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Structural characterization of lignin in wild-type versus COMT down-regulated switchgrass

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This study examined the chemical structural characteristics of cellulolytic enzyme lignin isolated from switchgrass focusing on comparisons between wild-type control and caffeic acid 3-*O*-methyltransferase (COMT) down-regulated transgenic line. Nuclear magnetic resonance techniques including ^{13}C, ^{31}P, and two-dimensional ^{13}C-^{1}H heteronuclear single quantum coherence as well as gel permeation chromatography were employed. Compared to the wild-type, the COMT down-regulated transgenic switchgrass lignin demonstrated a decrease in syringyl (S):guaiacyl (G) ratio and *p*-coumarate:ferulate ratio, an increase in relative abundance of phenylcoumaran unit, and a comparable content of total free phenolic OH groups along with formation of benzodioxane unit. In addition, COMT down-regulation had no significant effects on the lignin molecular weights during its biosynthesis process.

Keywords: switchgrass, COMT transgenic, lignin, NMR, molecular weights

INTRODUCTION

There is a growing focus on innovative technologies that shift bioethanol production to second generation cellulosic biofuels due to the competition between food resources and first generation biofuels derived from agricultural resources such as corn starch (Wyman, 1999; Wang et al., 2007; Rubin, 2008). Although lignocellulosic resources, such as energy crops and agricultural and forest residues, are readily becoming available for bioethanol production, their processing requires a costly pretreatment step to overcome their natural recalcitrance toward biological deconstruction to simple sugars (Sun and Cheng, 2002; Himmel et al., 2007; Pu et al., 2008; Somerville et al., 2010). Lignocellulosic biomass is a complex composite consisting primarily of three biopolymers (i.e., cellulose, hemicelluloses, and lignin) and its recalcitrance has been attributed to several factors such as cellulose accessibility to enzymes, lignin content/structure, lignin-carbohydrate complexes, as well as the presence and structure of hemicelluloses (Wyman et al., 2005; Li et al., 2012; Leu and Zhu, 2013; Pu et al., 2013). The goal of pretreatment is to reduce the recalcitrance of biomass by disrupting/modifying the lignin-polysaccharide matrix. In the past two decades, extensive research efforts have been directed at improving a diverse set of pretreatment technologies including: dilute acid, lime, hot water, steam explosion, ammonia, and organosolv pretreatments. While these pretreatments have achieved various level of success in overcoming the recalcitrance of lignocellulosic biomass, the pretreatment step still remains one of the most expensive steps in the current biomass to bioethanol production platform. Therefore, addressing biomass recalcitrance through alternative approaches is a crucial issue to the widespread, low-cost generation of cellulosic biofuels.

One of the promising approaches for reducing biomass recalcitrance is the development of genetically engineered plants involving down-regulation/overexpression of key enzymes involved in lignin biosynthesis that can achieve an improved sugar release performance with reduced pretreatment severities (Li et al., 2003; Chen and Dixon, 2007; Weng et al., 2008; Pu et al., 2011b; Shen et al., 2012; Tschaplinski et al., 2012). A recent report by Shen et al. (2012) has demonstrated that overexpression of PvMYB4 genes in switchgrass resulted in an approximately threefold increase in sugar release efficiency from transgenic cell wall residues. Chen and Dixon (2007) reported that *p*-coumarate 3-hydroxylase (C3H) and hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT) down-regulated alfalfa lines showed an improved fermentable sugar yield when compared to wild-type plants. The engineering of these enzyme genes is expected to generate a variation in lignin structures and thus impact the recalcitrance of biomass; therefore, there has been considerable interest in lignin structural characteristics in genetically engineered plants (Marita et al., 1999; Guo et al., 2001; Chen et al., 2004; Pu et al., 2009; Stewart et al., 2009; Lu et al., 2010; Tu et al., 2010; Vanholme et al., 2010; Ziebell et al., 2010). For example, Pu et al. (2009) and Ziebell et al. (2010) isolated ball-milled lignin from C3H and HCT transgenic alfalfa lines and studied their structural features. The results demonstrated that a substantial increase

in *p*-hydroxyphenyl (H) units and a decrease in syringyl (S) and guaiacyl (G) monolignol units were accompanied with a decrease in the presence of β-*O*-4 aryl ether.

Recently, Fu et al. (2011) generated caffeic acid 3-*O*-methyltransferase (COMT) down-regulated switchgrass and evaluated the impact of lignin pathway modification in these switchgrass lines on simultaneous saccharification and fermentation (SSF) efficiency and their response to consolidated bioprocessing using *Clostridium thermocellum*. The fermentation of the control and transgenic lines confirmed that transgenic lines produced more ethanol per gram than the control without pretreatment. Fermentation of select down-regulated switchgrass lines after a moderate dilute acid pretreatment resulted in a 38% increase in ethanol yield over the pretreated control sample under SSF. The reduced recalcitrance in transgenic lines was further demonstrated with a ~300–400% reduction in the required cellulase dosage with respect to the control for equivalent ethanol yield via SSF. In addition, the consolidated bioprocessing of mild acid pretreated COMT transgenic switchgrass was observed to lead to 18% more fermentation products than the control (Fu et al., 2011). The effects of COMT down-regulation on cellulose were investigated and the results showed that the cellulose content in the stems of COMT transgenic lines had small variations at −1 to 3% relatively, whereas the cellulose crystallinity index and degree of polymerization were essentially identical to the control, suggesting that COMT down-regulation had negligible impact on cellulose in switchgrass. Therefore, the lignin structural characteristics in the COMT transgenic switchgrass is of great interest as its alterations due to the COMT down-regulation could be related to the reduced recalcitrance in the transgenic switchgrass. As a continuation of this work, here we investigated and compared the structural features of lignin isolated from wild-type versus COMT down-regulated switchgrass in an attempt to explore the roles that the lignin related structural changes could play in the reduced recalcitrance of transgenic switchgrass.

MATERIALS AND METHODS

CHEMICALS

Dimethyl sulfoxide-d_6 (DMSO-d_6), pyridine, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, anhydrous diethyl ether, acetic acid, chloroform, and enzymes were purchased from Sigma-Aldrich and used as received. All other chemicals were obtained from VWR and 1,4-dioxane was distilled over $NaBH_4$ prior to use.

PLANT MATERIALS AND SAMPLE COLLECTION

A lowland-type switchgrass (*Panicum virgatum* L.cv. Alamo) was used for genetic transformation and lignin modification (Fu et al., 2011). A COMT RNAi vector was introduced into switchgrass calli by *Agrobacterium*-mediated transformation. The transgenics were grown in the greenhouse at 26°C with 16 h light (390 μE/m^2s). The vegetative development of switchgrass was divided into four elongation stages (E1, E2, E3, and E4) according to the criteria described by Moore et al. (1991) and Shen et al. (2009). Under the greenhouse conditions, switchgrass produced four visible internodes from basal to top before entering into reproductive development.

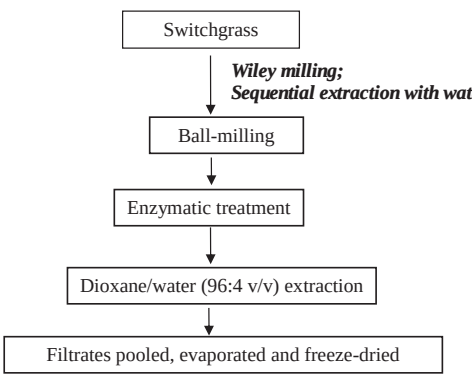
A transgenic line (T0–2) which had the greatest degree of COMT down-regulation was used to generate T1 progeny by crossing with a wild-type Alamo plant. The T1-generation materials were employed to study the structural differences in the COMT down-regulated transgenic and wild-type control plants. A null segregant (negative T1 plant identified from the progeny of the cross) was used as a control. The first internodes (hereafter referred as I1) from basal were collected for analysis when the plants had reached the E4 stage.

COMPOSITIONAL ANALYSIS

The compositional analysis was performed according to literature procedure (Davis, 1998; Cao et al., 2012). The switchgrass samples were sequentially extracted with water and dichloromethane 24 h each before analysis. The extractive-free switchgrass (~0.175 g) was subjected to a two-step hydrolysis with sulfuric acid (72% w/w, 1.5 mL) for 1 h at 30°C and then at 121°C for additional 1 h after diluting with the addition of water (4% w/w sulfuric acid). The samples were filtered and the filtrates were quantified for neutral monosaccharide contents using high performance anion exchange chromatography with pulsed amperometric detection (Dionex ICS 3000, Dionex Corp., Sunnyvale, CA, USA). The filtrates were diluted 25 times before analysis. Nanopure deionized water was used as the eluent and sodium hydroxide solution (0.4 M NaOH) as post-column rinsing effluent. Fucose was used as an internal standard. Flow rate was 1.0 mL/min. The residues were washed, dried, and weighed to give Klason lignin content. The measurements were carried out in duplicate and the results were reported as the average.

CELLULOLYTIC ENZYME LIGNIN ISOLATION

Cellulolytic enzyme lignin was isolated from control and transgenic switchgrass according to a slightly modified literature procedure (**Scheme 1**) (Chang et al., 1975; Capanema et al., 2004; Hu et al., 2006). In brief, the extractive-free, vacuum dried (40°C overnight) switchgrass samples were ball-milled in a porcelain jar with ceramic balls using a rotatory ball mill running at 96 rpm for 168 h under N_2. The milled fine cell wall powder was then subjected to enzyme treatment in acetic acid/ammonium acetate buffer (pH 4.8, 50°C) under continuous agitation at 200 rpm for 48 h. The residue was isolated by centrifugation and was



SCHEME 1 | Switchgrass cellulolytic enzyme lignin isolation.

hydrolyzed one more time with freshly added enzymes. The residue obtained was washed with deionized water, centrifuged, and freeze dried. The enzyme-treated residue was extracted with dioxane-water (96% v/v, 10.0 mL/g biomass) for 24 h × 2. The extracted mixture was centrifuged and the supernatant was collected. The extracts were combined, roto-evaporated to reduce the volume under reduced pressure (<45°C), and freeze dried. The obtained lignin samples were dried under vacuum at ~45°C for overnight before nuclear magnetic resonance (NMR) analysis.

NMR ANALYSIS

Nuclear magnetic resonance spectra of isolated lignin samples were acquired in a Bruker Avance/DMX 400 MHz spectrometer operating at a frequency of 100.59 MHz for the ^{13}C nucleus. The ^{13}C NMR acquisition was performed on a QNP probe using a 90° pulse with an inverse-gated decoupling pulse sequence, a 12-s pulse delay, and 12,288 scans at 50°C.

For quantitative ^{31}P NMR, lignin was dissolved in a solvent of pyridine/$CDCl_3$ (1.6/1.0 v/v) and derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. The spectrum was acquired using an inverse-gated decoupling pulse sequence (Waltz-16), 90° pulse, 25-s pulse delay, and 128–256 scans. *N*-hydroxy-5-norbornene-2,3-dicarboximide was used as an internal standard and the contents of hydroxyl groups were calculated on the basis of the internal standard. A standard Bruker heteronuclear single quantum coherence (HSQC) pulse sequence (hsqcetgp) was used on a BBO probe with the following acquisition parameters: spectra width 10 ppm in F2 (^{1}H) dimension with 2048 time of domain (acquisition time 256.1 ms), 210 ppm in F1(^{13}C) dimension with 256 time of domain (acquisition time 6.1 ms), a 1.5-s delay, a $^1J_{C–H}$ of 145 Hz, and 32 scans. The central DMSO solvent peak was used for chemical shifts calibration (δc 39.5 ppm, δ_H 2.50 ppm). Relative lignin monomer compositions and interunit linkage abundance were estimated semi-quantitatively using volume integration of contours in HSQC spectra (Ralph et al., 2004; Zhang and Gellerstedt, 2007; Kim et al., 2008; Yelle et al., 2008; Jiang et al., 2010; Kim and Ralph, 2010; Samuel et al., 2011; Mansfield et al., 2012). For monolignol compositions of S, G, H, *p*-coumarate (*p*CA), and ferulate (FA) measurements, the $S_{2/6}$, G_2, $H_{2/6}$, $pCA_{2/6}$, and FA_2 contours were used with G_2 and FA_2 integrals doubled (Higuchi et al., 1967; Marita et al., 2001; Ralph et al., 2001, 2004; Capanema et al., 2005; Zhang and Gellerstedt, 2007; Kim et al., 2008; Yelle et al., 2008; Jiang et al., 2010; Kim and Ralph, 2010; Mansfield et al., 2012). The Cα signals were used for contour integration for interunit linkages estimation. Bruker's TopSpin 2.1 software and MestReNova 8.0.0 software were employed for data processing and integrations.

MOLECULAR WEIGHT DISTRIBUTION ANALYSIS

The lignin molecular weight distribution analysis was performed with gel permeation chromatography (GPC). The lignin samples were acetylated before GPC analysis to dissolve in tetrahydrofuran. Dry lignin sample was dissolved a mixture of acetic anhydride/pyridine (1:1 v/v) and stirred at room temperature for 24 h. After the acetylation, the solvent was removed by rotary evaporation with adding of ethanol (95% v/v). The residue was dissolved in chloroform and precipitated with diethyl ether. The precipitate was centrifuged, washed with diethyl ether (×3), and dried under vacuum prior to GPC analysis. The molecular weight distributions of the acetylated lignin samples were analyzed on a GPC SECurity 1200 system (PSS-Polymer Standards Service, Warwick, RI, USA) operated on Agilent HPLC 1200 with four Waters Styragel columns (HR1, HR2, HR4, and HR6). An UV detector (270 nm) was used for detection. Tetrahydrofuran was used as the mobile phase (flow rate 1.0 mL/min). Polystyrene narrow standards were used for establishing the calibration curve.

RESULTS AND DISCUSSION

COMPOSITIONAL ANALYSIS

The compositional analysis results of switchgrass (internode I1) were presented in **Figure 1**. The COMT down-regulation resulted in a reduction of ca. 14% in Klason lignin content in the transgenic lines when compared to the wild-type control. The observed lignin content changes were consistent with the previous report that transgenic switchgrass had a lower lignin content estimated using acetyl bromide method (Fu et al., 2011). The neutral monosaccharides in both wild-type and transgenic switchgrass were primarily glucose and xylose as well as minor amounts of arabinose and galactose. While the COMT down-regulated transgenic line had slightly higher xylose content with comparison to the wild-type control, both lines appeared to have comparable contents of arabinose, galactose, and glucose which is consistent with an earlier study of COMT down-regulated switchgrass (Fu et al., 2011).

^{13}C NMR ANALYSIS

^{13}C NMR spectra for enzyme lignin samples isolated from the wild-type control and transgenic switchgrass (internode I1) were obtained, as presented in **Figure 2**. The signal assignments and quantitative analysis of ^{13}C NMR spectra of lignin were performed following literature reports (Higuchi et al., 1967; Marita et al., 2001; Ralph et al., 2001; Capanema et al., 2005; Pu et al., 2005; Hallac et al., 2010). The signals from both guaiacyl and syringyl units were readily observed from the G_2, G_6, and $S_{2/6}$ signals centered ~δ 109.5, 119.1, and 104.6 ppm, respectively. Significant

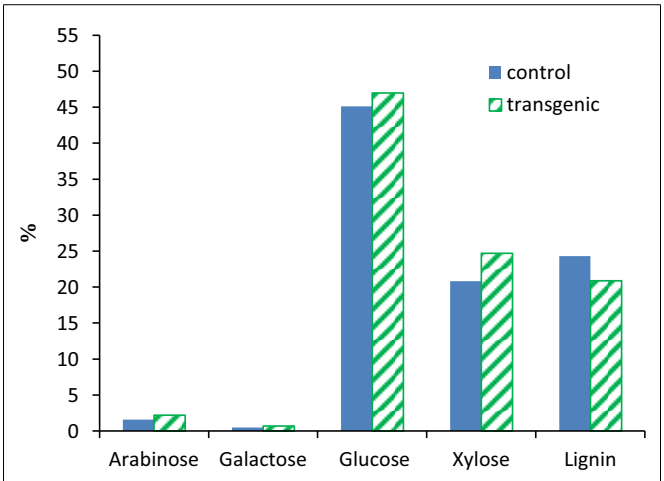


FIGURE 1 | Compositions of wild-type and COMT down-regulated switchgrass (internode I1). Contents expressed on a dry weight basis.

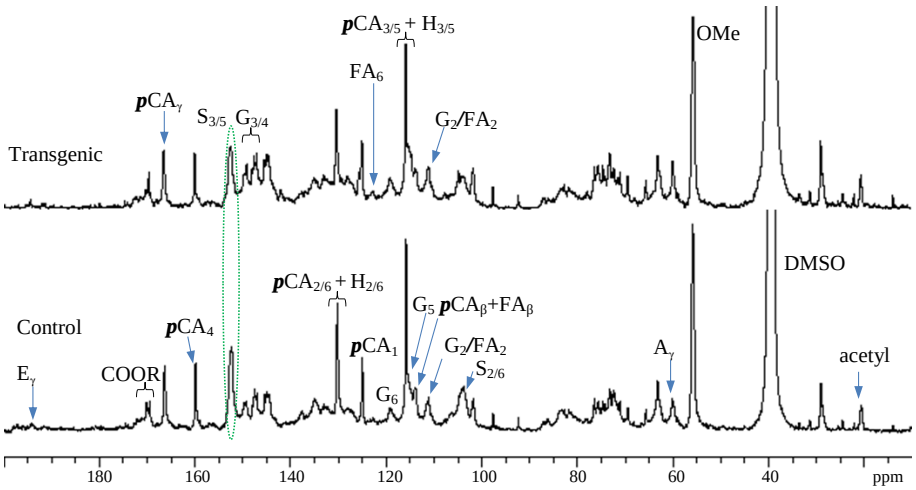


FIGURE 2 | Quantitative ^{13}C NMR spectra of enzyme lignins isolated from switchgrass internode I1 samples (Bottom: control; top: transgenic). A, β-O-4; G, guaiacyl; S, syringyl; H, *p*-hydroxyphenyl; *p*CA, *p*-coumarate; FA, ferulate; E, cinnamyl aldehyde; OMe, methoxyl.

amount of *p*-coumarates in both control and transgenic sample was observed from the presence of its carbonyl peak at δ 166.7 ppm and C_4 peak around δ 160.0 ppm. In addition, ^{13}C NMR spectra demonstrated a small peak around δ 122.9 ppm attributing to C_6 peak of ferulates, suggesting minor presence of ferulate units in the isolated enzyme lignin from wild-type and transgenic switchgrass. The signals of cinnamyl aldehyde (C_γ at ~194 ppm) and acetyl groups (methyl C around 20.1 ppm and carbonyl C ~170.2 ppm) were observed in both wild-type and COMT transgenic switchgrass lines. Compared to wild-type, the lignin in transgenic lines had a lower S/G ratio as revealed by a signal intensity reduction in $S_{2/6}$ peaks relative to G_2 peak as well as a reduction in $S_{3/5}$ peak intensity relative to $G_{3/4}$ peak intensity. The intense peak cluster in the spectral region of δ 79–65 ppm was largely due to the presence of carbohydrates in the isolated enzyme lignin. In addition, the peaks centered around 97.5 and 92.2 ppm arisen from anomeric carbons of xylan were further indicative of the noticeable presence of carbohydrates.

HSQC NMR ANALYSIS

The two-dimensional nature of ^{13}C-^{1}H HSQC NMR can provide valuable information about the chemical structures of lignin by deconvoluting or dispersing the overlapping carbon and proton resonances in the complex spectra over two spectral axes. **Figures 3** and **4** presented the aromatic and aliphatic regions of HSQC NMR spectra of lignin samples isolated from control and transgenic switchgrass I1 internodes. The observed lignin sub-units were presented in **Figure 5** and signal assignments were summarized in **Table 1** (Ralph et al., 2004; Zhang and Gellerstedt, 2007; Kim et al., 2008; Yelle et al., 2008; Jiang et al., 2009; Kim and Ralph, 2010; Mansfield et al., 2012). The correlation signals for various monolignols including syringyl, guaiacyl, *p*-hydroxyphenyl, *p*-coumarate, and ferulate units were evidently observed and distributed fairly well in the aromatic region of HSQC spectra. For example, the syringyl, guaiacyl, and *p*-hydroxyphenyl were readily identified due to the presence of their diagnostic cross peaks around 103.9/6.73 ($S_{2/6}$), 111.1/6.96 (G_2), 118.6/6.79 (G_6), and 127.5/7.13 ($H_{2/6}$) ppm, respectively. The strong cross peak centered at δ_C/δ_H 129.4/7.45 ppm was attributed to $C_{2/6}/H_{2/6}$ of *p*-coumarate (*p*CA), confirming the notable presence of *p*-coumarate with its $C_{3/5}/H_{3/5}$ correlation peaks overlapping with G_5 around 115.3/6.87 ppm. The signals for ferulate units were observed with its indicative cross peaks of FA_2 around 111.5/7.49 ppm. In addition, the cross peaks for vinyl carbons in *p*CA and FA structures were overlapped around C_α/H_α 144.5/7.47 and C_β/H_β 113.4/6.32 ppm. HSQC semi-quantitative estimation showed that the transgenic switchgrass lignin had a decrease in S/G ratio than the wild-type (0.45 vs. 0.78), supporting the observations from ^{13}C NMR analysis. Fu et al. (2011) investigated wild-type and transgenic switchgrass stem bulk (without separating the internodes) using GC/MS techniques and reported an S/G ratio decrease by ~32% for transgenic switchgrass. HSQC analysis revealed that *p*-hydroxyphenyl units were estimated to exhibit no significant changes accounting for 2–3% in wild-type and transgenic lignins. *p*-Coumarate units were observed to account for ca. 32 and 26% in lignins from wild-type versus transgenic switchgrass. In line with ^{13}C NMR analysis, HSQC spectra also demonstrated a weak signal of ferulate unit, which was estimated to account for ca. 6 and 11% in the isolated lignins of wild-type and transgenic lines, respectively. The *p*CA:FA ratio in the isolated enzyme lignin samples was observed to decrease (by ca. 50%) for the COMT transgenic switchgrass. Shen et al. (2012) reported a reduced ester-linked *p*CA:FA ratio in transgenic switchgrass with overexpression of PvMYB4 that demonstrated an increase in sugar release efficiency and suggested that saccharification efficiency was negatively correlated, in part, with cell wall ester-linked *p*CA:FA ratio.

In the side chain regions of HSQC spectra, the cross signals for methoxyl and β-*O*-4 substructures were among the most prominent ones. The phenylcoumaran substructure was evidenced by its C_α/H_α correlations centered at δ_c/δ_H 86.8/5.48 ppm. Resinol substructure was detected barely above the noise level

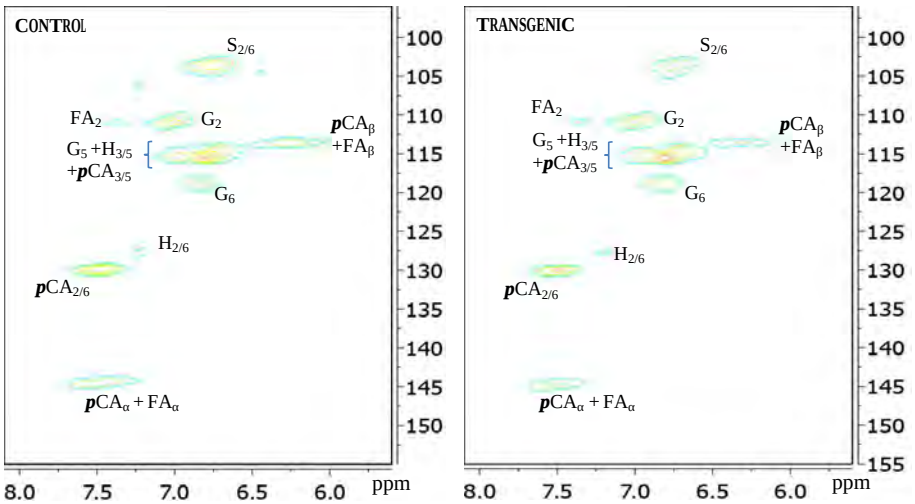


FIGURE 3 | Aromatic regions of ^{13}C-^{1}H HSQC spectra of enzyme lignins isolated from control and transgenic internode I1 switchgrass. G, guaiacyl; S, syringyl; H, *p*-hydroxyphenyl; *p*CA, *p*-coumarate; FA, ferulate.

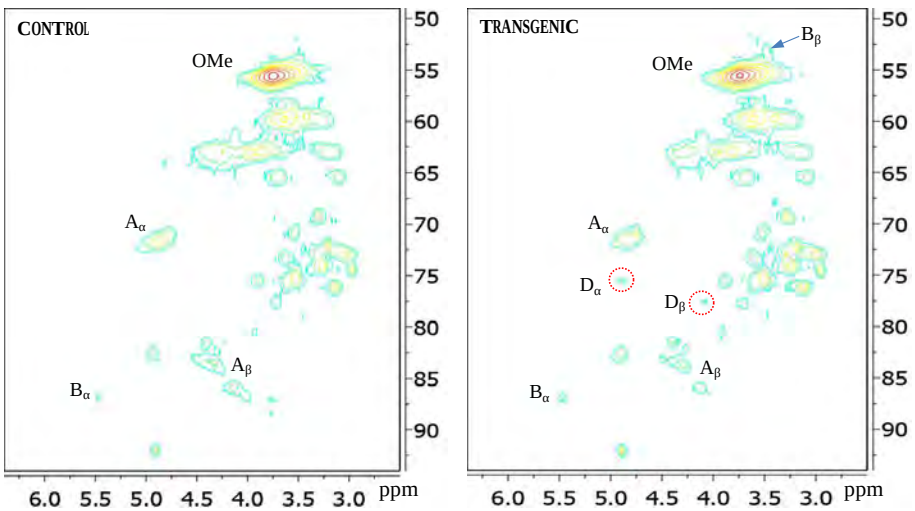


FIGURE 4 | Aliphatic regions of ^{13}C-^{1}H HSQC spectra of enzyme lignins isolated from control and transgenic internode I1 switchgrass. A, β-*O*-4 ether; B, phenylcoumaran; D, benzodioxane; OMe, methoxyl.

in the spectra. In addition, the presence of benzodioxane units (~5% of total interunit linkages) in the transgenic internode I1 lignin was apparently evidenced by the cross peaks at δ_C/δ_H 75.5/4.89 ppm (D_α) and 77.5/4.10 ppm (D_β), which were absent in the wild-type. The observation of benzodioxane substructure in COMT down-regulated switchgrass was consistent with previous reports that down-regulation of COMT during lignin biosynthesis resulted in the incorporation of 5-hydroxy coniferyl alcohol into lignin and generation of novel benzodioxane units (Lu et al., 2010; Vanholme et al., 2010). For example, Lu et al. (2010) reported that down-regulation of COMT in poplar led to the formation of benzodioxane units in lignin. Similarly, Vanholme et al. (2010) observed the newly formed benzodioxane units in COMT down-regulated *Arabidopsis*. The HSQC analysis showed that the isolated transgenic lignin had a decrease in β-*O*-4 linkages (by ~10%) and an increase in phenylcoumaran units (by ~57%) when compared to the wild-type.

^{31}P NMR ANALYSIS

The ^{31}P NMR is an effective tool for determining the presence of hydroxyl groups in lignin which can provide quantitative information for various types of major hydroxyl groups including aliphatic, carboxylic, guaiacyl, syringyl, C_5-substituted phenolic hydroxyls, and *p*-hydroxyphenyls (Pu et al., 2011a). The chemical structures of wild-type and COMT transgenic switchgrass lignins were characterized by phosphitylation followed-by ^{31}P NMR spectroscopy and the results were presented in **Figures 6** and **7**. **Figure 6** showed that the aliphatic OH appeared to be the dominant free

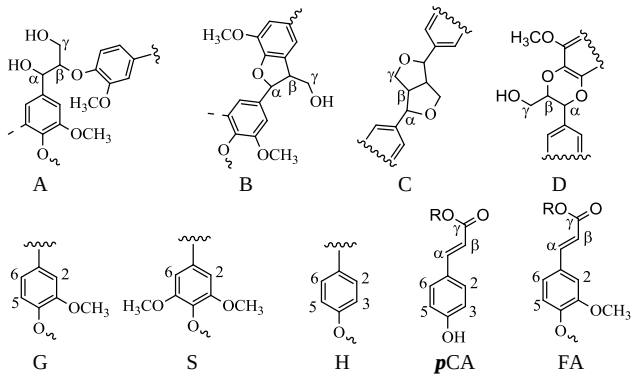


FIGURE 5 | Chemical structures in switchgrass lignin. G, guaiacyl; S, syringyl; H, *p*-hydroxyphenyl; A, β-*O*-4 ether; B, phenylcoumaran; C, resinol; D, benzodioxane; *p*CA, *p*-coumarate; FA, ferulate.

Table 1 | Signal assignments of chemical structures in ^{13}C-^{1}H HSQC NMR spectra of control and transgenic switchgrass lignin (Ralph et al., 2004; Zhang and Gellerstedt, 2007; Kim et al., 2008; Yelle et al., 2008; Kim and Ralph, 2010; Mansfield et al., 2012).

$\delta c/\delta_H$ (ppm)	Assignment
55.4/3.73	CH in methoxyl group
59.7/3.64	C_6 polysaccharide + A_γ
71.2/4.84	C_α/H_α in β-*O*-4 linkage (A)
73.1/4.48	C_2/H_2 in 2-*O*-Ac-β-D-Xylp
74.6/4.81	C_3/H_3 in 3-*O*-Ac-β-D-Xylp
82.8/4.45	C_β/H_β in β-*O*-4 linkage to G (A)
85.8/4.09	C_β/H_β in β-*O*-4 linkage to S (A)
86.8/5.48	C_α/H_α in phenylcoumaran (B)
75.5/4.89	C_α/H_α in benzodioxane (D)
77.5/4.10	C_β/H_β in benzodioxane (D)
103.9/6.73	$C_{2,6}/H_{2,6}$ in syringyl units
111.1/6.96	C_2/H_2 in guaiacyl units
115.3/6.87	C_5/H_5 in guaiacyl units
118.6/6.79	C_6/H_6 in guaiacyl units
122.2/7.07	C_6/H_6 in ferulate units
127.5/7.13	$C_{2,6}/H_{2,6}$ in *p*-hydroxyphenyl units
129.4/7.45	$C_{2,6}/H_{2,6}$ in *p*-coumarate
144.5/7.47	C_α/H_α in *p*-coumarate/ferulate

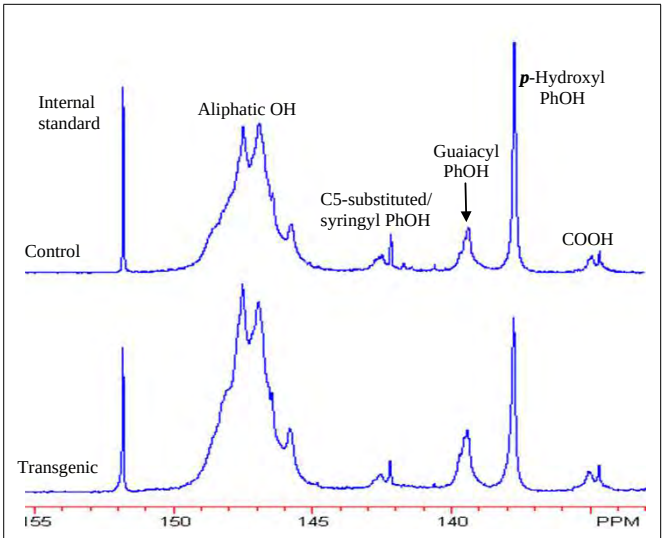


FIGURE 6 | ^{31}P NMR spectra of lignin samples isolated from wild-type and COMT transgenic switchgrass. Top: control; bottom: transgenic.

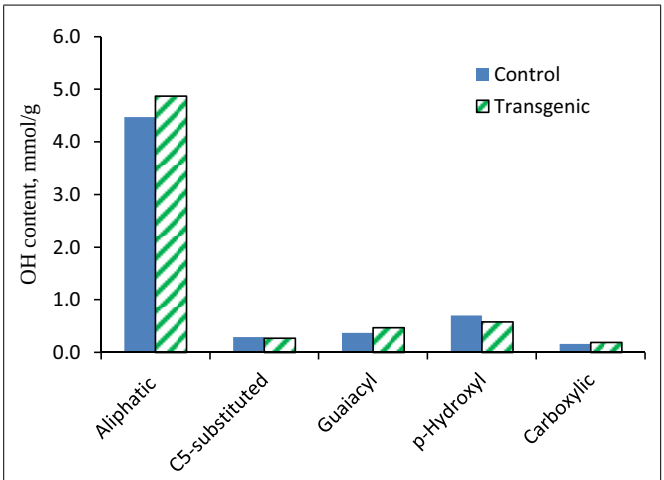


FIGURE 7 | Hydroxyl group contents (millimole per gram) in lignin samples isolated from the wild-type and COMT transgenic switchgrass as determined by ^{31}P NMR analysis.

hydroxyl type in both wild-type and transgenic switchgrass lignin, accounting for ca. 75% of total free hydroxyl groups, part of which can be attributed to sugar hydroxyl groups. The ^{31}P NMR spectra showed that the switchgrass lignin had a sharp and prominent signal of *p*-hydroxyl phenolic OH, which was mostly attributed from the *p*-coumarate substructures in switchgrass lignin. Among the free phenolic hydroxyls, the *p*-hydroxyl was observed to be the most prominent type (0.58 mmol/g in transgenic and 0.70 mmol/g in control), which accounted for 9.1% in transgenic lignin and 11.7% in wild-type control, respectively. Compared to the wild-type, the transgenic switchgrass lignin appeared to have an increase (ca. 8.9%) in aliphatic OH group content. The total amount of free phenolic OH groups in COMT transgenic switchgrass lignin appeared to be comparable with that in the wild-type control, while the amount of *p*-hydroxyl phenolic OH was observed to decrease in COMT transgenic lignin, in line with the HSQC results that COMT down-regulation resulted in a decrease in lignin *p*-coumarate substructure. In addition, both lignin samples had a small amount of free carboxylic hydroxyl groups and their contents remained relatively unchanged after COMT down-regulation.

GPC CHARACTERIZATION

To further understand the effects of COMT down-regulation on the lignin structures in the lignin biosynthesis process, GPC analysis was used to determine the molecule size and molecular weight distribution of lignin. **Figure 8** provided the GPC chromatograms

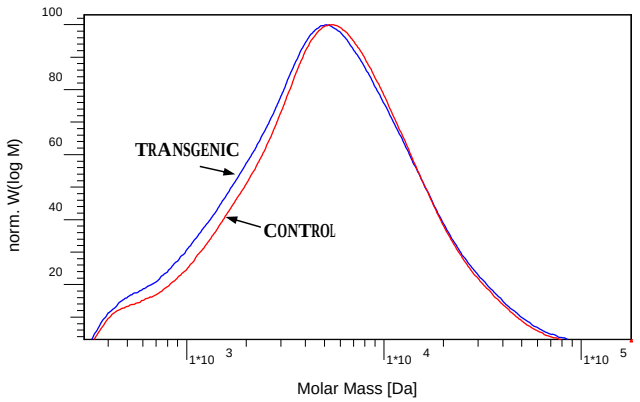


FIGURE 8 | Molecular weight distribution curves of acetylated lignin isolated from the wild-type and COMT transgenic switchgrass.

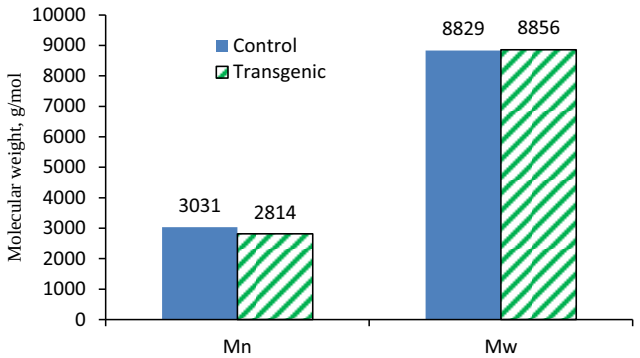


FIGURE 9 | The number-average (M_n) and weight-average (M_w) molecular weights of lignin isolated from the wild-type and COMT transgenic switchgrass as determined by GPC analysis.

of lignin samples isolated from the wild-type and COMT transgenic switchgrass. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of the lignin fractions were determined by GPC and the results were summarized in **Figure 9**. **Figure 8** showed that the lignin samples demonstrated a similar shape of molecular weight distribution curve, although the COMT transgenic lignin had a slightly higher portion in the low molecular weight range. The GPC analysis showed that the COMT transgenic lignin had comparable molar mass results compared to the wild-type control (**Figure 9**), indicating that COMT down-regulation under the conditions in this study appeared to have no significant impact on the molecule size and molecular weight distribution of lignin.

LIGNIN RELATED RECALCITRANCE REDUCTION

To date, several plants have been investigated for the effects of COMT down-regulation on lignin content, structure, and subsequent digestibility of these transgenic lines. For instance, COMT down-regulation of lignin in poplar has resulted in a decrease in lignin content, formation of benzodioxane, and a substantial decrease in syringyl units (Jouanin et al., 2000; Lu et al., 2010). In alfalfa, COMT down-regulation was reported to impact a marginal decrease in lignin content, a slight reduction in guaiacyl units, nearly total loss of syringyl units and the development of minor amount of 5-hydroxy guaiacyl units (Guo et al., 2001). Similarly, Sewalt et al. (1997) reported that COMT down-regulated tobacco had a decrease in S/G ratio and no change in lignin content and exhibited an improved cell wall digestibility. In COMT down-regulated transgenic maize, a strong decrease in lignin content, a decrease in S, H, and *p*-coumaric acid units, and the occurrence of 5-OH-guaiacyl units were reported to lead to improved digestibility (Piquemal et al., 2002). The lignin related changes observed in this study, such as the decrease in S/G and *p*CA:FA ratios and a decrease in total lignin content, might collectively contribute, in part, to the reduced recalcitrance in the COMT down-regulated switchgrass.

CONCLUSION

The COMT down-regulated transgenic switchgrass demonstrated a decrease in lignin content and structural alterations compared to the wild-type. A decreases in both S/G and *p*-coumarate:ferulate ratios, an increase in the relative abundance of phenylcoumaran units as well as the incorporation of benzodioxane units into lignin were observed in the transgenic switchgrass lignin. In addition, the COMT transgenic switchgrass lignin had an increase (ca. 8.9%) in aliphatic OH group content, while the total amount of free phenolic OH groups appeared to be comparable with that in the wild-type control. No significant effects of COMT down-regulation on the lignin molecular weights were observed. The reduction in recalcitrance of transgenic switchgrass appeared to be related, in part, to a combination of alterations in lignin content and its structures resulting from the COMT down-regulation.

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