Elucidation of Lignin and Polysaccharide Linkages in Wheat Straw by $^{2}H/^{13}C$ Isotopic Tracer

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To elucidate chemical linkages between lignin and polysaccharides, the aqueous mixed solutions of coniferin-[α-^{13}C], syringin-[α-^{13}C], D-glucose-[6-^{2}H], and phenylalanine ammonia-lyase inhibitor were injected into a living wheat stalk. Internode tissues with high abundance of ^{2}H-^{13}C were collected. The milled wood lignin, lignin-carbohydrate complex (LCC), and residual LCC (R-LCC) with enrichment of ^{2}H-^{13}C were isolated. The ^{13}C and ^{2}H abundances showed that the lignin and polysaccharides of internode tissues were labeled by ^{13}C and ^{2}H, respectively. Analysis with carbon-13 nuclear magnetic resonance (^{13}C-NMR) showed that ketal and benzyl ether bonds were formed between α-C of lignin and carbohydrates. The R-LCC and LCC were further treated with enzymes to obtain enzymatic degraded R-LCC (ED-R-LCC) and enzymatic degraded LCC (ED-LCC). ^{13}C-NMR spectra of ED-LCC showed that the α-C of lignin side chain was combined with 6-C of carbohydrates by ether, ester, and ketal linkages. ^{1}H-NMR differential spectra of ED-LCCs revealed an LC linkage of benzyl ether bond. Glucan-lignin (En-R-GL) and xylan-lignin (En-R-XL) complexes were separated from ED-R-LCC by ionic liquid. A part of lignin α-C was linked to cellulose 6-C by benzyl ether and α-ketal linkages. ^{13}C-NMR spectra of En-R-XL showed there were α-benzyl ether and α-ketal bonds between lignin and xylan.

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INTRODUCTION

The cell wall of lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin. Lignin-carbohydrate complexes (LCC) have chemical bonds between carbohydrates and lignin moieties, and these can seriously inhibit the separation of cellulose from lignin and hemicellulose (Björkman 1956). In the process of biorefinery and pulping, cellulose and lignin in plant raw materials are difficult to separate efficiently, which results in huge resource consumption and energy waste (Tribot *et al*. 2019).

LCC linkage plays a crucial role in wood structure, since all lignin moieties in softwoods and 47 to 66% of lignin fragments in hardwoods (Henriksson *et al*. 2007) are bound to carbohydrates, mainly to hemicellulose (Lawoko *et al*. 2005). Lignin and xylose in softwood are mainly linked by ether bonds, while lignin and mannose are mainly linked by phenyl glycosidic bond. Xyloses in hardwood LCC are mainly linked with lignin by benzyl ether bonds (Koshijima and Watanabe 2003), while lignin structures are linked to 30% glucuronic acid through the benzyl ester bond (Takahahsi *et al*. 1988). LCC from grass is structurally different from that in woods due to the incorporation of

hydroxycinnamates into the cell wall (You *et al*. 2015). It is well established that ferulic acid is ester-linked to carbohydrates and ether-linked to lignin in the cell wall of grass, forming a "lignin–ferulate–polysaccharide" (LFP) complex (Buranov and Mazza 2008). The LCC in wheat straw were particularly enriched with xylan and exhibited narrow polydispersity (Yao *et al*. 2015). Although previous work has studied many wood LCC and wheat LCC, the chemical linkage between cellulose and lignin in wheat LCC is still worthy of in-depth study, which may help develop the appropriate process to break the lignin-carbohydrate bond for efficient and selective separation of cellulose from biomass.

Many studies have shown that the chemical linkages of LCC belong to the following types: benzyl ether bond, benzyl ester bond, ketal bond, and phenyl glycosidic bond, *etc*. (Xie *et al*. 2000). The quinone methide intermediates in lignin biosynthesis couples with carbohydrate to form benzyl ether and benzyl ester bonds. The hydroxyl groups of alcohol and phenol on lignin moieties can be easily combined with the hydroxyl groups of glycosides on carbohydrates to form phenyl glycosidic bonds. Acidification (Eriksson *et al*. 1980), alkaline hydrolysis (Takahashi and Koshijima 1988), and other degradative methods can be used to study the structure of LCC. However, during its treatment process, it is inevitable to cause damage to the structure of lignin and carbohydrates. Therefore, non-destructive methods are required to better understand LCC structure without cleavage of lignin-carbohydrate (LC) bonds, and thus to extract lignocelluloses effectively and selectively (Tarasov *et al*. 2018).

Isotope labeling technique has a wide application in exploring the structure of LCC. Xie *et al*. (1991, 1993, 1994a,b) synthesized the lignin precursors of coniferin-[α-^{13}C], coniferin-[β-^{13}C], and coniferin-[γ-^{13}C] and successively carried out selective ^{13}C enrichments of ginkgo and rice straw lignin side chains (Cα, Cβ, and Cγ). The ^{13}C abundance of Cα of newly-formed xylem of ginkgo wood was 3.5 times more than that of natural abundance. Milled wood lignin (MWL) was prepared from the xylem of ginkgo, and the ^{13}C-NMR spectrum of lignin was analyzed. The results showed that the structure contained α-carbonyl, α-aldehyde, Cγ-carbonyl, Cγ-carboxyl, methylene, and phenylcoumaran. The connection between lignin and carbohydrates was also found. Xiang *et al*. (2013) studied the cellulose precursor, *i.e.*, uridine diphosphate glucose-[6-^{13}C], which was synthesized and injected into a living ginkgo tree together with a lignin inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP), and an exogenous lignin precursor. The ^{13}C-enriched LCC was isolated from the newly-formed xylem of ginkgo shoots. Then, it was degraded by cellulase and hemicellulase. Thus, the lignin-rich fractions, which were called enzyme-degraded LCC (ED-LCC), were obtained. Through determining their carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra, the bond formation between C6 position of glucose units in cellulose and carbons of lignin side chain were confirmed to be benzyl ether linkage. However, Xiang's study was able to prove the existence of LC bonds from the cellulose side only.

Enzymatic hydrolysis cuts the long chains of carbohydrates without changing the structure of the LC bonds due to its high selectivity and mild conditions (Karlsson *et al*. 2001). At present, researchers have proposed a general classification process for extracting LCC from lignocellulosic biomass, *i.e.*, the non-extractable biomass is first ground by ball milling and dissolved in DMSO/Tetra-Butyl-Ammonium Hydroxide (TBAH) solution, and three LCC fractions are further extracted as follows: a glucan-enriched fraction (glucan-lignin, GL), a mannan-enriched fraction (GML), and a xylan-enriched fraction (xylan-lignin, XL) (Du *et al*. 2013).

The inhibitor of phenylalanine ammonia lyase (PAL), *i.e.*, (carboxymethyl methoxy amine hydrochloride, AOA) was applied. The AOA can inhibit the transformation of D-glucose-[6-$^{2}H_2$] to lignin as shown in Fig. 1. The coniferin-[α-^{13}C] was degraded into the corresponding coniferyl alcohol by β-glucosidase in cells and deposited in the biosynthesis of lignin macromolecules.

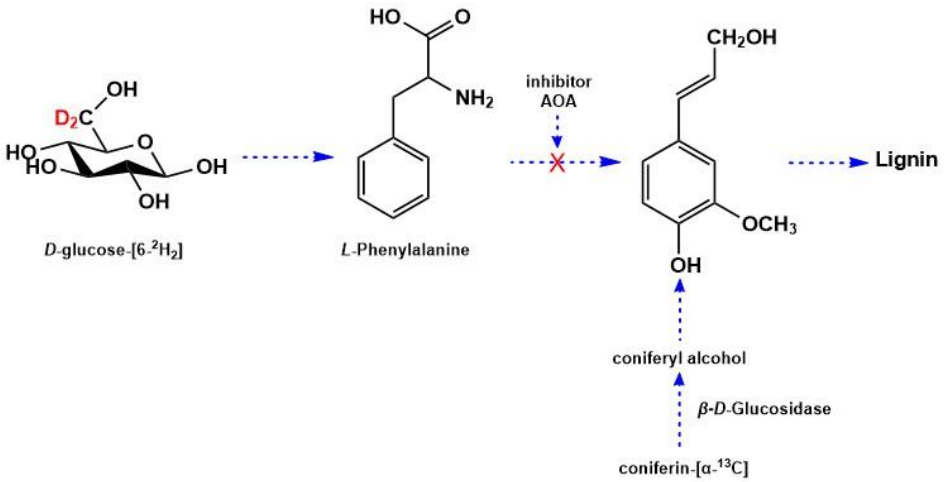

Fig. 1. Inhibition of the transformation of D-glucose-[6,6-$^{2}H_2$] to lignin and metabolism of coniferin-[α-^{13}C]

This research used ^{13}C-enriched lignin and ^{2}H-enriched carbohydrate precursors, respectively. The internode tissues with high abundances of ^{2}H-^{13}C from wheat straw were collected. As compared with previous studies, this research applied the Björkman's method (1957) and enzymatic hydrolysis to remove the fragments rich in hemicellulose, thereby enriching the fragments with LC bonds between cellulose and lignin. Then, the linkages between lignin and cellulose were elucidated clearly by ^{13}C-NMR and ^{1}H-NMR differential spectral analysis.

EXPERIMENTAL

Materials

Wheat *Emai 596* was provided by Hubei Academy of Agricultural Sciences (Wuhan, China). Sodium acetate-1-^{13}C and D-glucose- [6-$^{2}H_2$] were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The AOA was purchased from McLin (Shanghai, China). All other chemicals used were of analytical grade.

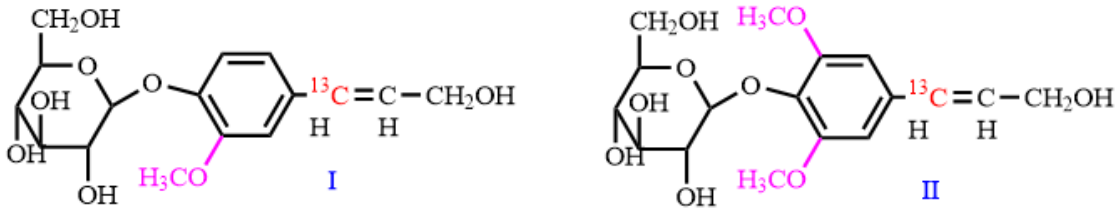

Fig. 2. Chemical structures of coniferin-[α-^{13}C] (I) and syringin-[α-^{13}C] (II)

Synthesis of Isotope Labeled Lignin Precursors

According to the method of Xie *et al*. (1991, 1993, 1994a, and 1994b), Sodium acetate-1-^{13}C was used as the starting material in the synthesis of coniferin-[α-^{13}C] and syringin-[α-^{13}C].

Administration of Lignin and Carbohydrate Precursors

Two batches of growing wheat *Emai 596* were selected as the experimental plant because of its wide internode cavities. Before wheat heading in early March, the aqueous solutions of D-glucose-[6-$^{2}H_2$] (5 mg/mL) mixed with coniferin-[α-^{13}C] (5 mg/mL) or syringin-[α-^{13}C] (5 mg/mL) were injected into the internodal cavities of the first, second, and third sections from the root to the top of two batches of wheat, as shown in Table 1,, Fig. 3 (a) and Fig. 3 (b). To inhibit the conversion of deuterium-labeled glucose to lignin, a solution of AOA (0.35 mg/mL), which was the inhibitor of phenylalanine ammonia-lyase, was also injected. After the injection, the plant was allowed to grow for 30 days (Imai and Terashima 1992).

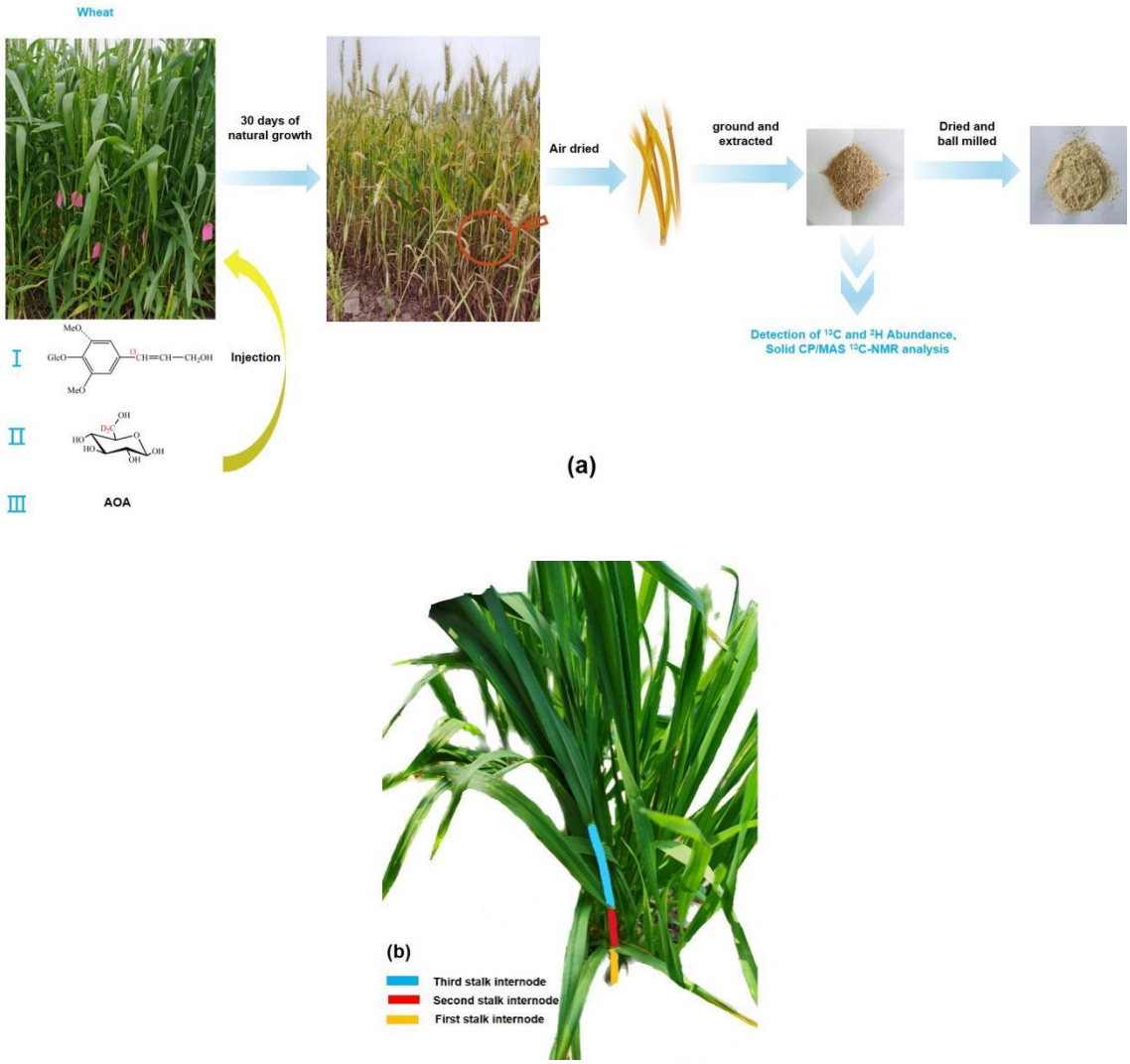

Fig. 3. Administration of Lignin and Carbohydrate Precursors: (a) Flow chart showing the injections of lignin precursors, carbohydrate precursor, and AOA into wheat internode cavities and the subsequent processing; (b) Stalk internodes from root to top

Table 1. Concentration of the Solution Injected to Wheat Internode

	Coniferin-[α-^{13}C]	Syringin-[α-^{13}C]	D-Glucose-[6-$^{2}H_2$]	AOA
Control	-	-	-	-
Group G	5 mg/mL	-	5 mg/mL	0.35 mg/mL
Group S	-	5 mg/mL	5 mg/mL	0.35 mg/mL

Preparation of Extractive-free Wheat Straw Mill

Wheat stalks fully absorbed the two types of lignin precursors and D-glucose-[6-$^{2}H_2$] samples and were harvested after their complete maturity. The internode tissues of group C, group G, and group S were collected. The wheat straw samples were then fully air-dried and ground using a Wiley mill to pass through 60-mesh screen. The milled straw was extracted by ethanol-benzene mixture (1/2, v/v) and hot water, and then air-dried.

Determination of ^{13}C and Deuterium Abundances

The ^{13}C and ^{2}H isotope abundances in 1.0 mg wheat tissue samples were determined by an elemental analyzer - isotope ratio mass spectrometer (EA-IRMS) equipped with FLASH2000 elemental analyzer (Thermo Fisher Scientific GmbH, Dreieich, Germany) and Delta V isotope mass spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany). Then, the value of $^{13}C\alpha/^{12}C\alpha$ was calculated using Eqs.1 to 3, while D6/H6 was calculated by Eqs. 4 to 5:

$$^{13}C/^{12}C = 1.11802\% \times (1 + \delta^{13}C \div 1000) \quad (1)$$

$$(^{13}C\alpha/^{12}C\alpha)_G = 1.07252\% + (^{13}C/^{12}C - 1.07252\%) \div 0.2073 \times 10 \quad (2)$$

$$(^{13}C\alpha/^{12}C\alpha)_S = 1.07252\% + (^{13}C/^{12}C - 1.07252\%) \div 0.2073 \times 11 \quad (3)$$

In Eqs. 1 to 3, $^{13}C/^{12}C$ is the ratio of ^{13}C and ^{12}C abundances in the sample; $\delta^{13}C$ is the relative ^{13}C isotope abundance value of the sample (Vienna Pee Dee Belemnite, VPDB‰); 1.11802% is the ^{13}C isotope abundance of the standard sample of VPDB; $^{13}C\alpha/^{12}C\alpha$ is the ^{13}C and ^{12}C isotopic ratios of Cα position in the lignin structural units of the sample; 1.07252% is the natural abundance of ^{13}C isotope of wheat straw; 0.2073 is the lignin content in wheat straw; 10 is the ratio of total ^{13}C content to α-^{13}C content in guaiacyl propane structural unit, 11 is the ratio of total ^{13}C content to α-^{13}C content in syringyl propane structural unit. The relative abundances of D are shown in Eqs. 4 and 5:

$$D/H = 0.015575\% \times (1 + \delta D \div 1000) \quad (4)$$

$$D6/H6 = (D/H - 0.01317\%) \div 0.445 \times 5 + 0.01317\% \quad (5)$$

In Eqs. 4 and 5: D/H is the ratio of D and H abundances in the sample; δD is the relative D isotope abundance value of the sample (Vienna Standard Mean Ocean Water, VSMOW‰); 0.015575% is the D isotope abundance of the standard sample VSMOW, D6/H6 is the abundance ratio of D to H on glucose 6-C position in the sample; 0.01317% is the natural abundance of D isotope of wheat straw; 0.445 is the cellulose content in wheat straw; 5 is the ratio of total D content in glucose unit to D content on 6-C.

Determination of CP/MAS ^{13}C-NMR Spectrum of Milled Wheat Straw

An Avance III HD 600 MHz wide-cavity solid-state NMR spectrometer (Bruker, Billerica, MA, USA) was used. The samples were continuously scanned at 150.6 MHz to obtain ^{13}C-NMR by conventional cross polarization (CP) and magic angle spinning (MAS)

methods. Experimental conditions were as follows: temperature 25 °C, pulse delay 3 s, acquisition time of 0.05 s, pulse width 75 kHz, and 5000 scans.

Preparation of LCC and R-LCC

As shown in Fig. 4， the extractives in milled wheat straw (20 g ground sample with a Wiley mill and sieved using 100-mesh) were removed. The extracted sample was dried *in vacuo* with P_2O_5 for 2 weeks. The extractive-free milled straw was further ground by a water-cooling vibration ball mill for 72 h, and it was extracted three times with aqueous dioxane (96/4, v/v).

The dioxane solution was subjected to rotary evaporation and freeze-drying to obtain crude MWL. A filtrate was obtained by dissolving in acetic acid aqueous (9/1, v/v) and followed by filtration. The filtrate was added dropwise to deionized water and followed by centrifugation. The obtained precipitate was dissolved in dichloroethane-ethanol (2/1, v/v) and centrifugated. The filtrate was added dropwise to absolute ether and then centrifugated. The precipitate was washed twice with absolute ether, and then it was washed once with petroleum ether. After vacuum drying, MWL 0.8 g was obtained with a yield of 4 %.

The Residue I was extracted with acetic acid-water (1/1, v/v) for three times, and the resulting precipitate was R-LCC 15.6 g with a yield of 78%.

The obtained acetic acid-water solution was freeze-dried by rotary evaporation, extracted with DMF, and added dropwise to dichloroethane-ethanol (2/1, v/v). After centrifugation, the precipitate was washed once with dichloroethane-ethanol (2/1, v/v) and washed three times with absolute ether. After vacuum drying, the product was dissolved in acetic acid-water (1/1, v/v), and added dropwise to acetone. The precipitate was obtained by centrifugation and washed once with acetone-acetic acid (96/1, v/v), three times with absolute ether, and once with petroleum ether. After vacuum drying, 2.66 g LCC was obtained with a yield of 13.3%.

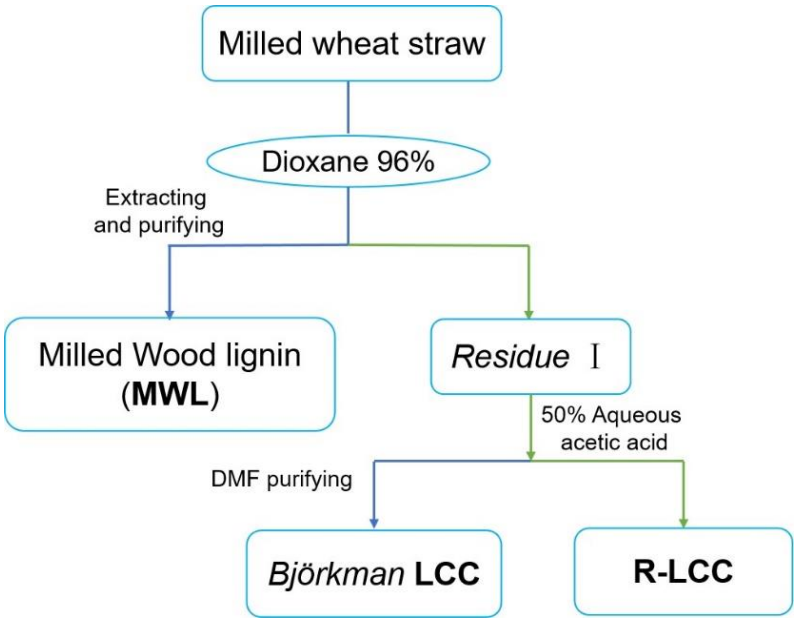

Fig. 4. Preparation processes of MWL, LCC and R-LCC

Enzymatic Hydrolysis of LCC and R-LCC

Cellulase (Onozuka RS, Yakult Pharmaceutical Industry Co., Nishinomiya, Japan, 16,000 units/g), hemicellulase (from *Aspergillus niger*, sigma, ≥ 1500 units/g), and xylanase (from *Thermomyces canuginosus*, sigma, ≥ 2500 units/g) were completely dissolved in 120 mL 0.5 M acetic acid/sodium acetate buffer (pH=4.5). The enzyme solution was filtered by a G4 glass filter and stored at 5 °C.

Approximately 2 g of LCC was added to 20 mL of the above enzyme solution and 80 mL of the acetic acid/sodium acetate buffer, and a little toluene was then dropped as a protective agent. The mixture was shaken in a water bath at 50 °C for 48 h, and centrifuged. After washing with deionized water 4 times, and then freeze -dried, ED-LCC 0.2 g was obtained with a yield of 10% (Lin *et al*. 1992).

The R-LCC (10 g) was added to 100 mL of the above enzyme solution and 400 mL of the acetic acid/sodium acetate buffer. Then the above enzymatic hydrolysis steps were repeated. After centrifugation and freeze-drying, 1.2 g En-R-LCC was obtained with a yield of 12%.

Classification of En-R-LCC Components by Ionic Liquid

As shown in Fig. 5, En-R-LCC (1.0 g) was added to the ionic liquid composed of DMSO/TBAH (5 mL/5 mL). The mixture was stirred continuously for 12 h until the sample was completely dissolved. The solution was then dropped into 100 mL deionized water with stirring and centrifuged to obtain a precipitate and supernatant. The precipitate was fully washed with deionized water to neutral. After freeze drying, the glucan-lignin fraction (En-R-GL) 150 mg was obtained with a yield of 15%. The supernatant was neutralized with dilute HCl, dialyzed (1000 Da) and freeze-dried to obtain 250 mg of xylan-lignin fraction (En-R-XL) with a yield of 25 %.

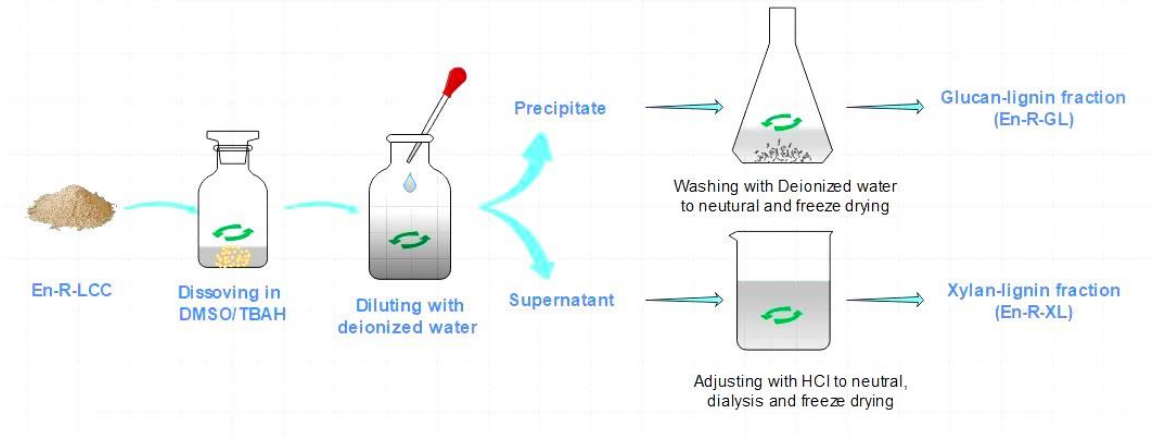

Fig. 5. Classification of wheat straw En-R-LCC with ionic liquid

Acetylation of Samples

A total of 150 mg of En-R-LCC or En-R-GL was added to the mixture of DMSO/N-methylimidazole (3 mL/1 mL). The mixture was stirred for 5 h until the sample was completely dissolved. Then, 0.6 mL of acetic anhydride was added and allowed to react for 1 h. The solution was dropped into 50 mL deionized water with stirring. After complete precipitation, the mixture was centrifuged, washed 4 times with water, and freeze dried to give acetylated products (Ac-En-R-LCC and Ac-En-R-GL).

Determination of Solution NMR Spectrum

A total of 90 mg of sample was completely dissolved in 0.6 mL DMSO-d_6 using a ϕ 5-mm NMR tube. The NMR spectrum was recorded by a Bruker Avance III 600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 294 K and scanned at 150 MHz for ^{13}C-NMR. Analysis conditions were as follows: pulse delay 2 s, acquisition time 0.9 s, and 20,000 scans. Spectrum with 2 Hz line broadening was used for processing.

^{1}H-NMR spectrum recording parameters included scanning at 600 MHz, pulse delay of 1.0 s, acquisition time of 2.7 s, and 500 scans with 12k acquisition data points.

RESULTS AND DISCUSSION

Analysis of ^{13}C/^{2}H Abundances in Wheat Internode Tissues

As shown in Tables 2 and 3, the abundances of ^{13}C and D (^{2}H) in the labeling groups were noticeably increased. The ^{13}Cα/^{12}Cα ratio of milled wheat straw sample labeled with ^{13}C and D was about three times that of natural isotope ratio. The D6/H6 ratio was about eight times that of natural isotope ratio, indicating that exogenous coniferin-[α-^{13}C], syringin-[α-^{13}C], and D-glucose-[6-$^{2}H_2$] were effectively absorbed and metabolized during the growth of wheat internode tissues. This resulted in lignin in the cell wall of wheat straw that was labeled with ^{13}C, while the polysaccharides were labeled with deuterium.

Table 2. ^{13}C Abundance of Milled Wheat Straw Administered with Coniferin-[α-^{13}C], Syringin-[α-^{13}C], and D-Glucose-[6,6-$^{2}H_2$]

Sample	δ^{13}C (VPDB)	^{13}C/^{12}C (%)	13Cα/^{12}Cα (%)
Control	-30.296	1.084	1.084
G1	24.216	1.145	4.573
G2	10.531	1.130	3.835
G3	-18.621	1.097	2.263
S1	26.705	1.148	4.708
S2	13.133	1.133	3.976
S3	-14.435	1.102	2.489
Sample labels: Control: Naturally grown milled wheat straw; G: guaiacyl propane moieties labeled with α-^{13}C structure by injection of the coniferin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$]; S: syringyl propane moieties labeled with α-^{13}C by injection of syringin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$]; and 1, 2, 3: Internode sequence from the root to the top.			

Table 3. ^{2}H Abundance of Milled Wheat Straw Administered with Coniferin-[α-^{13}C], Syringin-[α-^{13}C], and D-Glucose-[6,6-D_2]

Sample	δD (VSMOW)	D/H (%)	D6/H6 (%)
Control	-154.469	0.0132	0.0132
G1	377.346	0.0215	0.106
G2	224.132	0.0191	0.0794
G3	67.711	0.0166	0.0520
S1	501.841	0.0234	0.128
S2	282.716	0.0200	0.0897
S3	91.918	0.0170	0.0563

Solid-State CP/MAS ^{13}C-NMR Analysis of Wheat Straw Mill

As shown in Fig. 6, the CP/MAS ^{13}C-NMR spectra of milled wheat straw administered with syringin-[α-^{13}C], wheat straw mill administered with coniferin-[α-^{13}C], and natural wheat straw were analyzed. The results showed that there was no remarkable difference in the aromatic regions (δ 110 ppm to 160 ppm).

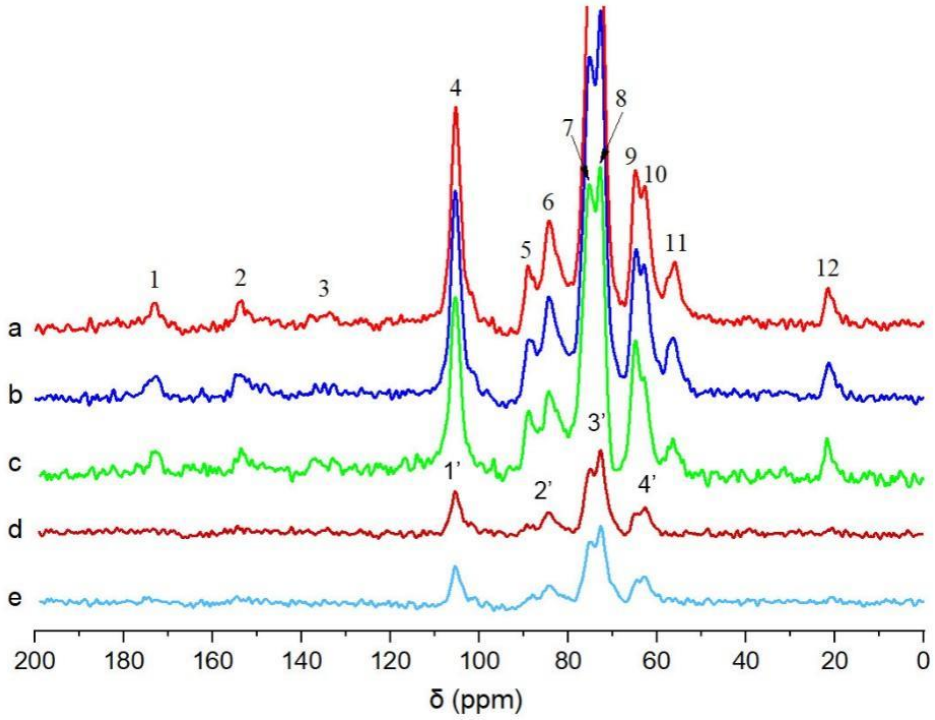

Fig. 6. CP/MAS ^{13}C-NMR spectra of milled wheat straw: a: administered with syringin-[α-^{13}C], b: administered with coniferin-[α-^{13}C], c: control group, d: difference spectrum obtained by subtracting spectrum (c) from spectrum (a), and e: difference spectrum obtained by subtracting spectrum (c) from spectrum (b)

To distinguish the signals of lignin side chain carbons labeled with ^{13}C isotope from that in unlabeled samples, the CP/MAS ^{13}C-NMR spectra of milled wheat straw were differentiated. In the differential spectra as shown in Fig. 6, all the other carbon signals were eliminated except for the ^{13}C enhanced signal. Therefore, the signals could be assigned more accurately to different types of α-C according to the chemical shifts. The peak of -OCH_3 at δ 56.0 ppm (No. 11) with stable content was used as the internal reference, and the tentative assignment of each signal is shown in Table 4. An enhanced signal at δ 100.5 to 110.2 ppm (No.1') is the signal of α-C with ketal linkages to carbohydrates (Xie *et al*. 2000). The peak at δ 93.1 to 80.7 ppm (No. 2') primarily arises from α-C in β-5, β-β, and benzyl ether linkage to carbohydrates (Xiang *et al*. 2014). The strong signal at δ 80.1 to 67.9 ppm (No. 3') are assigned to the α-C in the β-O-4 structure and α-C with ester linkage to carbohydrates (Xie *et al*. 1991), while the signal at δ 67.9 to 58.0 ppm (No. 4') arises from α-C in the β-1 structure between lignin moieties (Hafrén *et al*. 2002). Therefore, the connection between lignin structural units and LC bonds can be elucidated.

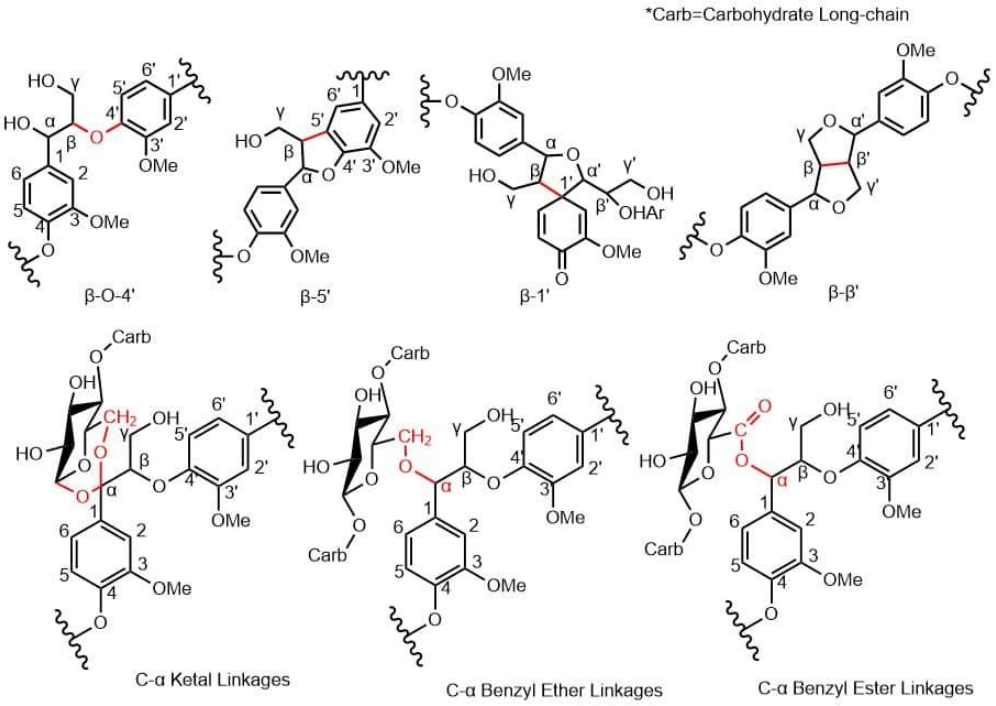

Fig. 7. Linkages between lignin structural units and LCC

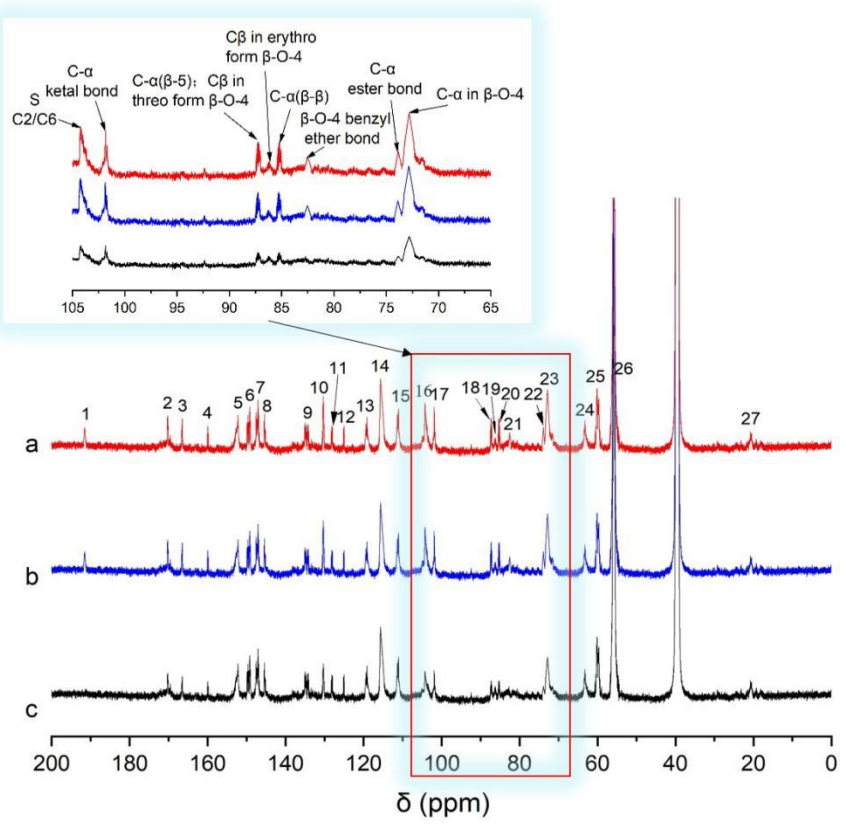

Fig. 8. ^{13}C-NMR spectra of ED-LCCs prepared from wheat straw
Legend: a: Sample administered with syringin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$].; b: sample administered with coniferin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$].; and c: control sample

Table 4. Tentative Assignment of Signals from CP/MAS ^{13}C-NMR Difference Spectrum of Wheat Straw Mill Administered with Syringin-[α-^{13}C] and Coniferin-[α-^{13}C], and D-Glucose-[6,6-$^{2}H_2$]

Signal	δ^{13}C (ppm)	Assignments
1’	100.5 to 110.2	Lignin C-α with ketal linkage to carbohydrates
2’	93.1 to 80.7	C-α in β-5, β-β; C-α in Cα-O-R, (R is carbohydrates)
3’	80.1 to 67.9	C-α in β-O-4, lignin C-α linked to carbohydrates by ester linkages
4’	67.9 to 58.0	C-α in β-1

Analyses of ^{13}C-NMR Spectra of ED-LCCs

The ^{13}C-NMR spectra of wheat straw ED-LCCs are shown in Fig. 8, and the tentative assignments of the signals are shown in Table 5.

Table 5. Assignments of ^{13}C-NMR Signals of ED-LCCs from Wheat Straw

Signal	δ^{13}C (ppm)			Assignments
	a	b	c	
1	191.5	191.4	191.4	α-CHO
2	170.2	170.2	170.1	C=O in aromatic acids
3	166.5	166.5	166.6	
4	159.9	159.9	160.0	C4 in etherified cinnamic acid and ferulic acid
5	152.2	152.2	152.3	C4 in etherified guaiacyl and C-α in cinnamaldehyde
6	149.3	149.3	149.1	C3/C4 in etherified guaiacyl
7	147.2	147.3	147.2	C3/C5 in syringyl, C4/C3 in guaiacyl
8	145.4	145.5	145.6	C4 in guaiacyl; C-α in cinnamic acid
9	134.7	134.7	134.8	C1 in guaiacyl
10	130.4	130.4	130.5	C-α in coniferyl alcohols; C-α in HC = C of guaiacyl;
11	128.1	128.1	128.2	C2 and C6 in *p*-hydroxyphenyl; C-β in cinnamaldehyde
12	125.0	125.1	125.1	etherified guaiacyl C6 with α-CO
13	119.2	119.3	119.3	C6 in guaiacyl
14	115.5	115.5	115.6	C5 in guaiacyl; C3/C5 in *p*-hydroxyphenyl
15	111.1	111.2	111.2	C2 in guaiacyl
16	104.3	104.3	104.4	C2/C6 in syringyl
17	101.8	101.8	101.9	α-C on the lignin side chain linked to the carbohydrates by ketal bond
18	87.2	87.3	87.2	C-α(β-5)；Cβ in threo form β-O-4
19	86.2	86.2	86.1	Cβ in erythro form β-O-4
20	85.1	85.2	85.2	C-α(β-β)
21	82.5	82.5	—	α-C on the lignin side chain linked to carbohydrates with benzyl ether bond
22	73.8	73.9	73.9	C-α linked with carbohydrates by ester bond
23	72.7	72.8	72.8	C-α in β-O-4
24	63.3	63.4	63.3	C-α(β-1), C-γ in β-5
25	60.0	60.1	60.1	C-γ in β-O-4
26	55.8	55.8	55.9	-OCH_3
27	20.7	20.8	20.7	-CH_3 in acetyl group

Legend: a: Sample administered with syringin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$]; b: sample administered with coniferin-[α-^{13}C] and D-glucose-[6,6-$^{2}H_2$].; and c: control sample

The -OCH_3 peak with stable content at δ 55.8 ppm (No. 25) was used as the internal reference. An enhanced signal at δ 191.5 ppm (No. 1) is from α-CHO. After α-^{13}C labeling, an enhancement signal can be observed at δ 101.8 ppm (No. 17) and assigned to α-C of lignin linked to the carbohydrates by ketal bond (Zhang *et al*. 2021). The enhanced signal at δ 87.2 ppm (No. 18) is from α-C in phenylcoumaran. The signal at δ 85.2 ppm (No. 20) is from α-C in pinoresinol. After labeling with α-^{13}C, a weak signal at δ 82.5 ppm (No. 21) is enhanced and assigned to the α-C of the lignin side chain linked to the carbohydrates *via* benzyl ether bond (Gu *et al*. 2001). An enhanced signal at δ 73.9 ppm (No. 22) is from α-C connected with carbohydrates through ester bond (Xie *et al*. 2020). The resonance signal at δ 72.8 ppm (No. 23) is the α-C in the structure of β-O-4 linkage. A peak at δ 60.1 ppm (No. 25) comes from the γ-C in the β-O-4 substructure (Xiang *et al*. 2014).

Analyses of ^{1}H-NMR Differential Spectra of ED-LCCs

To further understand the connection between lignin side chain α-C and glucan C6 from polysaccharide side, ^{1}H-NMR differential spectra of the ED-LCCs administered with D-glucose-[6-$^2H_2$] together with coniferin-[α-^{13}C] or syringin-[α-^{13}C] were analyzed. A proton on the lignin aromatic ring (δ 7.2 ppm) was used as the internal standard. The ^{1}H-NMR differential spectrum of ED-LCC was obtained by subtracting the ^{1}H-NMR spectrum of the D-labeled group from that of control group, as shown in Fig. 9. The tentative signal assignment is shown in Table 6.

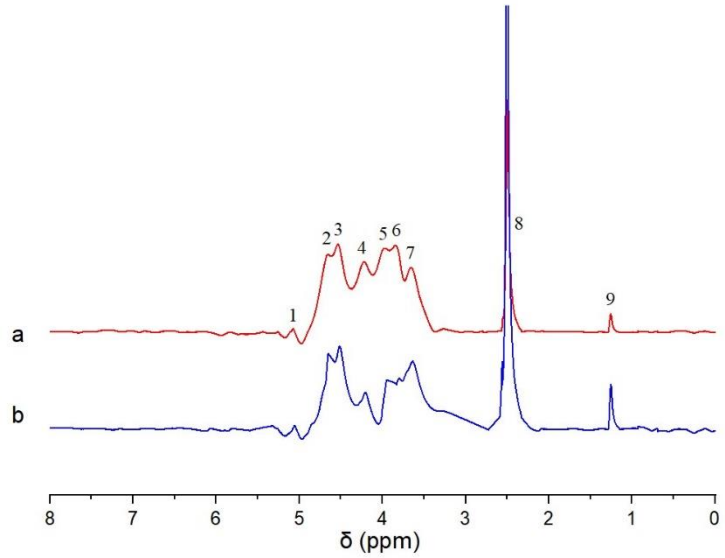

Fig. 9 ^{1}H-NMR differential spectra of EDLCCs
Legend: a: Control group subtracted wheat straw labeled with syringin-[α-^{13}C] and D-glucose-[6-$^2H_2$]; b: Control group minus wheat straw labeled with coniferin-[α-^{13}C] and D-glucose-[6-$^2H_2$]

A pair of peaks appeared at δ 4.65 ppm (No. 2) and 4.53 ppm (No. 3), which arise from a pair of H signals on the 6-C of the polysaccharide connected with the lignin side chain α-C by benzyl ether bond. The characteristic signals at δ 4.22 ppm (No. 4) and 3.97 ppm (No. 5) were assigned to H on 6-C of carbohydrates connected with ester bond to lignin side chain (Nishida *et al*. 1984). A signal at δ 3.63 ppm arises from carbohydrates 6-H,H' without linkage with lignin, which indicates that some carbohydrates were linked with lignin not through C-6 position.

Table 6. Assignments of signals of ^{1}H-NMR differential spectra of ED-LCCs from Wheat Straw

Signal	δ^{13}C (ppm)		Assignments
	a	b	
1	5.05	5.08	α-H, β-H in lignin β-5, β-O-4, β-1
2	4.61	4.65	H of 6-C of carbohydrates linked with lignin by benzyl ether bond
3	4.52	4.53	
4	4.21	4.22	H of 6-C of carbohydrates linked with lignin by ester bond
5	3.95	3.97	H of 6-C of carbohydrates linked with lignin by ester bond
6	3.80	3.83	Methoxy H, carbohydrates 6-H, H'
7	3.63	3.65	Carbohydrates 6-H, H'
8	2.50	2.50	DMSO-d_6 solvent peak
9	1.25	1.25	Highly obscured aliphatic H

Analyses of ^{13}C-NMR Spectra of Ac-En-R-GL Fractions

The ^{13}C-NMR spectra of Ac-En-R-GL fractions of wheat straw are shown in Fig. 10. A stable -OCH_3 peak at δ 55.5 ppm (No. 20) is used as an internal reference. The enhanced signals at δ 194.5 ppm (No. 1) and 191.5 ppm (No. 2) are primarily assigned to α-CHO. The signal at δ 170.8 ppm (No. 3) arises from -COO- of ferulic acid structure. The signal at δ 134.2 ppm (No. 8) is assigned to C1 on the aromatic nucleus. The signal at δ 128.1 ppm (No. 9) is assigned to α-C in lignin side chain -CαH =CH- structure. The peak at δ 105.8 ppm is significantly enhanced after α-^{13}C labelling due to the formation of α-ketal bond between lignin side chain α-C and cellulose.

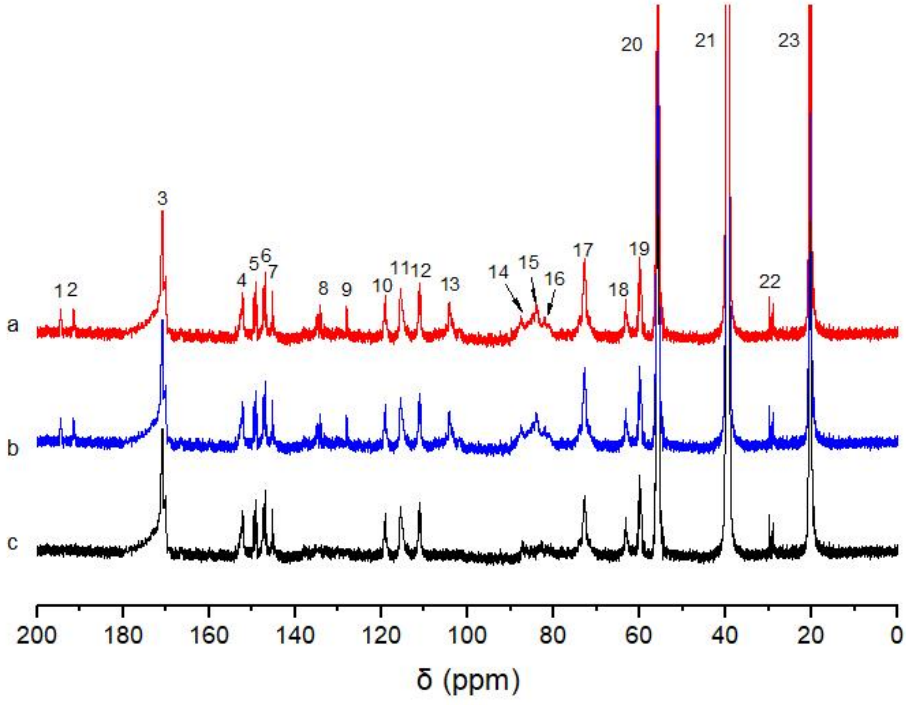

Fig. 10. ^{13}C-NMR spectra of Ac-En-R-GL prepared from En-R-LCC fractions of wheat straw Legend: a: Sample administered with syringin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]; b: sample administered with coniferin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]; and c: control sample

The enhanced signal at δ 87.6 ppm (No. 14) is assigned to α-C of β-5. The signal at δ 84.0 ppm (No. 15) could be assigned to enhanced α-C of β-β. Through ^{13}C labeling, the signal at δ 82.2 ppm (No. 16) is enhanced and assigned to α-C linked to cellulose with benzyl ether bond. The peak at δ 72.8 ppm (No. 17) can be assigned to enhanced α-C of β-O-4.

Analysis of ^{1}H-NMR Differential Spectrum of Ac-En-R-GL Fractions

Figure 11 shows the ^{1}H-NMR differential spectra of Ac-En-R-GLs. The peaks at δ 4.71 ppm (No. 2) and 4.59 ppm (No. 3) arise from a pair of hydrogen on cellulose6-C connected with lignin side chain α-C. The H of glycosyl 6-C has an benzyl ester bond signals in the 4.20 ppm~4.00 ppm region of the spectra, and ether bond signals in the 4.65 ppm~4.40 ppm region (Nishida *et al*. 1984). The 2D-NMR analysis of birch LCC shows that there was an ether bond between lignin side chain α-C and glucan 6-C (Balakshin *et al*. 2011). Combined with the ^{13}C-NMR analysis of Ac-En-R-GL, it is considered that the signals at 4.71 ppm (No.2) and 4.59 ppm (No.3) are from cellulose 6-C linked to lignin side chains α-C, which was connected by benzyl ether bond. Because the acetylation of the sample will lead to the formation of ester bonds, the resonance signals at δ 4.28 ppm (No. 4) and 4.03 ppm (No. 5) in the spectra cannot prove the ester bond connection between cellulose 6-C and lignin side chain α-C (Hikichi *et al*. 1995).

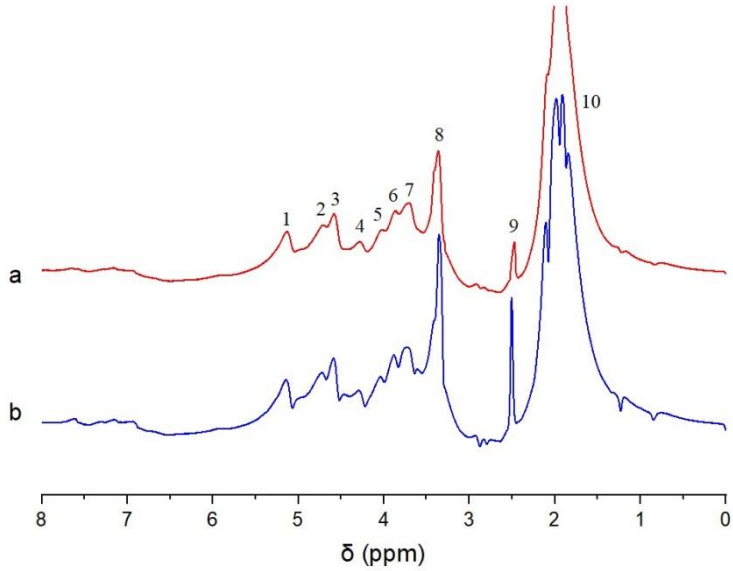

Fig. 11. ^{1}H-NMR differential spectra of Ac-En-R-GLs prepared from En-R-LCC fractions of wheat straw
Legend: a: Control group subtracting wheat straw labeled with syringin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]; b: Control group minus wheat straw labeled with coniferin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]

Analyses of ^{13}C-NMR Spectra of En-R-XL Fractions

As shown in Fig. 12, in the ^{13}C-NMR spectra of xylan-lignin fractions, the peak due to -OCH_3 at δ 55.9 ppm (No. 24) with stable content was used as the internal reference. The enhanced signal at δ 191.2 ppm (No. 1) was primarily assigned to α-CHO. After labeling, a signal at δ 130.1ppm (No. 10) was enhanced and assigned to α-C of coniferyl alcohol subunits. The signal intensities of the ^{13}C-enriched samples and the control group did not exhibit remarkable difference in the range of δ 110 to 120 ppm (No. 12 to No. 14),

which primarily arise from aromatic carbons (C2, C5, and C6). The signal at δ 101.9 ppm (No. 16) was enhanced after ^{13}C labeling, which was assigned to the α-C of the lignin side chain linked to the xylan subunit by ketal bond (Xie *et al*. 2000). It was found that there were two enhanced signals No.17 and No.18 at δ 86 ppm and 88 ppm in the ^{13}C labeled samples and assigned to α-C in phenylcoumaran and pinoresinol, indicating the low contents of these two structures. The enhanced peak at δ 81.5 ppm (No. 20) after ^{13}C labeling arises from the α-C of lignin linked to xylan by an ether bond. The signal intensity shows that the content of this kind of ether bond in wheat straw En-R-XL was low (Gu *et al*. 2001). The enhanced signal at δ 71.5 to 72.3 ppm (No. 21) was from the α-C of β-O-4. From the peak intensity, it can be concluded that β-O-4 is the main connection between lignin moieties of En-R-XL (Besombes *et al*. 2003). The signal at δ 62.7 ppm (No. 22) comes from α-C of β-1, while that at δ 60.2 ppm (No. 23) is γ-C of β-O-4.

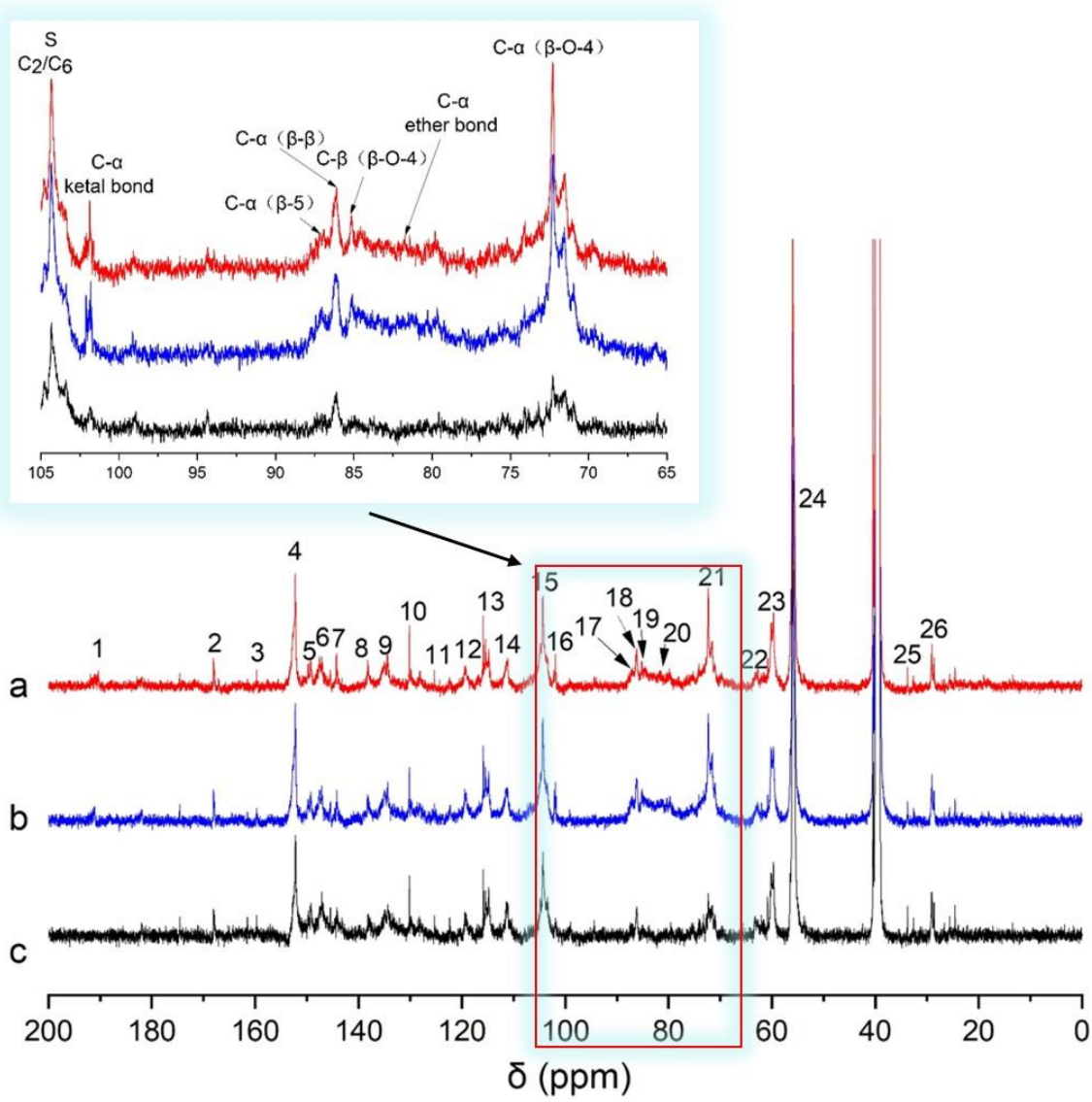

Fig. 12. ^{13}C-NMR Spectra of En-R-XL fractions prepared from R-LCC fractions of wheat straw Legend: a: Sample administered with syringin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]; b: sample administered with coniferin-[α-^{13}C] and D-glucose-[6-$^{2}H_2$]; and c: control sample

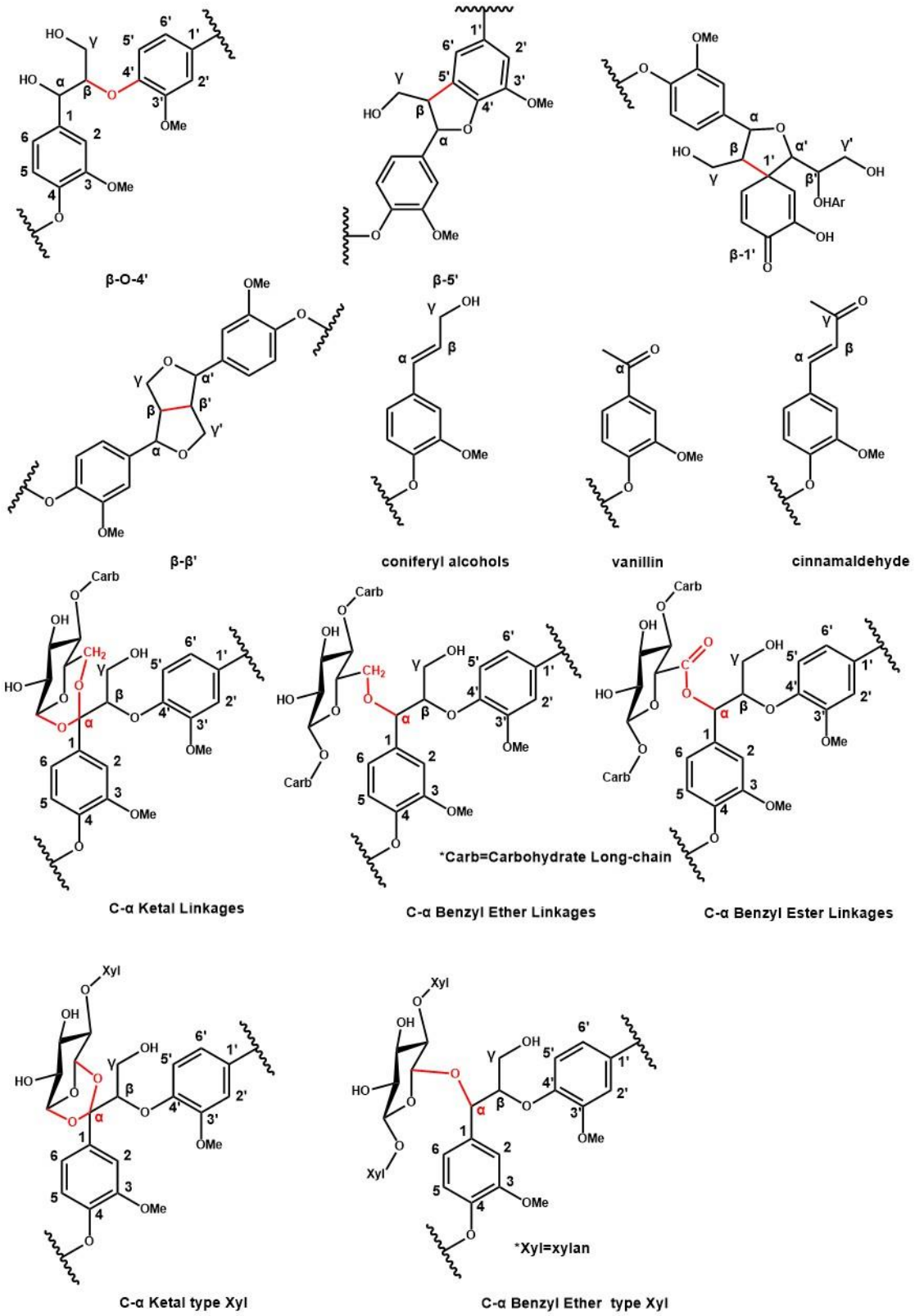

Fig. 13. Linkages between lignin structural units, lignin-glucan, and lignin-xylan

Neish (1958) confirmed that the first step of xylan biosynthesis in plant cell wall was enzymatic oxidation of D-glucose to form glucuronic acid. Then, a series of metabolisms were conducted to form xylan. In this process, the ^{2}H isotope on C6 of D-glucose-[6-$^{2}H_2$] in plants will be eliminated due to enzymatic hydrolysis. Therefore, no difference is found in the ^{1}H-NMR differential spectra of xylan-lignin fractions.

In summary, there is an LCC structure linked by benzyl ether bond and ketal bond between lignin and xylan in the En-R-XL fraction of wheat straw. Figure 13 shows the linkage between lignin in wheat straw and the LC bond between lignin, xylan, and glucan.

CONCLUSIONS

1. Through the analyses of the abundances of ^{13}C and D atoms in the labeling and control groups, it was found that the $^{13}C\alpha/^{12}C\alpha$ ratio of wheat straw labeled with ^{2}H-^{13}C was about three times that of natural isotope ratio, while the D6/H6 ratio was about eight times that of natural isotope ratio, indicating that exogenous coniferin-[α-^{13}C], syringin-[α-^{13}C], and D-glucose-[6-$^{2}H_2$] were effectively absorbed and metabolized during the growth of wheat internode tissues. These results indicate that lignin in the cell wall of wheat straw was labeled with ^{13}C, while the polysaccharides were labeled with deuterium isotopes.
2. Analysis of CP/MAS ^{13}C-NMR spectra and their differential spectra of extractive-free milled wheat straw showed that enrichment of stable isotope (^{2}H-^{13}C) had no obvious interference on the normal lignification process of wheat cell wall. The lignin structural units are mainly composed of β-O-4, β-5, β-β, and β-1 subunits, and also contain a small amount of coniferyl alcohols substructure. The α-C of lignin phenylpropane side chain is connected with carbohydrates by ketal bond and benzyl ether linkages.
3. To further understand the structure of LC linkages in wheat straw, LCC and R-LCC were isolated and treated by enzymatic hydrolysis. It was found that the side chain α-C of lignin in $^{13}C/^{2}H$-enriched ED-LCC of wheat straw was combined with 6-C of carbohydrates by benzyl ether, ester, and ketal bonds. It was further confirmed that the lignin was primarily composed of β-O-4, β-β, β-5, and β-1 subunits, and also contained a small amount of coniferyl alcohol subunits.
4. The ^{13}C-NMR spectra of En-R-GL showed that a part of lignin α-C was connected with cellulose by benzyl ether and α-ketal linkages. The ^{1}H-NMR differential spectrum of Ac-En-R-GL fraction showed that a part of lignin side chain α-C was bound with cellulose 6-C *via* benzyl ether bond. The ^{13}C-NMR spectra of En-R-XL showed that there was a small amount of lignin α-C was connected with xylose by α-benzyl ether and α-ketal linkages. The lignin structural units were primarily composed of β-O-4, and also included a small amount of β-β, β-5, and β-1 linkages.

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