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Increase in 4-coumaryl alcohol (H) units during lignification in alfalfa (*Medicago sativa*) alters extractability and molecular weight of lignin

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The lignin content of biomass can impact the ease and cost of biomass processing. Lignin reduction through breeding and genetic modification therefore has potential to reduce costs in biomass processing industries (e.g. pulp and paper (1,2), forage (3) and lignocellulosic ethanol (4)). We here investigate compositional changes in two low lignin alfalfa (*Medicago sativa*) lines with antisense down-regulation of *p*-coumarate 3-hydroxylase (C3H) or hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT). We investigate whether the difference in reactivity during lignification of H monomers, versus the naturally dominant S and G lignin monomers, alters the lignin structure. Sequential base extraction readily reduced the H monomer content of the transgenic lines, leaving a residual lignin greatly enriched in H subunits; the extraction profile highlighted the difference between the controls and the transgenic lines. Gel permeation chromatography (GPC) of isolated ball milled lignin indicated significant changes in the weight averaged molecular weight distribution of the control versus the transgenic lines (CTR1a=6,000 Da.; C3H4a=5,500 Da.; C3H9a=4,000 Da.; HCT30a=4,000 Da.).

Key Words: Lignin, C3H, HCT, molecular weight
Abbreviations: C3H - *p*-coumarate 3-hydroxylase, HCT - hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase

The advent of large scale liquid fuel production from biomass has served to highlight how difficult it is to commercially process biomass effectively and efficiently. Much of the difficulty is due to the recalcitrant nature of lignocelluloses (4), a complex interlinking structure composed of cellulose, hemicelluloses, and lignin that makes up the bulk of terrestrial biomass. Accessibility to the cell wall is influenced by lignin which provides structural integrity to the cell wall. Both total lignin content and lignin monomer composition may impact the ease with which biomass is processed. This study examines whether lignin molecular weight is altered by changing the lignin monomer composition, and if these changes affect the ease in which lignin can be removed by chemical processing.

Three monomers (Figure 1), 4-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S), polymerize in what is thought to be a combinatorial fashion to form the bulk of the lignin polymer (5,6). The amount of each unit depends on the species, age, cell type and tissue type (7,8). The presence of each additional methoxy group on a lignin unit results in one less reactive site (S < G < H), and therefore, less possible combinations during the polymerization reaction. For example, the S unit has no vacant 5-position, therefore 5,5' cross linking is unavailable for lignin S subunits. As a result, lignin rich in S subunits is more easily depolymerized than lignin rich in G subunits.

The relative level of S and G lignin subunits is expressed as the S/G ratio, an important measurement used in the assessment of biomass. H lignin subunits are present in low levels in natural materials (4.9% of lignin in wild type alfalfa (4), 0.8% in Norway Spruce(9)); consequently, less is known about lignin high in H subunits and its impact on biomass processing.

In addition to the core H/G/S polymer, lignin includes a range of other structures commonly

bonded to the main lignin structure through ether or ester linkages. Such modifications are especially frequent in herbaceous species and usually involve the addition of p-coumaric acid, ferulic acid and occasionally sinapic acid. The modifications can take the form of cross-linking groups between lignin and carbohydrate (10-12), or pendant groups at the γ-position of the lignin inter-linkage unit (13-16). A detailed review of these structures, as they apply to herbaceous biomass, can be found in Buranov and Mazza (13).

In the present work, transgenic lines of alfalfa (*M. sativa*) antisense down-regulated in expression of *p*-coumarate 3-hydroxylase (C3H) and hydroxycinnamoyl CoA; shikimate hydroxycinnamoyl transferase (HCT) were used as models. Both *C3H* and *HCT* genes are involved in the production of S and G lignin monomers but not H monomers in the lignin biosynthetic pathway. Their down-regulation is, therefore, expected to reduce production of S and G monomers and lead to an increased incorporation of H monomers in the lignin constructed by the plant (1,2).

Ralph et al. (17) reported decreases in lignin content (70-76% of the control) and large increases in H monomer units (from 20 to 65 times depending on the analysis) with no change in S/G ratio in C3H down-regulated alfalfa. Previous work on isolated lignin from the same alfalfa C3H and HCT lines used here has shown the lignin content to be lower than the control, and the percentage of H units, relative to S and G units, to be greater than the controls (3,4,17-19). Structurally, the lignin in alfalfa C3H and HCT transgenic lines has been shown to contain a greater proportion of phenylcoumaran- and resinol-type linkages, while aryl ether interunit linkages were less prevalent compared to the control (17,20). Ralph et al. also demonstrated that, in the C3H line, phenylcoumarans were formed almost solely from coupling reactions involving *p*-coumaryl alcohol, while coupling reaction in the control resulted from the combination of *p*-coumaryl, coniferyl, and sinapyl alcohols(17). These three most common interunit linkages are shown in Figure 2.

Using the same plant materials, Chen and Dixon (4) showed that the decrease in lignin content was between 30-50% (dependant on analysis method and use of pretreatment). This decrease in lignin content after transformation was accompanied by a 56 and 100% increase in enzymatic saccharification efficiency for acid pretreated and non-pretreated samples, respectively. This highlights the significant positive influence that decreasing lignin content can have on the efficiency of saccharification of biomass.

To better understand the changes in lignin in these transgenic alfalfa lines, we here utilize sequential extraction to chemically dissect the cell wall material. In sequential extraction, progressively more aggressive treatments are used to fractionate the biomass. In the case presented here, three extractions were used; methanol (MeOH) extraction to remove non-structural cell wall components, 0.1 M NaOH (overnight, 4 °C) to remove highly labile cell wall bound phenolics, and 2 M NaOH (10 minutes, 110 °C oil bath) to remove less labile cell wall-bound phenolics. We observed a higher removal of cell wall components under the base extraction conditions for the transgenic lines compared to the controls, indicating differences in the chemical and/or the physical construction of the cell wall after transgenic modification of the lignin biosynthetic pathway. In addition GPC of isolated ball milled lignin indicated smaller lignin in the transgenic lines.

EXPERIMENTAL PROCEDURES

Materials: Methanol (HPLC grade), tetrahydrofuran (THF, HPLC grade), glacial acetic acid (reagent grade) and diethyl ether (anhydrous, reagent grade) were from JT Baker, Phillipsburg, NJ. The 1,4-dioxane (anhydrous, 99.80%), 1,2-dichloroethane (≥99.8% HPLC grade), acetic anhydride (reagent grade) and pyridine (anhydrous, ≥99.8%) where sourced from Sigma-Aldrich, MO, USA. The generation of lignin-modified transgenic lines of *M. sativa* is presented in detail elsewhere (3,17). The independent transgenic lines investigated were: CTR1a, CTR49a, C3H4a, C3H9a, HCT3a, and HCT30a. CTR1a and CTR49a are empty vector controls used to account for potential changes to the cell wall chemistry due to the genetic transformation process itself. Two vegetatively propagated replicates of each independent line were used to account for potential variation in cell wall

chemistry due to environmental effects. Mature stems were harvested (the first seven internodes from the top were discarded) for each line when the plants had reached the late flowering stage. Samples were dried at 55 °C. The compositional analyses on untreated whole stems discussed here have been published previously (21).

Methanol Extraction: Dried stem material (1.0 g batches) was weighed into 120 mL vials and MeOH (50 mL) added. The vials were capped and shaken at 5 °C overnight. A filtering crucible with a type A/D glass fibre filter was used to filter the solutions (the same arrangement was used in each step). The solids were washed with 50 mL of MeOH. The methanolic washes and filtrates were combined, collected, and dried. The solids were then washed with 50 mL of 18.2 MΩ water. The solid was freeze dried and weighed, and the MeOH was evaporated and freeze dried.

0.1 M Sodium Hydroxide (NaOH) Extraction: The above freeze dried solids (0.7 g batches) were weighed into 120 mL vials, and 0.1 M NaOH (50 mL) added. The vials were capped and shaken at 5 °C overnight then filtered. The vials were then washed six times with 5.0 mL of water, and the solid residue was washed four times with 5.0 mL of water. The washes and filtrates were collected. The crucible was washed with water until the flow through was neutral (this second wash was not saved for use). The solid was freeze dried and weighed, and the filtrate was neutralized with acidified CG50 Amberlite resin and stored at 4 °C.

2 M NaOH Extraction: Samples were split in two for reaction in the oil bath due to volume constraints. Two ASE vials were prepared with 0.2 g each of the 0.1 M NaOH extracted residue and 25 mL each of 2 M NaOH. These were reacted simultaneously in a 110 °C oil bath for ten min with stirring. Samples were cooled in a water bath at room temperature. The split samples were recombined and washed until the filtrate was neutral (~200 mL). The filtrate was acidified to pH 2.0 with concentrated hydrochloric acid (HCl). The solution was allowed to rest at 4 °C overnight before the precipitate was filtered off, freeze dried and weighed.

NMR: Solid-state NMR spectra were collected using high-resolution ^{13}C cross-polarization/magic angle spinning (CP/MAS) with a Bruker Avance 200 MHz spectrometer (50.13 MHz, room temperature). The spinning speed was 7000 Hz, a contact time of 2 ms with a 1 db ramp on the proton spin locking field was applied during cross polarization. The acquisition time was 32.8 ms and the recycle delay was 1 s.

Thioacidolysis: The thioacidolysis method used to determine the lignin composition of treated and untreated cell wall material as well as extracted material was as described by Lapierre et al. (22).

Lignin Isolation: Ball-milled lignin was isolated according to modified literature methods described previously (20,23,24). Wiley milled alfalfa samples (60 mesh) were Soxhlet extracted with ethanol:H_2O (95:5, v/v) for 48 h and then dried under vacuum overnight. The dried alfalfa (5-10.0 g) was ball milled in a Roalox ceramic jar (0.35 L) with zirconia balls using a rotatory ball mill running at 96 rpm for 8 days. A zirconia balls:biomass weight ratio of 30:1 was used. The ball milled residue was then extracted (2 x 48 hrs stirring, in the dark) with dioxane-water (96:4, v/v) (10 mL/g alfalfa). The extracted mixture was centrifuged, and the supernatant was collected and evaporated at 35 °C under reduced pressure to obtain the crude ball milled lignin. The crude lignin was dissolved in acetic acid-water (90:10, v/v) (50 mg/mL) and precipitated in de-ionized water (200 mL), centrifuged and dried under vacuum at room temperature for 24 h**.** For further purification, the solid product was dissolved in minimum quantity of 1,2-dichloroethane:ethanol (2:1, v/v) and precipitated in diethyl ether (200 mL), centrifuged, washed with diethyl ether (3 x 20 ml), dried under vacuum (40 ^{o}C, overnight) and stored in a refrigerator. The ball milled lignin yield was 7.7%, 14.2%, 9.4% and 10.1%, for the control, C3H4a, C3H9a and HCT30a samples respectively.

GPC: The isolated lignin samples were weighed into Reacti-vials and acetylated by dissolving in pyridine (10 uL / 1 mg sample), stirring at 40 ^{o}C for 1 h, adding acetic anhydride (10 uL / 1 mg sample) and stirring overnight at room temperature. The reaction was terminated by addition of methanol (4 uL / 1 mg sample). The acetylation reagents were evaporated from the samples at 40 °C under a stream of nitrogen gas, and the samples were then dried in a vacuum oven at 40 °C overnight, then (1 torr) at room temperature for 1 h. The dried acetylated samples were dissolved in THF and filtered (0.45 µm nylon membrane syringe filters) before GPC analysis.

GPC analysis was performed using an Agilent HPLC with three polystyrene-divinyl benzene GPC columns (Polymer Laboratories, 300 x 7.5 mm, 10 µm beads) having nominal pore diameters of 10^4, 10^3, and 10^2 Å. The eluent was THF, the flow rate 1.0 mL/min, the sample concentration was ~2 mg/mL and an injection volume of 25 µL was used. The HPLC was attached to a diode array detector measuring absorbance at 260 nm (band width 40 nm). Polystyrene calibration standards were used with molecular weights ranging from 580 Da. to 2.95 million Da. Toluene was used as the monomer calibration standard.

RESULTS

Extractions: Methanol extraction was first used to ensure the removal of small molecules which are structurally unrelated to the plant cell wall. Gravimetric variations in methanol extracts of the non-cell wall components were not statistically significant across the lines. Upon further extraction with 0.1 M NaOH and then 2.0 M NaOH, there was significantly more material removed from the two transgenic lines compared to the control. The 2.0 M NaOH solution was acidified and the precipitates collected. A higher mass of precipitate was collected from the transgenic lines compared to the control, in agreement with a decrease in the extracted residue in the transgenic lines.

Solid-state NMR of the cell wall residue: Solid-state ^{13}C cross polarization nuclear magnetic resonance spectroscopy (^{13}C CP/MAS) was used to investigate the lignin structural characteristics in plant cell walls. The advantage of this technique over liquid state NMR is the inclusion of the whole biomass (treated or untreated) instead of only the components that can be solubilized in applicable solvents. This allows a greater proportion of the biomass to be examined; however solid-state NMR has the disadvantage of lower resolution. Background measurements of the whole insoluble cell wall residue (post methanol extraction) were used to compare the control with the C3H and HCT down-regulated lines (Figure 3).

By adjusting the solid-state NMR spectra of the six lines so they are equal at 89 ppm (assigned to the C4 carbon in interior crystalline cellulose) the relative amount of lignin in each sample can be compared (see Figure 1 for carbon labeling convention). A comparison of the three alfalfa lines after methanol extraction (Figure 3) showed that the control had greater intensity in the aromatic region (110-165 ppm) than the transgenic lines, relative to the carbohydrate region (60-110 ppm). The aromatic region of the cell wall residue of plant materials is generally assigned to lignin although other compounds such as proteins, tannins, and phenolic compounds (such as ferulates and coumarates) cross linked to either lignin or hemicellulose fractions can also contribute to the aromatic intensity. The decrease in the aromatic intensity relative to the carbohydrate intensity, suggesting decreased lignin content in the transgenic materials compared to the control, is consistent with previous determination of lignin content in these lines as determined by the acetyl bromide method (25).

The spectra of the whole plant cell wall residue for the C3H and HCT transgenic lines show an increase in intensity at ~160 ppm, assigned to the lignin H_4 (Figure 3 insert), and ~130 ppm, assigned to H_2 and H_6. We believe that the expected increase in intensity at ~115 ppm due to the H_3 and H_5 is offset by the decrease in lignin content, although close examination of the HCT transgenic alfalfa spectra (Figure 3 insert) indicates that a possible broad peak can be observed. Concurrent with an increase in intensity assigned to H-type units are decreases in resonances assigned to S- and G- type units (for example, 152 ppm assigned to the S_3 and S_5(26)). The lower abundance of S- and G-type lignin units can also be determined by the decrease of the methoxyl peak at 56 ppm, although decreases in total lignin content can also contribute to the loss in signal intensity. A more complete summary of reference lignin chemical shifts can be found elsewhere (17,26-29).

Base extraction: The treatment of biomass with base removes both phenolic and hemicellulosic components of the plant cell wall making it difficult to compare spectra. In order to compare two treatment conditions of the same sample, we assume that the crystalline cellulose concentration of the sample is constant between treatments. The C4 carbon of the crystalline cellulose peak appears at 88-89 ppm (30-32) and appears distinct from the

amorphous region. By assuming that there is no change in crystalline cellulose during the base extractions, the crystalline cellulose peak can be used as an internal standard for relative comparisons between samples. Although there could be some change in crystalline cellulose due to the conversion of type I cellulose to type II cellulose, the spectra do not show any type II cellulose peaks indicating no large changes in cellulose morphology. Use of the crystalline cellulose peak as an internal standard is not absolute but gives an indication of the change in the aromatic intensity relative to the crystalline cellulose.

0.1M NaOH Extraction: The solid-state NMR representation of the sequential extraction of the control, C3H and HCT lines is shown in Figure 4. Black represents the whole cell wall (extracted with methanol to remove small molecules and metabolites from the cell), red represents the 0.1 M NaOH extracted cell wall (overnight, 5 °C) and blue the 2 M NaOH extracted (10 min, 110 °C) cell wall. As shown by the minimal change in the intensity of the aromatic region of the spectrum (110-165 ppm), the control sample does not lose a significant amount of aromatic material during either the 0.1 M NaOH or the 2.0 M NaOH extraction. This result is expected given the relatively mild treatments used in this work.

However, material is removed from both the C3H and HCT transgenic lines by 0.1 M NaOH extraction, especially in the regions between 122-140 ppm (representing $H_{2/6,}$ $S_{1/4}$ and G_1) and 151-161 ppm (representing $S_{3/5}$, G_3 phenolic and G_4 non-phenolic). The $H_{2/6}$ region (126-129 ppm) does not overlap with the $S_{1/4}$ and G_1 (133-136 ppm) indicating that, at the very least, both H and S or G lignin subunits are being lost on extraction in the transgenic lines compared to the control. Loss of methoxyl content represented by a decrease in the peak intensity at 55 ppm (Figure 3) is also indicative of loss of either S or G subunits of lignin.

2.0 M NaOH Extraction: Treatment with 2.0 M NaOH results in a ^{13}C NMR signal reduction similar to that seen on treatment with 0.1 M NaOH. The control was only slightly changed, whereas major changes were observed in the spectra of the transgenic lines. Similar to what was observed in the 0.1 M NaOH extraction, the signal reduction was seen mostly in the regions 122-140 ppm and 151-161 ppm, with additional decreases seen in the region at 55 ppm (Figure 3). As with the 0.1 M NaOH treatment, this reduction indicates a decrease in $H_{2/6,}$ $S_{1/4,}$ $G_{1,}$ $S_{3/5}$, G_3 phenolic and G_4 non-phenolic resonances signifying the removal of lignin and lignin-like components. A concomitant decrease in methoxyl groups at 55 ppm can also be seen (Figure 3), confirming that G and/or S lignin subunits are being removed as well as H subunits.

Solid state NMR spectra of the 2.0 M NaOH precipitate were compared after normalizing to the carbohydrate peak at 101 ppm (Figure 6). Although this method of comparison does not allow quantification, it is clear that there is very little aromatic material (125-165 ppm) in the 2.0 M NaOH extract of the control. There are, however, large aromatic signals present in the extracts from both of the transgenic lines, corresponding to $H_{3/5}$ (114-118 ppm), $H_{2/6}$ (126-132 ppm) and H_4 (156-163 ppm). This indicates that the aromatic containing material in the precipitate is rich in H subunits rather than S or G subunits.

Thioacidolysis: The methanol, 0.1 M NaOH and the 2.0 M NaOH extracted biomass, the 0.1 M NaOH extractives and 2.0 M NaOH precipitate were subjected to thioacidolysis to further confirm the distribution of the H, G and S lignin subunits and their tendency to be extracted.

The thioacidolysis data support the NMR findings (Figure 5) and, in addition, allow quantitative comparison of the lines. The thioacidolysis of the methanol extracted residues indicates H:G:S ratios are similar to previously published results (Table 1). The methanol extracted controls show lignin high in G and S subunits and little H monomer contribution while the transgenic lines show high levels of H monomer content. The HCT down-regulated line had low levels of G and S lignin subunits while the C3H line still retained more than half of the G/S lignin subunits found in the control. This indicated that the lignin structure in C3H is less altered compared to the control than is that of the HCT line. Note that thioacidolysis values are lower for the 0.1 M NaOH extracted material than they are for either the methanol or 2 M NaOH extracted residue. The 0.1 M NaOH extraction appeared to liberate phenolic compounds which releases S, G, and H monomers less readily than the methanol or

2 M NaOH treated material. A possible explanation is that the low molecular weight phenolic compounds contain a higher number of C-C bonds that are not cleaved under the thioacidolysis conditions employed, and therefore were not detected**.**

Thioacidolysis analysis confirmed that lignin with high proportions of H monomers has been removed from the cell wall residue of the transgenic lines on extraction with 2.0 M NaOH, in agreement with the solid state NMR analysis of the residues (Figure 6). This analysis also indicates that the control lines contain very small amounts of the H lignin subunits (< 10 umol), as would be expected given the low natural abundance of H monomers in the control, and the small amount of material extracted from the control. The thioacidolysis results of the 2.0 M NaOH extracted residues show a greatly reduced lignin content for the transgenic lines (especially HCT), in agreement with the ^{13}C CP/MAS (Figure 4).

Analysis of the percentage of H lignin monomers (Figure 7), which are removed by either the 0.1 M or 2.0 M NaOH treatments, demonstrates how the extractability of lignin high in H subunits changes radically from the control to the transgenic lines. Analysis of the data based on the percentage of H monomer units extracted removes the bias of different lignin contents and the different monomer ratios from the results. Using this data the increased tendency of H monomer units to be preferentially extracted from the transgenic lines can be seen (Figure 7). The HCT lines can be seen to have the highest percentage of H lignin monomers extracted with around 90% extracted compared to <20% for the controls. The percent H lignin monomers extracted is intermediate for the two C3H lines (ranging from 57 to 80%) as expected from their intermediate total lignin and H monomer contents.

GPC Analysis: The lignin isolated from HCT30a has a peak averaged molecular weight (Mp) of 2,800, a weight averaged molecular weight (Mw) of 4,000 and an H monomer content of 74%. At the other extreme the control has an Mp = 4,100, Mw = 6,000 and an H monomer content of 2%. Interestingly, even the difference between the two C3H lines is captured by the size distribution of the isolated lignin. C3H4a, which is less down-regulated than C3H9a, behaves most similarly to the control. The Mw of 4,000, Mp of 3,000 and H monomer content of 48% for C3H9a reflect a strong down-regulation of C3H and are similar to the values in the HCT line. C3H4a instead has values part way between C3H9a and the control (Mw = 5,500, Mp = 3,600 and H monomer content of 27%) reflecting a partial down regulation of C3H expression.

DISCUSSION

Radical polymerization and the formation of the lignin polymer: Lignin is thought to form via oxidative coupling of the mono-lignol metabolites (5,33). Any variable that influences these coupling reactions therefore influences the resultant lignin. Russell et al. (34,35) reported trends in the oxidative coupling patterns of different mono-lignols which indicate that the mono-lignol distribution of a lignin influences both the interunit linkage distribution, and, possibly, the size of the resultant lignin. Insights can be gleaned from studying this artificial system.

Using electron spin resonance (ESR) spectroscopy of peroxidase catalyzed phenylpropanoid oxidation and molecular modeling, Russell et al. investigated the distribution of the unpaired electron in lignin precursor radicals (34). They found that methoxyl substitution increases the unpaired electron density on the phenolic oxygen and therefore the tendency of the oxidative coupling reaction to involve reactions through the phenol. As the H lignin subunit forming mono-lignol (coumaryl alcohol) has no methoxy substituents on the ring, the unpaired electron density is greatest on the carbon nuclei, not the phenolic oxygen, and consequently the preference is for C-C reactions. This is exactly what was observed by Pu et al. (20) in lines where lignin is high in H subunits. 2D NMR showed a decrease in β-O-4 linkages (a phenolic linkage) and an increase in both resinol and phenylcoumaran linkages (C-C linkages). Furthermore, both resinol and phenylcoumaran linkages are in greater abundance in HCT (H lignin subunits, 75.5 %) than in C3H (H lignin subunits, 50.0 %) down-regulated lines (4). Thus these results follow the trend that lignin with a higher H lignin subunit content is concomitantly higher in C-C linkages. Ralph et al. additionally found that dibenzodioxocin (a linkage involving three monomer units, two phenolic linkages and

one C-C linkage) was doubled in C3H-downregulated alfalfa (17).

In a follow up report, Russell et al.(36) showed that (for horseradish peroxidase) the ease with which lignin precursors undergo oxidation to their free radical form is also dependant on their degree of methoxylation. In this *in vitro* example, additional methoxy groups help stabilize the free radical, aid oxidation and therefore produce a greater number of reactive species. Russell et al. point out that this phenomenon likely explains why the non-methoxylated lignin subunits make up such a small amount of natural lignin. It is not known exactly how these changes in the tendency to form radicals impact the incorporation of the resultant lignin into the plant cell wall, but given the changed extraction profile of the C3H and HCT lines it is apparent that it does. One possibility is that due to a reduced number of reactive monomer species, chain growth of the lignin polymer may be reduced, leading to a decrease in molecular weight of lignin. In fact Russell et al. suggest that the non-methoxylated monomer may have a role in terminating chain growth (36). Smaller lignin polymers would presumably be less cross linked (per total length), potentially having less bonds tying them into the lignocellulosic ultra-structure. Smaller aromatic chains would also have greater solubility, as they are not limited by the phenomenon of hydrophobic hydration which impacts large compounds of the same unit composition.

GPC of isolated lignin confirms that there is a trend towards lower molecular weight lignin in the transgenic lines versus the control (Figure 8), supporting the idea that lignin high in H content may be more easily extracted due to decreased chain length. Moreover, the trend in molecular weight decrease corresponds with the increase in the percentage of H lignin subunits present in the methanol extracted cell wall residue.

Impact on Lignin Structure: Our results indicate there are extractable and non-extractable fractions of polyphenolic compounds present in the transgenic materials. Thus, the lignin rich in H subunits behaves significantly differently to the lignin present in the controls. Russell et al. established that a polymer formed primarily from H monomers has a reduced tendency to react, tends to form C-C bonds and has a tendency to cap. That, in conjunction with the GPC of isolated lignin indicating lower average molecular weights, points towards the difference between the control and transgenic lignin as being, at least partly, one of polymer molecular weight. This is not to rule out the possibility that alterations are also occurring due to an inability of the plant to cope with the large increase in flux along the p-coumaryl alcohol portion of the lignin biosynthetic pathway.

Lignin high in H subunits has been observed to be laid down in the cell wall very early in the lignification process and may therefore be associated with initiation of lignification. Possibly the material that is not removed by base extraction is serving that purpose and is laid down via a different mechanism from the lignin formed with the increased H content due to down-regulated expression of *C3H* and *HCT*. The extractable material is then the lignin synthesized by the plant as it attempts to compensate for the loss of the G and S monomers. It is not surprising then, that the extractable polyphenolic material is different in these transgenic materials compared to the control materials. It is a matter for further investigation as to whether the polyphenolic material produced by the transgenic plants conforms to our current definition of lignin.

Additional work is underway to characterize the structure of the components of the extracted materials to increase our understanding of how the change in the lignin monomer ratio has altered lignin incorporation into the cell walls of the C3H and HCT downregulated transgenic alfalfa plants. Understanding more about this process will help further detail what occurs *in planta*, in the time between initiation of lignin monomer biosynthesis and the generation of the final lignin product within the plant cell wall.

CONCLUSIONS

The increased extractability of the lignin-like material composed predominately of H subunits is due (at least partly) to a decrease in molecular weight of the polymer. The decrease in molecular weight is explained by the reduced stability of the 4-coumaryl alcohol building block during radical coupling, compared to the more prevalent sinapyl and coniferyl alcohols. As a result of the radical tending to stabilize on the ring instead of the phenolic oxygen, the interunit-linkage distribution

of the materials high in H lignin subunits is also altered. Our findings suggest that the lignin molecular weight, and as a result, the ease with which it can be removed during chemical processing, can be influenced by altering monomer ratio.

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FIGURE LEGENDS

Table 1: S/G ratio and lignin H, G and S content of the three alfalfa lines. As determined by thioacidolysis(4) and ^{13}C CP/MAS NMR of the cell wall residue.

Figure 1: Structure labeling convention. A lignin carbon (left) is denoted by H_x, G_x or S_x where H (4-coumaryl), G (coniferyl) or S (sinapyl) denote the lignin subunit type and $_x$ denotes the position on the ring. A cellulose carbon (right) is denoted by CX, where X denotes the position on the sugar ring.

Figure 2: β-O-4 (A) interunit linkages decrease in HCT and C3H alfalfa where as phenylcoumaran (B) and resinol (C) interunit linkages increase (17,20).

Figure 3: ^{13}C CP/MAS NMR spectra of methanol extracted alfalfa stems CTR49a (———), C3H9a (———), and HCT30a (———). The inserted spectra highlights the change in intensity at 157-165 ppm assigned to H_4 demonstrating the increase in H-lignin.

Figure 4: ^{13}C CP/MAS NMR spectra from alfalfa control, C3H and HCT down-regulated alfalfa. Methanol (———), 0.1 M NaOH (———) and 2.0 M NaOH (———) extracted biomass.

Figure 5: Lignin monomer compositions by thioacidolysis of extracts from control and transgenic alfalfa. H, G and S lignin monomer levels (umol/g) from Methanol, 0.1 M NaOH and 2.0 M NaOH extracted residues, 0.1 M NaOH extractives and 2.0 M NaOH precipitate. Thioacidolysis analysis of extracted residues were performed in triplicate, extractives were performed in duplicate.

Figure 6: Solid-state ^{13}C CP/MAS NMR spectra of the 2.0 M NaOH precipitate. Alfalfa control, C3H and HCT down-regulated alfalfa (top) control, (middle) C3H9a, and (bottom) HCT30a.

Figure 7: Percent thioacidolysis yield. H monomer content from extracted material; 0.1 M NaOH extraction (■), or the 2 M NaOH extraction (■).

Figure 8: GPC of isolated lignin. Isolated from alfalfa CTR1a, C3H4a, C3H9a and HCT30a

Table 1

Sample type	Line	H %	G %	S %	Total lignin (mg/g)	S/G Ratio
Thioacidolysis and acetyl bromide (Whole plant cell wall residue)(4)*	**Control**	4.9	56.0	39.2	207.2	0.70
	C3H	50.0	27.4	22.6	131.7	0.82
	HCT	75.5	13.0	11.5	99.8	0.88
Solid-state ^{13}C NMR#	**Control**				149.5	
	C3H				117.4	
	HCT				89.7	

* - averaged from n lines, control n=4, C3H n=3, HCT n=2

- averaged from the two relevant lines presented in this work

Figure 1

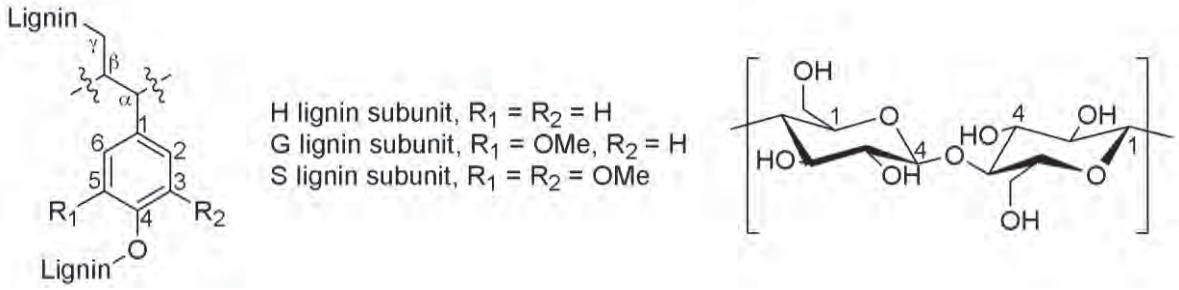

Figure 2

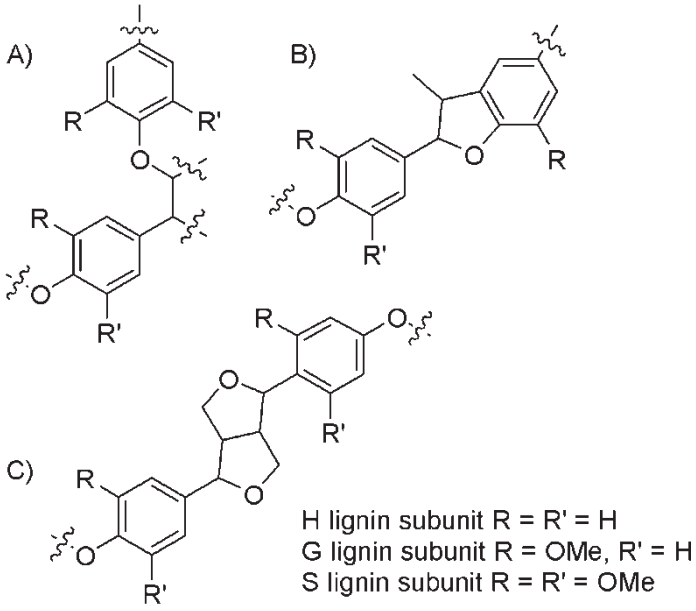

Figure 3

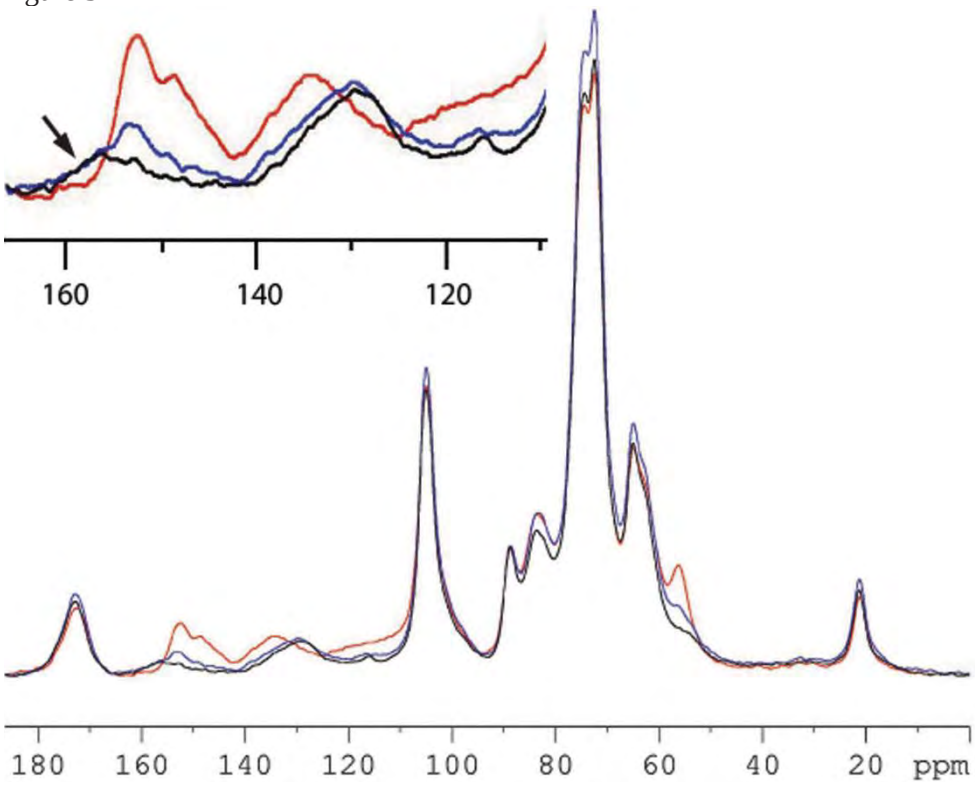

Figure 4

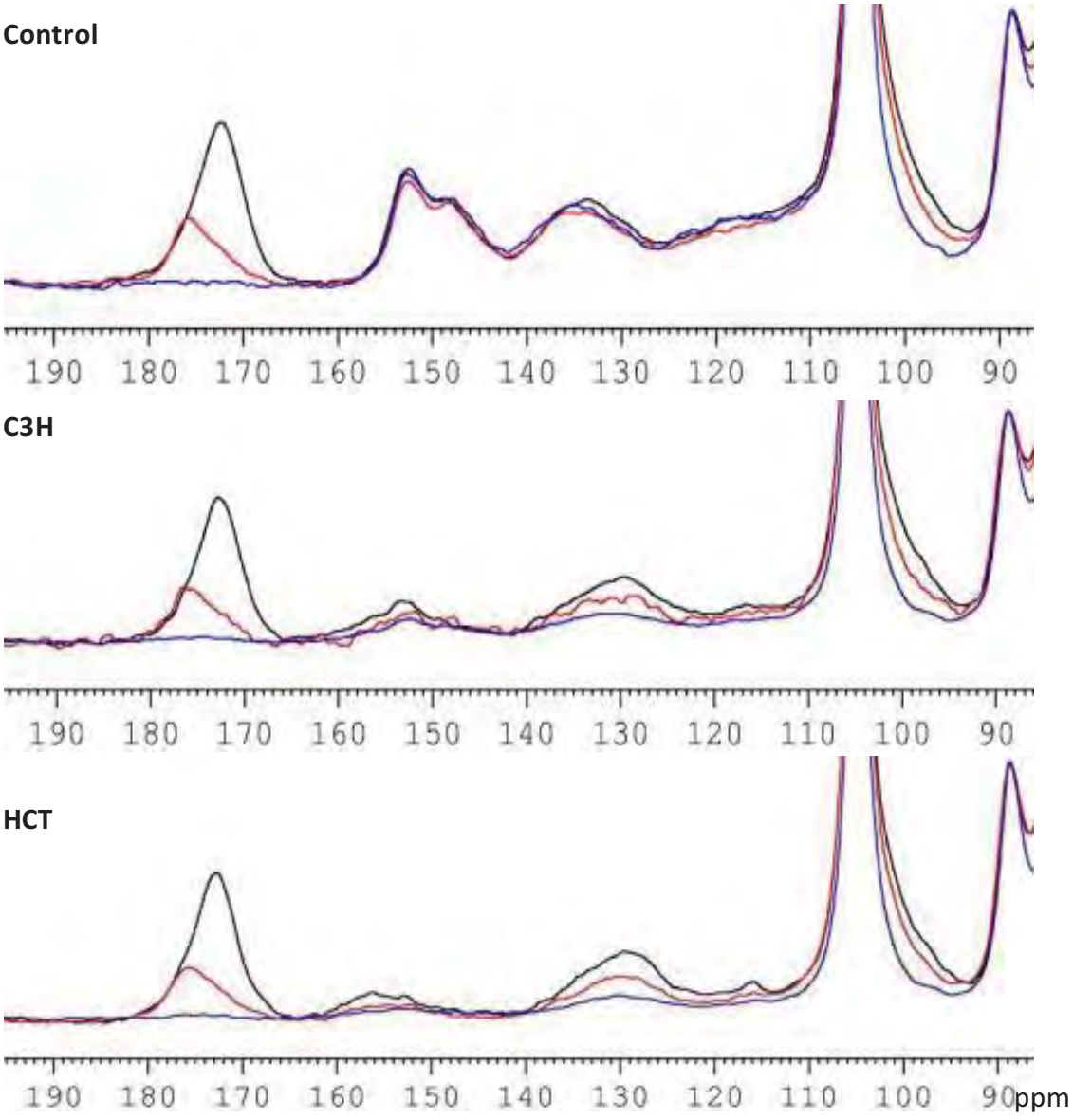

Figure 5

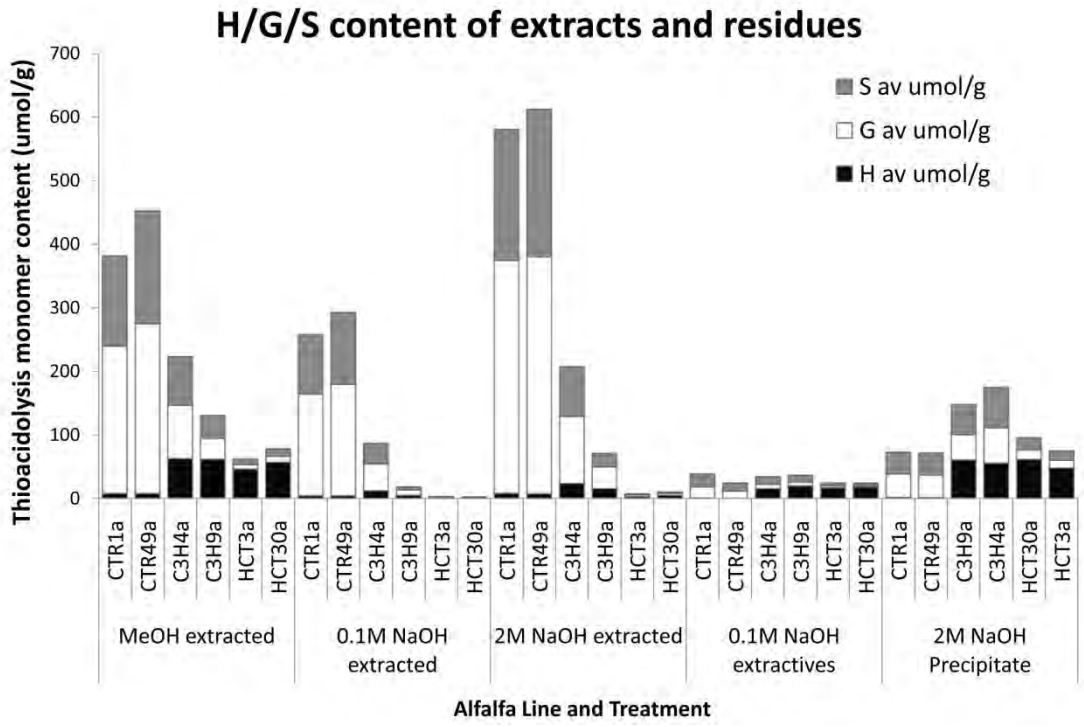

Figure 6

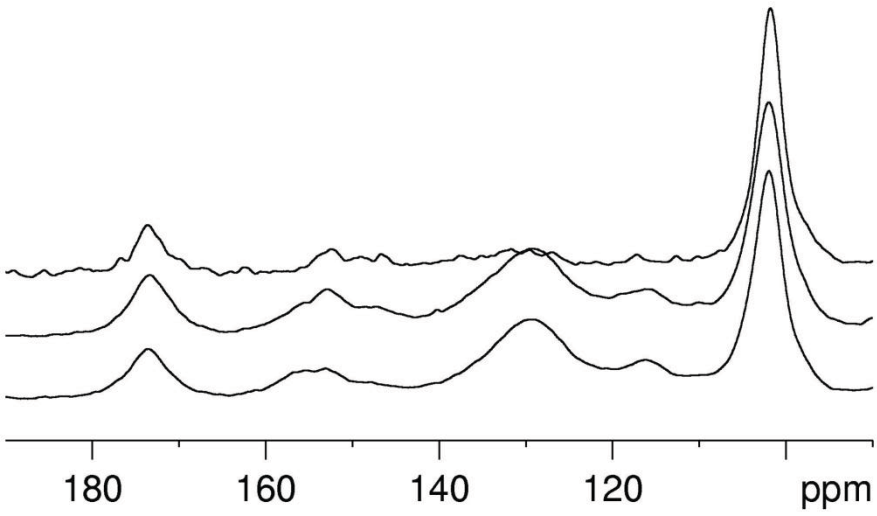

Figure 7

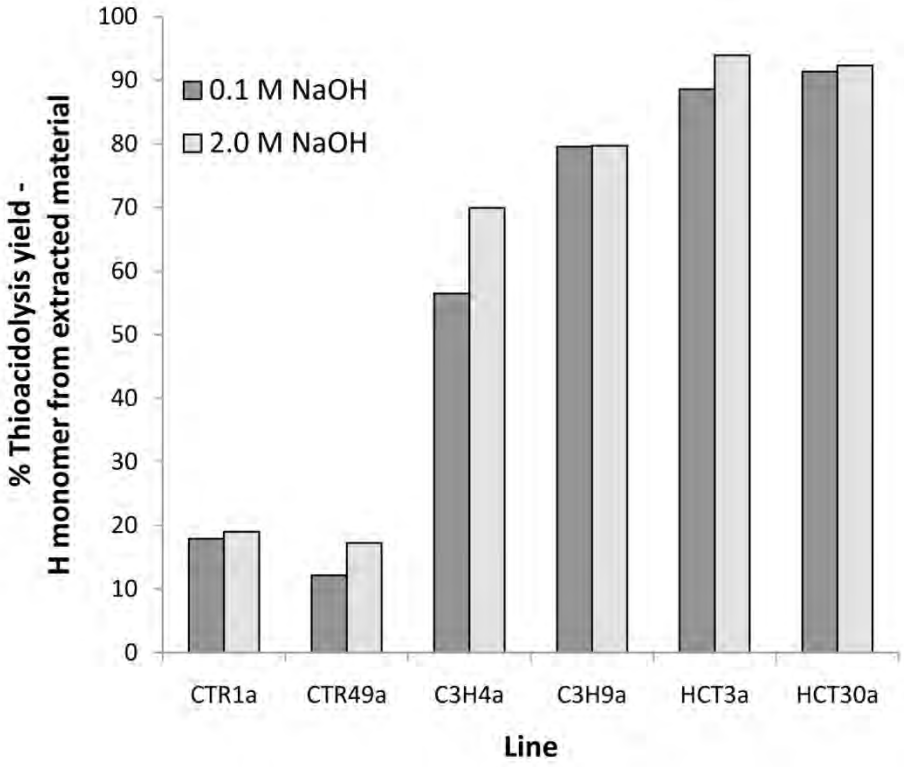

Figure 8