl (5OHG) units as well as the usual G units by downregulation of the gene encoding the enzyme caffeic acid *O*-methyltransferase (COMT).

In order to assess the changes produced in the lignin composition (H:G:S) as result of modifying the expression of genes involved in monolignol biosynthesis, it is important to have appropriate analytical tools that can be used for quick screening as well as for detailed structural analysis. Nowadays, there are several analytical techniques and methodologies that can provide valuable information about the composition of lignin, including spectroscopic methods such as FT-IR [11] or nuclear magnetic resonance (NMR) [12], chemical degradative methods such as permanganate and nitrobenzene oxidations [13,14], and thioacidolysis [15] or derivatization followed by reductive cleavage (DFRC) [16]. However, these methods often require tedious and time-consuming procedures and need significant amounts of plant sample, which sometimes is a major limitation in plant genetic engineering. In this context, analytical pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS) is a rapid and highly sensitive analytical tool that allows the analysis of very small amounts of sample without any prior manipulation and/or isolation. Pyrolysis offers some important advantages that include: (i) easy sam-

ple preparation; (ii) small sample amount requirement (less than 1 mg, typically ~100 μg); (iii) short analysis times (20–30 min); (iv) allows for direct *in situ* analysis of the lignin polymer, *i.e.*, without requiring prior lignin isolation, and (v) high sensitivity. Py-GC/MS has already proven to be a reliable analytical technique for the rapid characterization of lignins from many different wild-type plants, including both woody and non-woody varieties [17–23]. It is also an excellent tool to analyze the variations in the lignin composition of genetically modified plants [24–29]. In addition, Py-GC/MS analysis can be applied to young plants without requiring destructive sampling, as it only needs a minimal amount of sample. Given its moderate throughput and easy sampling method, it is a useful procedure to assess the alterations produced in the lignin polymer of a large number of genetically modified plants. In the present work, the value of Py-GC/MS analysis for revealing the key features of three genetically modified plants including *C3H*-downregulated alfalfa, an Arabidopsis *comt* mutant, and an *F5H*-upregulated poplar, is presented.

2. Material and methods

2.1. Plant materials

The samples used in the present work consisted of woody (poplar) and non-woody (Arabidopsis and alfalfa) angiosperms, including wild-type controls and their respective genetically modified lines used in previous studies.

2.1.1. Wild-type, comt-deficient and comt C4H:F5H1 chs Arabidopsis

The Arabidopsis lines, Columbia (Col-0) were from an earlier study [30]. The *comt* mutant (At5g54160, SALK_002373) was obtained from the SALK collection. To obtain the *comt C4H:F5H1 chs* mutant, a chalcone synthase (*chs*) mutation was crossed into a *comt C4H:F5H1* background. Plants were germinated directly in Saniflor soil (Van Israel; http://vanisrael.testvds.com) supplemented with 10% v/v vermiculite (16 h light, 22 °C, 55% humidity). Senesced inflorescence stems of Arabidopsis were ground in a Retsch MM400 mixer mill, equipped with a 10 mL ZrO_2 vessel and ZrO_2 ball-bearings (2 × 12 mm diameter), for 1 min at 30 Hz, for subsequent analysis.

2.1.2. Wild-type and C3H-deficient alfalfa

Wild-type and transgenic alfalfa (*Medicago sativa* cv. *Regen SY*) plants down-regulated in *C3H* transcripts, having ~5% residual C3H activity, were from material described elsewhere [10]. Stems (internodes 4–10) were harvested from the control (wt) and C3H-deficient (C3H) alfalfa lines, ground and Soxhlet-extracted sequentially with water, methanol, acetone, and chloroform. The isolated cell walls were ball-milled using a stainless steel jar containing a stainless steel ball bearing (5 mm) for 2.5 h, with 30 min breaks after every 30 min of milling to avoid excessive sample heating.

2.1.3. Wild-type and F5H-overexpressed poplar

F5H transgenic hybrid poplar (*Populus tremula* × *Populus alba*) was as previously described [8]. Both wild-type and the transgenic line were selected based on previous studies [7]. Stems were harvested from 2.5-year-old poplar wild-type and transgenic trees, manually debarked, and the pith was removed. Stems were ball-milled using a Retsch PM100 mill at 600 rpm with ZrO_2 jar (50 mL) and ball bearings (3 × 30 mm, 7 × 10 mm), for 3 h (10 min breaks after every 20 min of milling).

2.2. Pyrolysis-GC/MS

Pyrolysis of samples (~100 μg) was performed with a 2020 microfurnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC–MS system equipped with a DB-1701 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass-selective detector (EI at 70 eV, mass range *m/z* 50–550, 2.89 scans/s). The pyrolysis was performed at 500 °C during 1 min. The oven temperature was programmed from 50 °C (1 min) to 100 at 20 °C min^{-1} and then to 280 °C (5 min) at 6 °C min^{-1}. Helium was the carrier gas (1 mL min^{-1}). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in the literature [20,21] and, when possible, by comparison with retention time and mass spectra of authentic standards. Peak molar areas were calculated for the lignin degradation products, the summed areas were normalized, and the data for two repetitive analyses were averaged and expressed as percentages. We report the compositional data on an S + G + H = 100% basis. The relative standard deviation for the pyrolysis data was <5%.

Wild-type, COMT-deficient and *comt C4H:F5H1 chs* Arabidopsis were also analyzed on a Py-GC/MS instrument equipped with a Curie-Point pyrolyzer (Horizon Instruments) to confirm that 5-hydroxyguaiacyl units can be released from benzodioxane structures that are characteristic of these lignins. Samples were pyrolyzed for 5 s at 600 °C. The pyrolysis unit was connected to a Carlo Erba GC8060 gas chromatograph and the products were separated by a fused silica column (Varian, 25 m, 0.32 mm i.d.) coated with CP-Sil5 (film thickness 0.40 μm). Helium was used as carrier gas. The oven was initially kept at 40 °C for 1 min, then was heated at a rate of 7 °C min^{-1} to 320 °C and maintained at that temperature for 15 min. The column was coupled to a Fisons MD800 mass spectrometer (mass range *m/z* 45–650, ionization energy 70 eV, cycle time 0.7 s).

3. Results and discussion

The genetically modified plants selected for this study were *COMT*-downregulated Arabidopsis [30], *C3H*-downregulated alfalfa [10], and *F5H*-overexpressed poplar [8]. The alterations produced in the lignin polymers in these genetically modified plants were analyzed by Py-GC/MS, and the results were compared to those from the respective wild-type plants (Figs. 2–4). The identities and relative molar abundances of the compounds released upon pyrolysis from the plants analyzed are detailed in Table 1. Significant differences were observed among the pyrograms of genetically modified plants compared to their respective wild-type controls, revealing important structural modifications produced in the lignin polymer as a consequence of misregulating genes involved in the biosynthesis of monolignols.

3.1. Wild-type and COMT-deficient Arabidopsis

Caffeic acid *O*-methyltransferase (COMT) is an enzyme that methylates the 5-hydroxyl on the aromatic ring of monolignol precursors (Fig. 1) and is therefore crucial for the biosynthesis of sinapyl alcohol and ultimately of syringyl groups in lignin. Previous studies have shown that COMT-deficient plants have a higher cell-wall degradability under both acid pretreatment and enzymatic digestion [31,32] but are generally much worse for pulping [33]. The pyrograms of the wild-type and the *COMT*-downregulated mutant Arabidopsis stems are shown in Fig. 2 and the identities and relative molar abundances of the released compounds are listed in Table 1. Remarkable differences could be observed among them. Pyrolysis of the stem from wild-type Arabidopsis released phe-

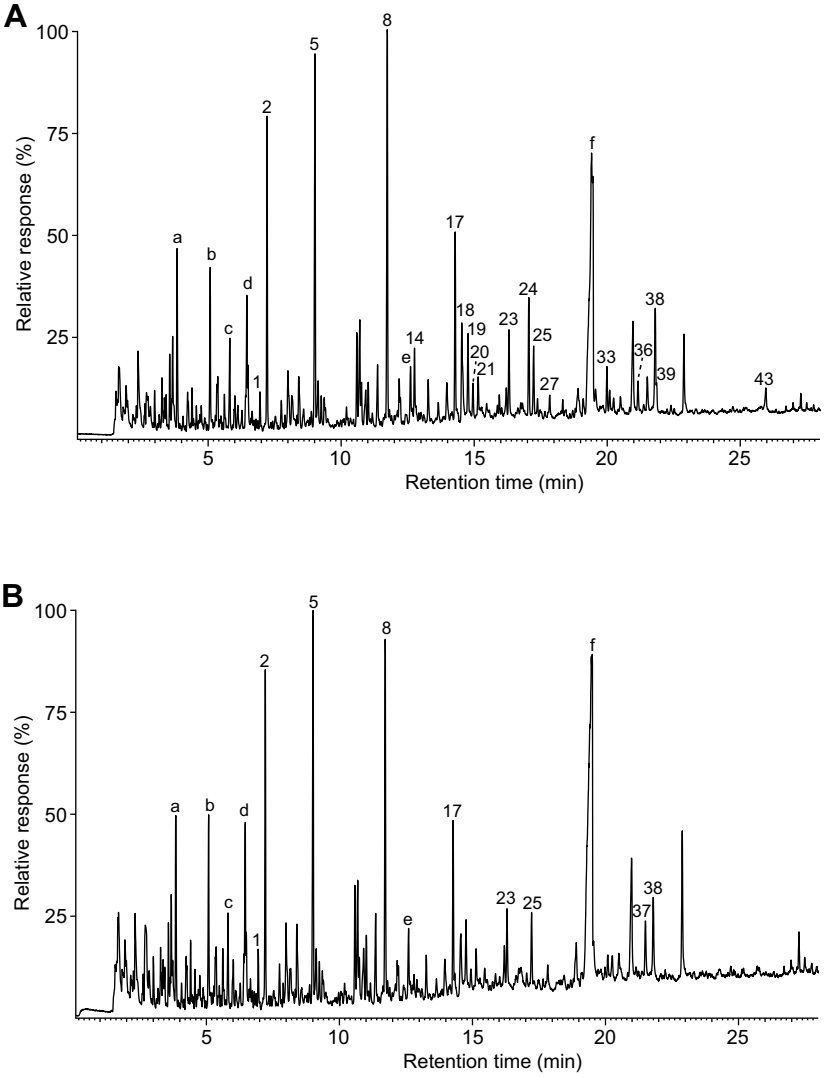

Fig. 2. Pyrograms (Total-Ion Chromatograms) from stems of Arabidopsis wild-type (A) and COMT-deficient mutant (B), obtained using a Frontier 2020 pyrolyzer. The identities and relative abundances of the compounds released by Py-GC/MS are listed in Table 1. Carbohydrate peaks: furfural (a), 2,3-dihydro-5-methylfuran-2-one (b), (5*H*)-furan-2-one (c), 2-hydroxy-3-methyl-2-cyclopenten-1-one (d), 5-hydroxymethyl-2-furfuraldehyde (e), and levoglucosan (f).

nolic compounds that are derived from H, G and S lignin units, together with other compounds derived from carbohydrates. The predominant lignin-derived aromatic (phenolic) compounds were guaiacol (2), 4-methylguaiacol (5), 4-vinylguaiacol (8), syringol (14), *trans*-isoeugenol (17), 4-methylsyringol (18), 4-vinylsyringol (24) and *trans*-coniferaldehyde (38). The pyrolysis revealed a predominance of G- over S-lignin units, with an S/G ratio of 0.35. On the other hand, pyrolysis of the COMT-deficient Arabidopsis mutant released mostly phenolic compounds derived from G lignin units, such as guaiacol (2), 4-methylguaiacol (5), 4-ethylguaiacol (7), 4-vinylguaiacol (8), *trans*-isoeugenol (17), *trans*-coniferyl alcohol (37) and *trans*-coniferaldehyde (38), with only traces of S-derived lignin units, resulting in an extremely low S/G ratio of 0.01. These results are produced because the enzyme COMT is directly involved in the biosynthesis of sinapyl alcohol, catalyzing the penultimate step that consists of the methylation of 5-hydroxyconiferaldehyde to sinapaldehyde before its reduction to sinapyl alcohol. Therefore, a strong decrease in COMT activity leads to plants with lignin depleted in S-units, as already observed by 2D NMR spectroscopy [30,34]. In addition, Py-GC/MS data analysis shows that the relative abundances of the compounds derived from lignin and carbohydrates remained almost unchanged (L/C ratios: 0.93 for wild type *vs* 0.91 for *comt* mutant), which indicates that *COMT* downregulation mainly altered the lignin composition without significantly altering the lignin content.

Another interesting aspect of *COMT* mutants is that they can incorporate 5-hydroxyconiferyl alcohol into the lignin polymer giving rise to benzodioxane substructures that are readily detectable by NMR [30,35], derivatization followed by reductive cleavage (DFRC) [36] and thioacidolysis [37]. The occurrence of lignins incorporating alternative monomers [30,38], usually derived from products of incomplete monolignol biosynthesis such as 5-hydroxyconiferyl alcohol, is another demonstration of the flexibility of the lignification process [39].

3.1.1. *Influence of the pyrolysis system on the release of 5-hydroxyguaiacyl units from benzodioxane structures upon Py-GC/MS*

The incorporation of 5-hydroxyconiferyl alcohol into the lignin polymer produces benzodioxane structures as a diagnostic feature of *COMT*-downregulated plants. These benzodioxane structures, under pyrolysis condition, should give rise to 5-hydroxyguaiacyl compounds as already reported when a Curie-Point pyrolyzer was employed [40,41]. Surprisingly, however, but as has been noted

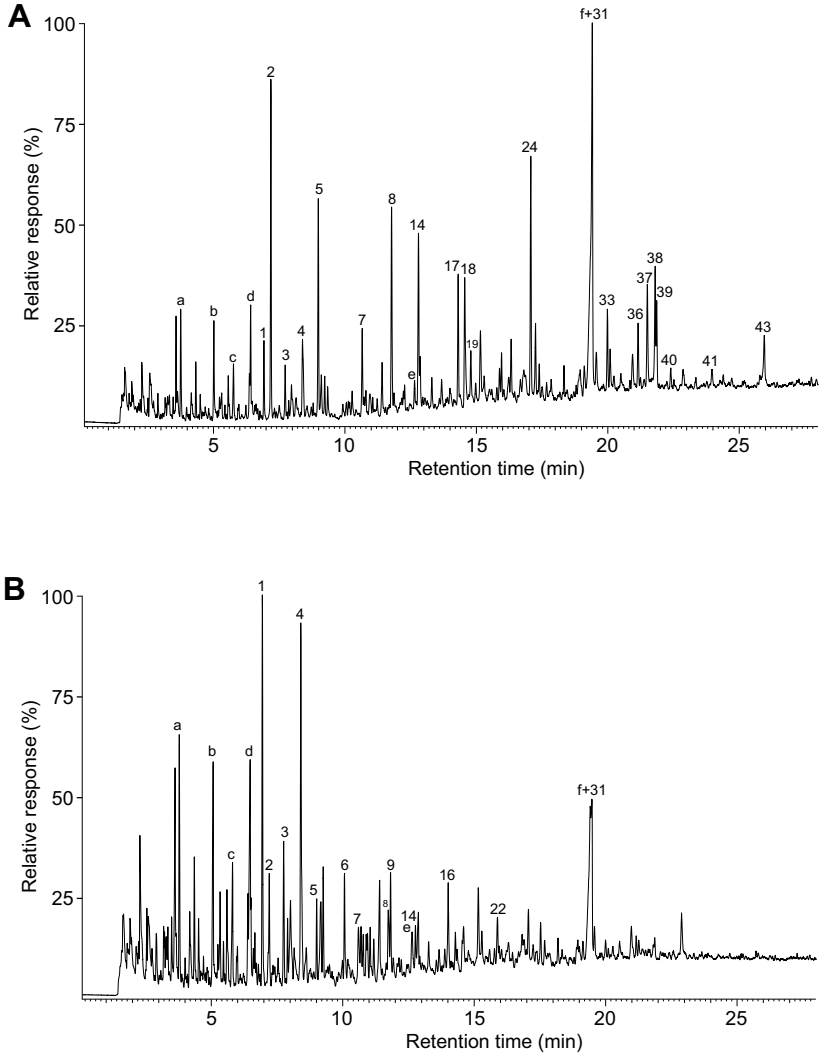

Fig. 3. Pyrograms (Total-Ion Chromatograms) of alfalfa stem material from wild-type (A) and a C3H-deficient transgenic (B), obtained using a Frontier 2020 pyrolyzer. The identities and relative abundances of the compounds released by Py-GC/MS are listed in Table 1. Carbohydrate peaks: furfural (a), 2,3-dihydro-5-methylfuran-2-one (b), (5*H*)-furan-2-one (c), 2-hydroxy-3-methyl-2-cyclopenten-1-one (d), 5-hydroxymethyl-2-furfuraldehyde (e), and levoglucosan (f).

in our labs and others previously, 5-hydroxyguaiacyl compounds were absent from our pyrograms that were obtained using a microfurnace pyrolyzer. Likewise, caffeyl alcohol-derived peaks were similarly difficult to find in pyrograms in OMT-deficient softwoods using another microfurnace pyrolyzer [29]. Numerous repetitions of the analyses under different pyrolysis conditions (using a wide range of pyrolysis temperatures from 400 to 600 °C and pyrolysis times from 15 to 120 s) also failed to detect these compounds. However, it is important to note here that 5-hydroxyguaiacol can be readily detected using the Frontier microfurnace pyrolysis system used in this work, and that an authentic standard of 5-hydroxyguaiacol has been analyzed without any problem in this pyrolysis system. Hence, the absence of 5-hydroxyguaiacyl units upon pyrolysis of COMT-deficient Arabidopsis seems to indicate that the microfurnace pyrolyzer is not able to release such monomers from benzodioxane structures. Therefore, and in order to assess whether the pyrolysis system used is essential for the release of 5-hydroxyguaiacyl compounds from benzodioxane structures, this Arabidopsis *comt* mutant, together with another Arabidopsis mutant sample (*comt C4H:F5H1 chs*), generated by concomitant downregulation of *comt* and upregulation of *F5H* using a *C4H*-promoter and characterized by an extremely high content of 5-hydroxyconiferyl alcohol involved in benzodioxane structures [30], was analyzed using a different Py-GC/MS system equipped with a Curie-Point pyrolyzer. Interestingly, and contrary to what occurred when using a microfurnace pyrolyzer, pyrolysis employing a Curie-point unit released significant amounts of 5-hydroxyguaiacol and related compounds, including the vinyl- and propenyl- counterparts, especially from the Arabidopsis *comt C4H:F5H1 chs* mutant. Fig. 5 shows a comparison of the selected-ion chromatograms of the main monomeric compounds (guaiacol, *m*/*z* 124; 5-hydroxyguaiacol, *m*/*z* 140 and syringol, *m*/*z* 154) obtained upon pyrolysis of Arabidopsis *comt C4H:F5H1 chs* mutant using microfurnace and Curie-Point pyrolysis systems. Hence, significant amounts of 5-hydroxyguaiacol, arising from the cleavage of benzodioxane substructures, could only be released when a Curie-point system was used. This unequivocally reveals that the pyrolysis system employed is critical to release these compounds. Although H:G:S values 22:23:55 (Curie point system) and 21:21:58 (microfurnace pyrolyzer) can be derived as usual, the fact that potentially significant and diagnostic new components from these lignins, 5-hydroxyguaiacyl (5OHG) units, do not produce diagnostic monomers is an important limitation that should be taken into consideration when a microfurnace pyrolyzer is used.

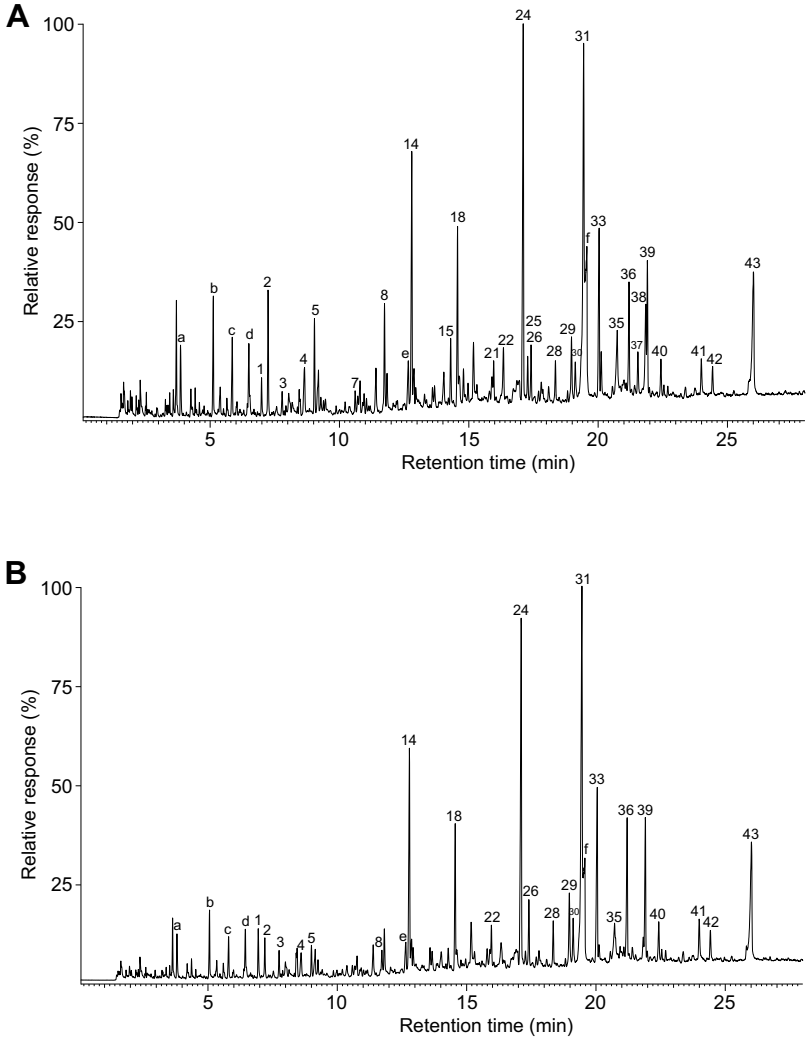

Fig. 4. Pyrograms (Total-Ion Chromatograms) of hybrid poplar (*Populus tremula* x *Populus alba*) wild-type (A) and the *F5H*-upregulated transgenic (B), obtained using a Frontier 2020 pyrolyzer. The identities and relative abundances of the compounds released by Py-GC/MS are listed in Table 1. Carbohydrate peaks: furfural (a), 2,3-dihydro-5-methylfuran-2-one (b), (5*H*)-furan-2-one (c), 2-hydroxy-3-methyl-2-cyclopenten-1-one (d), 5-hydroxymethyl-2-furfuraldehyde (e), and levoglucosan (f).

3.2. Wild-type and C3H-deficient alfalfa

The design of plants deficient in the enzyme C3H has its origin in the search for plants with lower lignin content and higher degradability compared to wild-type plants. C3H is an enzyme that acts early in the biosynthesis of lignin monomers, catalyzing the incorporation of the hydroxyl group at the 3-position of *p*-coumarate to produce caffeate, which is a key step in the biosynthesis of coniferyl alcohol and sinapyl alcohol (Fig. 1). The pyrograms of the wild-type and *C3H*-downregulated alfalfa revealed striking differences between the S/G lignin in normal wild-type alfalfa vs the H-rich lignin in the *C3H*-downregulated plant (Fig. 3, Table 1). Pyrolysis of the wild-type alfalfa released phenolic compounds derived from H-, G- and S-lignin units, with the most abundant pyrolysis products being guaiacol (2), 4-methylguaiacol (5), 4-vinylguaiacol (8), syringol (14), *trans*-isoeugenol (17), 4-methylsyringol (18), 4-vinylsyringol (24) and *trans*-4-propenylsyringol (31), with an H:G:S ratio of 8:54:38. Pyrolysis of the stems from C3H-deficient alfalfa released a completely different set of phenolic compounds that were mostly derived from H-lignin units, including 4-methylphenol (3), 3-methylphenol (4), 4-ethylphenol (6), 4-vinylphenol (9) and *trans*-4-propenylphenol (16), with a striking H:G:S ratio of 71:18:11. As mentioned above, C3H is an essential enzyme for the eventual formation of coniferyl and sinapyl alcohols, and thereby *C3H* downregulation blocks the biosynthesis of both lignin precursors (Fig. 1), keeping the S/G ratio almost constant but producing extraordinarily high levels of H-units. A decrease in the relative abundance of lignin-derived compounds compared to those derived from carbohydrates (peaks a–f) was also observed in the pyrograms, as indicated by the decrease of the lignin/carbohydrate ratio from 2.4 in the wild-type alfalfa to 0.8 in the *C3H* transgenic. These data are in agreement with previous studies, based on DFRC and 2D-NMR analysis, that reported an increase in H-lignin units in the *C3H* transgenic and Klason lignin contents of 9.7% and 6.8% for the wild-type and the *C3H* line, respectively [42].

3.3. Wild-type and F5H-overexpressed poplar

Another ingenious strategy to produce lignins more amenable to chemical degradation consists of reducing the level of condensed linkages and branch-points within the polymer. This can be achieved by increasing the proportion of S-lignin units, in which the aromatic 3- and 5-positions are methoxylated. S-lignin units result from the incorporation of sinapyl alcohol into the lignin polymer during the lignification process. Therefore, an

Table 1
Identities and relative molar abundances of the lignin-derived compounds identified in the pyrograms, obtained using a microfurnace pyrolyzer, of the samples analyzed in the present work.

No.	Compound	Origin[a]	Arabidopsis WT	Arabidopsis COMT	Alfalfa WT	Alfalfa C3H	Poplar WT	Poplar F5H
1	phenol	LH/C	2.7	3.7	3.5	25.0	1.8	3.2
2	guaiacol	LG	10.7	15.8	11.3	4.8	4.8	1.8
3	4-methylphenol	LH	1.8	2.9	2.2	8.9	1.3	1.6
4	3-methylphenol	LH	4.8	6.8	4.6	25.8	0.7	1.7
5	4-methylguaiacol	LG	13.1	18.3	7.0	3.4	3.4	1.3
6	4-ethylphenol	LH	0.6	0.7	0.5	5.5	0.0	0.0
7	4-ethylguaiacol	LG	3.6	5.0	2.9	1.7	0.7	0.5
8	4-vinylguaiacol	LG	10.1	15.4	7.0	2.0	3.8	1.4
9	4-vinylphenol	LH	0.0	0.0	0.5	5.6	0.0	0.0
10	4-allylphenol	LH	0.0	0.0	0.0	0.6	0.0	0.0
11	eugenol	LG	0.8	1.4	0.5	0.0	0.2	0.1
12	4-propylguaiacol	LG	0.9	1.3	0.5	0.2	0.2	0.1
13	*cis*-4-propenylphenol	LH	0.0	0.0	0.0	0.7	0.0	0.0
14	syringol	LS	3.4	0.2	5.7	1.6	9.1	10.1
15	*cis*-isoeugenol	LG	1.4	2.0	1.1	0.2	0.3	0.1
16	*trans*-4-propenylphenol	LH	0.0	0.0	0.0	4.1	0.0	0.0
17	*trans*-isoeugenol	LG	5.3	7.3	4.2	1.0	2.1	1.0
18	4-methylsyringol	LS	4.5	0.3	5.4	0.7	5.4	5.5
19	vanillin	LG	3.2	3.0	1.8	0.0	1.1	0.3
20	propyne-G	LG	0.8	0.9	0.8	0.0	0.6	0.3
21	propyne-G	LG	1.5	1.8	0.7	0.0	0.5	0.2
22	4-ethylsyringol	LS	0.5	0.0	1.2	0.5	1.2	1.4
23	acetovanillone	LG	3.7	3.4	1.8	0.0	2.0	0.5
24	4-vinylsyringol	LS	4.0	0.5	6.8	2.0	12.7	14.5
25	guaiacylacetone	LG	3.0	2.9	2.2	0.0	1.3	0.4
26	4-allyl-syringol	LS	0.4	0.0	0.7	0.2	1.4	2.1
27	propiovanillone	LG	0.7	0.5	0.3	0.0	0.1	0.1
28	*cis*-4-propenylsyringol	LS	0.5	0.0	0.7	0.2	1.2	1.5
29	propyne-S	LS	0.0	0.0	1.0	0.0	2.3	2.6
30	propyne-S	LS	0.0	0.0	0.7	0.0	1.3	1.6
31	*trans*-4-propenylsyringol	LS	3.4	0.0	5.0	1.7	10.1	14.2
32	dihydroconiferyl alcohol	LG	0.0	0.0	1.5	0.0	0.0	0.0
33	syringaldehyde	LS	2.4	0.0	2.3	0.0	5.7	7.4
34	*cis*-coniferyl alcohol	LG	0.0	0.0	1.1	0.0	0.0	0.0
35	4-hydroxybenzoic acid	PB	0.0	0.0	0.0	0.0	4.2	2.9
36	acetosyringone	LS	1.7	0.0	1.8	0.4	3.3	5.2
37	*trans*-coniferyl alcohol	LG	2.9	2.6	3.4	0.0	1.4	0.0
38	*trans*-coniferaldehyde	LG	5.4	3.8	3.6	0.0	2.9	0.0
39	syringylacetone	LS	1.5	0.0	1.8	0.5	3.2	4.5
40	propiosyringone	LS	0.0	0.0	0.6	0.0	1.0	1.4
41	dihydrosinapyl alcohol	LS	0.0	0.0	0.6	0.0	1.1	1.6
42	*trans*-sinapyl alcohol	LS	0.0	0.0	0.4	0.0	0.9	1.1
43	*trans*-sinapaldehyde	LS	1.1	0.0	2.2	0.0	7.0	7.8
	%H[b]		7.7	11.2	8.1	71.0	2.1	3.5
	%G		68.4	87.8	53.5	18.4	27.0	8.7
	%S		23.9	1.1	38.4	10.6	70.9	87.8
	%PB[c]		–	–	–	–	4.4	3.1
	Syringyl/Guaiacyl ratio		0.3	0.0	0.7	0.6	2.6	10.1
	Lignin/carbohydrate ratio (L/C)[d]		0.9	0.9	2.4	0.8	2.0	3.4
	Lignin content (%)[e]		12.0	10.5	9.7	6.8	22.7	21.8

[a] LH: H-lignin units, LG: G-lignin units, LS: S-lignin units, PB: *p*-hydroxybenzoates and C: carbohydrates.
[b] Phenol content has not been considered for calculation of %H because it could also be derived from carbohydrates under pyrolysis conditions.
[c] %PB is expressed as a fraction of (S + G + H).
[d] Obtained using the molar areas of the carbohydrates peaks a-f (see Figs. 2–4).
[e] From references [8], [10] and [30].

increase in the proportion of sinapyl alcohol within the pool of monolignols would lead to lignin enriched in S-units that is less-branched and less recalcitrant for pulping and saccharification purposes [43]. Syringyl-rich lignins can be generated by the overexpression of the gene encoding the enzyme F5H, which is the responsible of the hydroxylation of coniferaldehyde to form 5-hydroxyconiferaldehyde that is subsequently reduced to sinapyl alcohol (Fig. 1) [8,44]. The pyrograms from the stems of wild-type and *F5H*-overexpressed transgenic poplar are shown in Fig. 4, and the identities and relative molar abundances of the main pyrolysis products are detailed in Table 1. The pyrolysis of wild-type poplar released mainly guaiacol (2), 4-methylguaiacol (5), 4-vinylguaiacol (8), syringol (15), 4-methylsyringol (18), 4-vinylsiringol (24), *trans*-propenylsyringol (31), syringaldehyde (33) and *trans*-sinapaldehyde (43), with a H:G:S composition of 2:26:67 and an S/G ratio of 2.63. In addition, some amounts of *p*-hydroxybenzoic acid (35), arising from *p*-hydroxybenzoate residues that acylate the γ-hydroxyl group of S-lignin units [45], were also released. A fraction of the *p*-hydroxybenzoates might decarboxylate upon pyrolysis producing phenol, in the same way as *p*-coumarates and ferulates decarboxylate generating 4-vinylphenol and 4-vinylguaiacol. However, phenol may also be produced from polysaccharides [21], so we do not use the phenol peak for quantification of any of the *p*-hydroxyphenyl-derived products. The pyrogram from the poplar *F5H*-upregulated line was markedly different compared to that of the wild-type as the pyrolysis released overwhelming amounts of phenolic compounds derived from S-lignin units, whereas compounds derived from G-

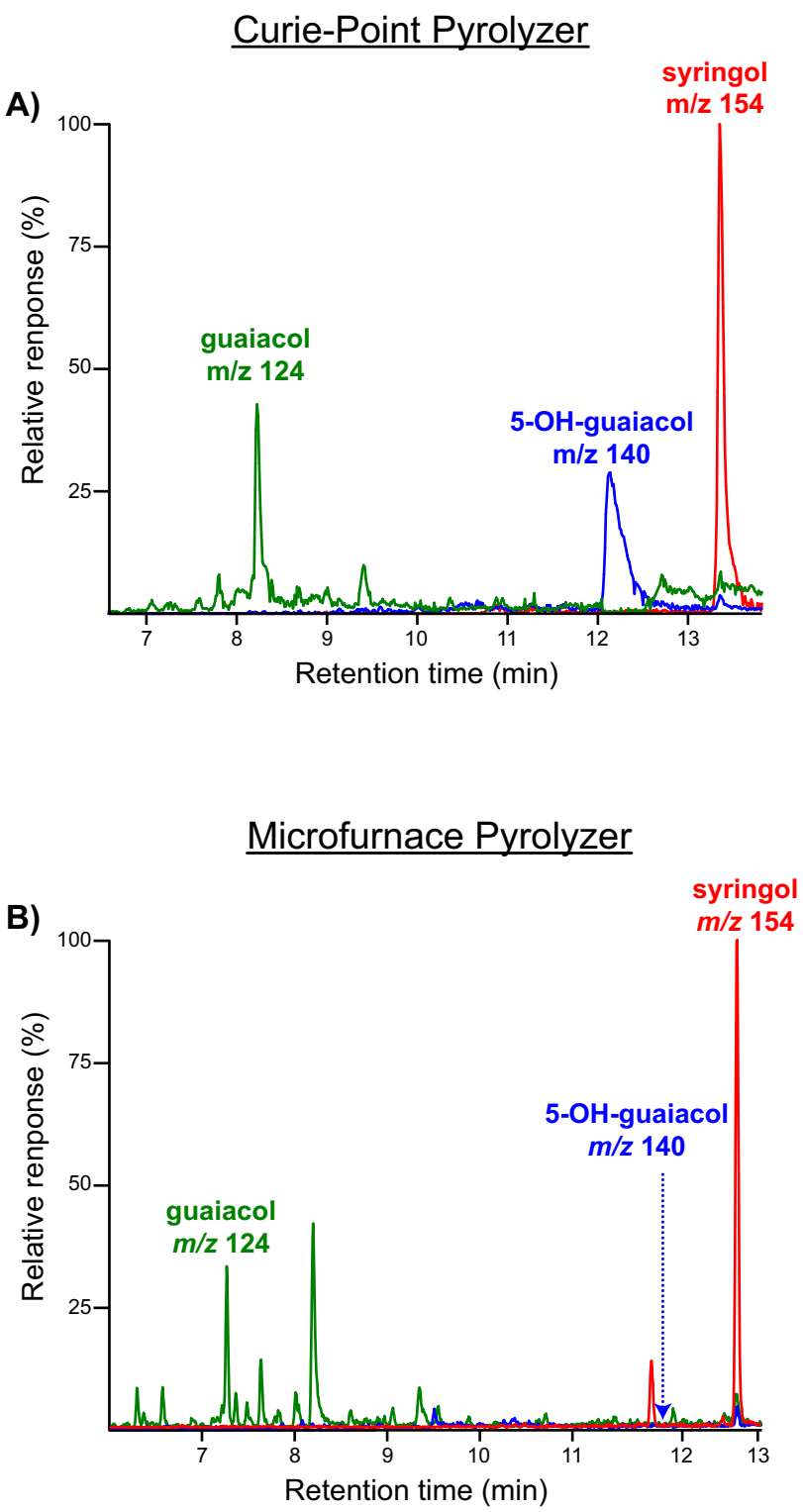

Fig. 5. Comparative selected-ion chromatograms of the lignin monomers guaiacol (*m*/*z* 124), 5-hydroxyguaiacol (*m*/*z* 140) and syringol (*m*/*z* 154) released from Arabidopsis *comt C4H:F5H1 chs* mutant upon pyrolysis using a Curie-Point pyrolyzer (A) and a microfurace pyrolyzer (B). The arrow indicates the retention time at which 5-hydroxyguaiacol should elute according to a standard compound.

lignin units were released only in lower amounts. Consequently, the *F5H* transgenic poplar presented an H:G:S composition of 3:9:88 with an extremely high S/G ratio of 10.12. Prior NMR analyses [8] suggested that the S level might be as high as 97.5% (with a corresponding S/G of ~40); each method measures the components with different sensitivities and therefore the discrepancy is not of concern as both methods show the remarkable enhancement in the S-level in the transgenic. In addition, a decrease in the content of *p*-hydroxybenzoic acid (35) from *F5H* upregulated compared to wild-type poplar was observed, in agreement with a previous study [8].

4. Conclusions

Py-GC/MS has proven to be a useful and convenient tool for the rapid evaluation of compositional changes in lignin from genetically modified plants. Small amounts of samples (<1 mg), short analysis times (less than 30 min), and no tedious or time-consuming isolation processes are required. In addition, the high sensitivity of Py-GC/MS allows the *in situ* characterization of plants with relatively low lignin contents, such as in the C3H alfalfa transgenic plant, with a lignin content as low as 6.8%. Py-GC/MS analyses indicated that the lignin of the COMT-deficient Arabidopsis mutant is mostly composed of G-units, with an extremely low S/G ratio of 0.01 in comparison to that of the wild-type plant (S/G 0.35). In addition, the data indicated that C3H-deficient alfalfa presented a lower lignin content than the wild-type plant, and that was extremely enriched in H-lignin units. Finally, pyrolysis of the *F5H*-overexpressed transgenic poplar indicated that its lignin is mostly made of S-units compared to the wild-type plant that also presented significant amounts of G-units. However, a major limitation exists for the release of 5-hydroxyguaiacyl units from benzodioxane structures in COMT-deficient plants, which seems to be highly dependent of the pyrolysis system used; virtually no traces of 5-hydroxyguaiacyl units could be detected with a micro-furnace pyrolyzer but significant amounts were released when a Curie-point pyrolyzer was used. Nevertheless, the dramatic H:G:S shifts observed by Py-GC/MS imply that significant changes have occurred in the lignin composition. Py-GC/MS, therefore, has significant value as a rapid screening method.

Acknowledgements

This study has been funded by the Spanish projects CTQ2014-60764-JIN and AGL2014-53730-R (co-financed by FEDER funds), the CSIC project 2014-40E-097 and the EU-project INDOX (KBBE-2013-7-613549. John Ralph was funded through the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-FC02-07ER64494). We are grateful to various labs for samples that had been used in prior studies: WT alfalfa and *C3H*-downregulated transgenics [10] from R. A. Dixon, U. North Texas, USA; WT Arabidopsis and *comt* and *comt C4H:F5H1 chs* mutants from W. Boerjan, Ghent University, Belgium; and for WT and *F5H*-overexpressed high-S poplar [22] from S. Mansfield, UBC, Canada, and Clint Chapple, U. Purdue, USA.

References

[1] R. Vanholme, K. Morreel, C. Darrah, P. Oyarce, J. Grabber, J. Ralph, W. Boerjan, Metabolic engineering of novel lignin in biomass crops, New Phytol. 196 (2012) 978–1000.
[2] J. Ralph, K. Lundquist, G. Brunow, F. Lu, H. Kim, P.F. Schatz, J.M. Marita, R.D. Hatfield, S.A. Ralph, J.H. Christensen, W. Boerjan, Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids, Phytochem. Rev. 3 (2004) 29–60.
[3] J.C. del Río, P. Prinsen, J. Rencoret, L. Nieto, J. Jiménez-Barbero, J. Ralph, Á.T. Martínez, A. Gutiérrez, Structural characterization of the lignin in the cortex and pith of Elephant grass (*Pennisetum purpureum*) stems, J. Agric. Food Chem. 60 (2012) 3619–3634.
[4] R. Vanholme, K. Morreel, J. Ralph, W. Boerjan, Lignin engineering, Curr. Opin. Plant Biol. 11 (2008) 278–285.
[5] R. Vanholme, K. Morreel, J. Ralph, W. Boerjan, Lignin biosynthesis and structure, Plant Physiol. 153 (2010) 895–905.
[6] J. Ralph, G. Brunow, P.J. Harris, R.A. Dixon, P.F. Schatz, W. Boerjan, Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template replication, in: F. Daayf, A. El Hadrami, L. Adam, G.M. Ballance (Eds.), Recent Advances in Polyphenol Research, Vol 1, Wiley-Blackwell Publishing, Oxford, UK, 2008, pp. 36–66.
[7] S.K. Huntley, D. Ellis, M. Gilbert, C. Chapple, S.D. Mansfield, Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins, J. Agric. Food Chem. 51 (2003) 6178–6183.
[8] J.J. Stewart, T. Akiyama, C.C.S. Chapple, J. Ralph, S.D. Mansfield, The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar, Plant Physiol. 150 (2009) 621–635.
[9] O. Skyba, C.J. Douglas, S.D. Mansfield, Syringyl-rich lignin renders poplars more resistant to degradation by wood decay fungi, Appl. Environ. Microbiol. 79 (2013) 2560–2571.
[10] M.S.S. Reddy, F. Chen, G.L. Shadle, L. Jackson, H. Aljoe, R.A. Dixon, Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.), Proc. Natl. Acad. Sci. 102 (2005) 16573–16578.
[11] O. Faix, Holzforschung Int. J. Biol. Chem. Phys. Technol. Wood (1991), p. 21.
[12] J. Ralph, L.L. Landucci, NMR of lignins, in: C. Heitner, D.R. Dimmel, J.A. Schmidt (Eds.), Lignin and Lignans; Advances in Chemistry, CRC Press (Taylor & Francis Group), Boca Raton, FL, 2010, pp. 137–234.
[13] M. Tanahashi, T. Higuchi, Chemical Degradation Methods for Characterization of Lignins, in: Methods in Enzymology, Academic Press, 1988, pp. 101–109.
[14] Y. Xie, S. Yasuda, Difference of condensed lignin structures in eucalyptus species, Nordic Pulp Pap. Res. J. 19 (2004) 18–21.
[15] C. Rolando, B. Monties, C. Lapierre, Thioacidolysis, in: in: Methods in Lignin Chemistry, Springer Series in Wood Science, Springer, 1992, pp. 334–349.
[16] F. Lu, J. Ralph, Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers, J. Agric. Food Chem. 45 (1997) 2590–2592.
[17] J. Rencoret, A. Gutierrez, J.C. del Río, Lipid and lignin composition of woods from different eucalypt species, Holzforschung 61 (2007), 165–164.
[18] J.C. del Río, J. Rencoret, P. Prinsen, Á.T. Martínez, J. Ralph, A. Gutiérrez, Structural characterization of wheat straw lignin as revealed by analytical pyrolysis 2D-NMR, and reductive cleavage methods, J. Agric. Food Chem. 60 (2012) 5922–5935.
[19] J.C. del Río, A. Gutierrez, A.T. Martinez, Identifying acetylated lignin units in non-wood fibers using pyrolysis-gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 18 (2004) 1181–1185.
[20] O. Faix, D. Meier, I. Fortmann, Thermal degradation products of wood, A collection of electron-impact (EI) mass spectra of monomeric lignin derived products, Holz Roh-Werkstoff 48 (1990) 351–354.
[21] J. Ralph, R.D. Hatfield, Pyrolysis-GC–MS characterization of forage materials, J. Agric. Food Chem. 39 (1991) 1426–1437.
[22] T. Ohra-aho, M. Tenkanen, T. Tamminen, Direct analysis of lignin and lignin-like components from softwood kraft pulp by Py-GC/MS techniques, J. Anal. Appl. Pyrol. 74 (2005) 123–128.
[23] K.-i. Kuroda, T. Ozawa, T. Ueno, Characterization of sago palm (*Metroxylon sagu Rottb.*) lignin by analytical pyrolysis, J. Agric. Food Chem. 49 (2001) 1840–1847.
[24] D. Meier, I. Fortmann, J. Odermatt, O. Faix, Discrimination of genetically modified poplar clones by analytical pyrolysis-gas chromatography and principal component analysis, J. Anal. Appl. Pyrol. 74 (2005) 129–137.
[25] A. Wagner, Y. Tobimatsu, L. Phillips, H. Flint, B. Geddes, F. Lu, J. Ralph, Syringyl lignin production in conifers: proof of concept in a Pine tracheary element system, Proc. Natl. Acad. Sci. 112 (2015) 6218–6223.
[26] A. Wagner, Y. Tobimatsu, G. Goeminne, L. Phillips, H. Flint, D. Steward, K. Torr, L. Donaldson, W. Boerjan, J. Ralph, Suppression of CCR impacts metabolite profile and cell wall composition in *Pinus radiata* tracheary elements, Plant Mol. Biol. 81 (2013) 105–117.
[27] A. Wagner, L. Donaldson, H. Kim, L. Phillips, H. Flint, D. Steward, K. Torr, G. Koch, U. Schmitt, J. Ralph, Suppression of 4-coumarate-CoA ligase in the coniferous gymnosperm *Pinus radiata*, Plant Physiol. 149 (2009) 370–383.
[28] A. Wagner, J. Ralph, T. Akiyama, H. Flint, L. Phillips, K.M. Torr, B. Nanayakkara, L. Te Kiri, Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase in *Pinus radiata*, Proc. Natl. Acad. Sci. 104 (2007) 11856–11861.
[29] A. Wagner, Y. Tobimatsu, L. Phillips, H. Flint, K. Torr, L. Donaldson, L. Pears, J. Ralph, CCoAOMT suppression modifies lignin composition in *Pinus radiata*, Plant J. 67 (2011) 119–129.
[30] R. Vanholme, J. Ralph, T. Akiyama, F. Lu, J. Rencoret Pazo, J. Christensen, A. Rohde, K. Morreel, R. DeRycke, H. Kim, B. Van Reusel, W. Boerjan, Engineering traditional monolignols out of lignins by concomitant up-regulation *F5H1* and down-regulation of *COMT* in Arabidopsis, Plant J. 64 (2010) 885–897.
[31] F. Chen, R.A. Dixon, Lignin modification improves fermentable sugar yields for biofuel production, Nature Biotechnol. 25 (2007) 759–761.
[32] Y. Barrière, C. Riboulet, V. Méchin, S. Maltese, M. Pichon, A. Cardinal, C. Lapierre, T. Lübberstedt, M. Jean-Pierre, Genetics and genomics of lignification in grass cell walls based on maize as model species, Gene Genom. Genomics 1 (2007) 133–156.
[33] L. Jouanin, T. Goujon, V. de Nadaï, M.-T. Martin, I. Mila, C. Vallet, B. Pollet, A. Yoshinaga, B. Chabbert, M. Petit-Conil, C. Lapierre, Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity, Plant Physiol. 123 (2000) 1363–1373.
[34] J.-K. Weng, H. Mo, C. Chapple, Over-expression of F5H in COMT-deficient Arabidopsis leads to enrichment of an unusual lignin and disruption of pollen wall formation, Plant J. 64 (2010) 898–911.
[35] J.M. Marita, J. Ralph, C. Lapierre, L. Jouanin, W. Boerjan, NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity, J. Chem. Soc. Perkin Trans. 1 (2001) 2939–2945.
[36] F. Lu, J.M. Marita, C. Lapierre, L. Jouanin, K. Morreel, W. Boerjan, J. Ralph, Sequencing around 5-hydroxyconiferyl alcohol-derived units in caffeic acid

O-methyltransferase-deficient poplar lignins, Plant Physiol. 153 (2010) 569–579.
[37] C. Lapierre, M.T. Tollier, B. Monties, A new type of constitutive unit in lignins from the corn *bm3* mutant, Comptes rendus de l'Académie des Sci. Série III 307 (1988) 723–728.
[38] J. Ralph, C. Lapierre, J. Marita, H. Kim, F. Lu, R.D. Hatfield, S.A. Ralph, C. Chapple, R. Franke, M.R. Hemm, J. Van Doorsselaere, R.R. Sederoff, D.M. O'Malley, J.T. Scott, J.J. MacKay, N. Yahiaoui, A.-M. Boudet, M. Pean, G. Pilate, L. Jouanin, W. Boerjan, Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR, Phytochemistry 57 (2001) 993–1003.
[39] C.G. Wilkerson, S.D. Mansfield, F. Lu, S. Withers, J.-Y. Park, S.D. Karlen, E. Gonzales-Vigil, D. Padmakshan, F. Unda, J. Rencoret, J. Ralph, Monolignol Ferulate Transferase introduces chemically labile linkages into the lignin backbone, Science 344 (2014) 90–93.
[40] J.C. del Río, Á.T. Martínez, A. Gutiérrez, Presence of 5-hydroxyguaiacyl units as native lignin constituents in plants as seen by Py-GC/MS, J. Anal. Appl. Pyrol. 79 (2007) 33–38.
[41] S. Suzuki, T.B.T. Lam, K. Iiyama, 5-Hydroxyguaiacyl nuclei as aromatic constituents of native lignin, Phytochemistry 46 (1997) 695–700.
[42] J. Ralph, T. Akiyama, H. Kim, F. Lu, P.F. Schatz, J.M. Marita, S.A. Ralph, M.S.S. Reddy, F. Chen, R.A. Dixon, Effects of coumarate-3-hydroxylase downregulation on lignin structure, J. Biol. Chem. 281 (2006) 8843–8853.
[43] J.C. del Río, A. Gutiérrez Suárez, M. Hernando, P. Landín, Á.T. Martínez Ferrer, J. Romero Sánchez, Determining the influence of eucalypt lignin composition in paper pulp yield using Py-GC/MS, J. Anal. Appl. Pyrol. 115 (2005) 110–115.
[44] K. Meyer, A. Shirley, J. Cusumano, D. Bell-Lelong, C. Chapple, Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in Arabidopsis, Proc. Natl. Acad. Sci. 95 (1998) 6619–6623.
[45] F. Lu, S. Karlen, M. Regner, H. Kim, S. Ralph, R.-C. Sun, K.-i. Kuroda, M. Augustin, R. Mawson, H. Sabarez, T. Singh, G. Jimenez-Monteon, S. Zakaria, S. Hill, P. Harris, W. Boerjan, C. Wilkerson, S. Mansfield, J. Ralph, Naturally *p*-hydroxybenzoylated lignins in palms, Bioenergy Res. 8 (2015) 934–952.