The Existence of Cellulose and Lignin Chemical Connections in Ginkgo Traced by ^{2}H-^{13}C Dual Isotopes

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To elucidate the covalent association between the celluloses and lignins found in gymnosperms, they were labeled with stable isotopes (deuterium and carbon-13) at specific positions and traced *via* mass spectroscopy and nuclear magnetic resonance (NMR). Both the ^{2}H-labeled cellulose precursor (UDP-glucose-[6-$^{2}H_2$]) and the ^{13}C-labeled lignin precursor (coniferin-[α-^{13}C]) were added to a growing ginkgo plant, in combination with a 4-coumarate-CoA ligase inhibitor. The detection of abundance of ^{13}C and ^{2}H revealed that the lignin precursor and cellulose precursor deposited more actively in 300 to 1300 μm and 100 to 900 μm distance from cambium, respectively. The lignin-carbohydrate complexes (LCCs) were isolated from the newly-formed ginkgo shoot xylem and further degraded with cellulase and hemicellulase to obtain enzymatically degraded lignin-carbohydrate complexes (EDLCCs). Analysis of the solid-state cross polarization / magic angle spinning (CP/MAS) ^{13}C-NMR of the newly-formed xylem, liquid-state ^{13}C-NMR, and ^{1}H-NMR of the EDLCCs confirmed that the major connection between celluloses and lignins was a benzyl ether bond (between cellulose C6 and lignin Cα). A minor ester bond was also found between the hydroxyl group (at the 6-position of cellulose) and ferulic acid (at the γ position in lignins).

Keywords: Lignin-cellulose complexes; Isotope tracer; Ginkgo; NMR; Benzyl ether linkage

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INTRODUCTION

There are chemical bonds between the celluloses and lignins found in plant cell walls, which severely hinders the complete removal of lignins during the pulping process (Karlsson *et al*. 2001; Oinonen *et al*. 2015). This results in the consumption of an increased amount of chemicals and energy during the pulping and bleaching process (Daljeet *et al*. 2019; Sharma *et al*. 2020). In the 1990s, Karlsson and Westermark (1996) used chromatographic methods to prove that there was a strong lignin-cellulose chemical bond in pine kraft pulp. Isogai (2001) found that pure cellulose could not be obtained through repeated refining treatments when studying high-purity viscose cellulose; rather, it was usually accompanied by lignin components. Therefore, Isogai (2001) suggested that there might be a chemical bond between cellulose and lignins. Through endoglucanase-catalyzed hydrolysis and repeated purification, it was found that less than 10% of the lignin-carbohydrate complexes (LCCs) in unbleached kraft softwood pulp had a linkage between the lignins and celluloses (Lawoko *et al*. 2003). Du *et al*. (2013) carried out deep delignification of pulp through a laccase-mediator and found that lignins always existed in the form of a glucan-lignin complex after deep treatment. Some researchers have also shown that there might be phenylglycoside bonds in the lignin-cellulose complexes (Kondo

and Sarkanen 1984; Joseleau and Kesraoui 1986).

As an important method for studying the biosynthetic pathways, structures, and reaction mechanisms of polymers, isotope labeling technology has been widely used in the study of lignin-carbohydrate complexes (Ong *et al*. 2002; Tugarinov *et al*. 2006). Imai and Terashima (1990) made use of the cellulose precursor of D-glucose-[U-^{14}C] and a phenylalanine amonia-lyase (PAL) inhibitor to visualize the deposition process of cellulose. Xie *et al*. (2000) injected coniferin with ^{13}C-labeled at the α, β, and γ-positions of side chain into growing ginkgo to separate the LCC and the enzymatically degraded LCC. By this means they found that there were benzyl ether linkages, benzyl ester bonds, and acetal bonds. Gu (2002a) also synthesized a ^{13}C-labeled lignin precursor and injected it into the growing internode tissue of rice, so that the side chain of the lignin of rice straw was labeled with ^{13}C and the chemical structure of the lignin-carbohydrate complexes could be elucidated. Zhou *et al*. (2001) used the natural abundance of the stable isotope ratio (^{18}O/^{16}O) to analyze the chemical bonds between the celluloses and lignins in the plant cell walls and found γ-ester bonds and α-ether bonds between the lignins and celluloses. Yang *et al*. (2007) put ^{13}C-labeled coniferin into natural plant rice straw and analyzed it with high-resolution liquid ^{13}C-NMR and found that the lignins were associated with cellulose and hemicellulose by benzyl ether connections. The above results indicated that there were chemical connections between the lignins and celluloses. However, the formation mechanism, the proportion of linkages, and the position of the bond have not yet been described.

In this study, the ^{2}H/^{13}C dual stable-isotope-labeling method was used to trace both the celluloses and lignins, as a means to determine the association between the celluloses and lignins found in the ginkgo tree. A cellulose precursor (UDP-glucose-[6-$^{2}H_2$]) and lignin precursor (coniferin-[α-^{13}C]) were added to the ginkgo plant, and the levels of ^{13}C and ^{2}H were determined by elemental analysis combined with isotopic mass spectroscopy to understand the deposition of celluloses and lignins and elucidate the distribution of isotopic abundance in the newly-formed xylem. The possibility of conversion of the UDP-glucose-[6-$^{2}H_2$] to hemicellulose was also investigated. The newly-formed xylem with relatively high isotopic abundance was determined *via* CP/MAS ^{13}C-NMR. The milled wood lignin (MWL), lignin-carbohydrate complexes (LCCs), and enzymatically degraded LCCs (EDLCCs) labeled with 6-^{2}H/α-^{13}C were isolated from the newly-formed xylem of the ginkgo tree, and these were analyzed *via* ^{13}C-NMR and ^{1}H-NMR to determine the cellulose-lignin linkages of ginkgo. The proposed metabolic map of the present work is shown in Fig. 1. The 4CL inhibitor prevented the conversion of glucose to lignin, while coniferin-[α-^{13}C] was hydrolyzed into coniferyl alcohol by β-glucosidase.

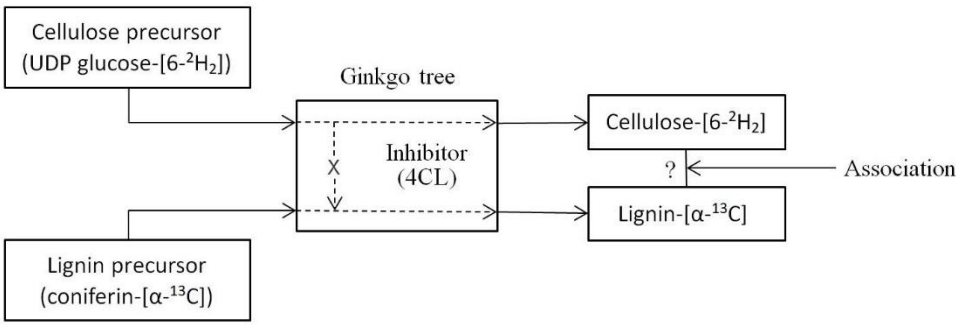

Fig. 1. The proposed metabolism route of the isotope labeled cellulose precursor and lignin precursor in a ginkgo tree

Then the coniferyl alcohol was further converted to lignin, as shown in Fig. 2. Therefore, the lignin content in the ginkgo shoots administered with the coniferin was similar to that of intact ginkgo tree.

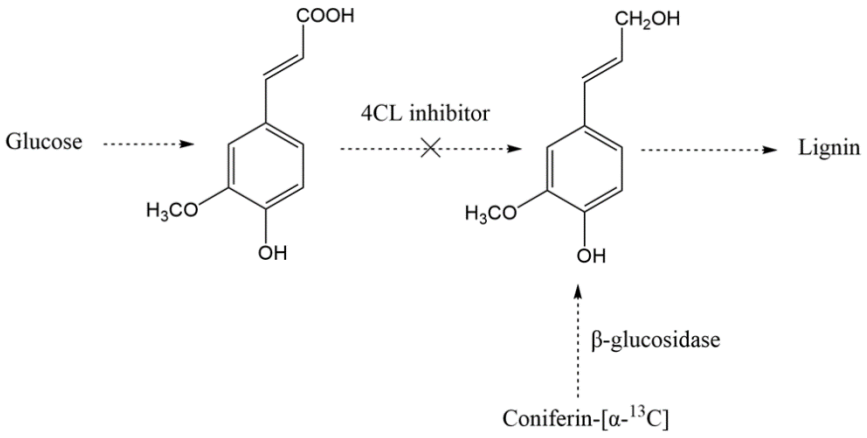

Fig. 2. Inhibiting of glucose transformation to lignin and metabolism of coniferin-[α-^{13}C]

EXPERIMENTAL

Materials

Five-year-old *Ginkgo biloba* L. trees were obtained from the Wuhan Botanical Garden (Wuhan, China). Sodium acetate-1-^{13}C and (6-$^{2}H_2$) D-glucose were purchased from Sigma-Aldrich (Saint Louis City, MO), and 3,4-(methylenedioxy)cinnamic acid was purchased from Aladdin (Shanghai City, China). All other chemicals were analytical grade.

Synthesis of the Isotope-Labeled Lignin and Cellulose Precursors

Coniferin-[α-^{13}C] was synthesized using sodium acetate-1-^{13}C according to previously described methods (Xie *et al.* 1994a,b). The cellulose precursor uridine diphosphoglucose-[6-$^{2}H_2$] was synthesized as previously described by Dinev *et al*. (2006). The chemical structures of the isotope enrichment precursors are shown in Fig. 3.

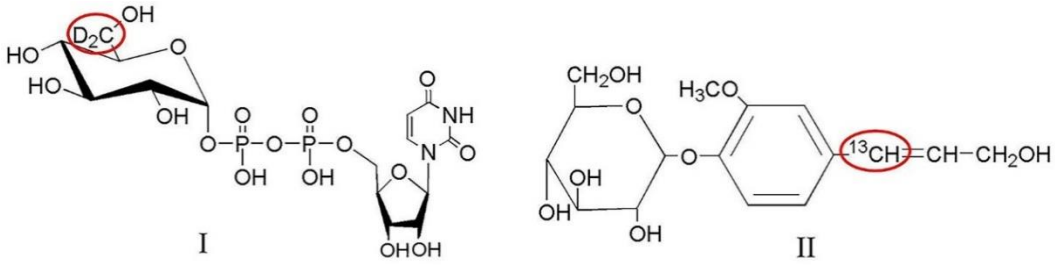

Fig. 3. Chemical structures of UDP glucose-[6-$^{2}H_2$] (I) and coniferin-[α-^{13}C] (II)

Administration of the Precursors to the Ginkgo Tree

In early June, the five-year-old ginkgo tree was cut into shoots with a length of approximately 30 cm, and 20 to 30 leaves were retained on each shoot. The culture solution of three ginkgo shoots was composed of UDP-glucose-6,6-D_2 (100 mg/100 mL), coniferin-^{13}Cα (100 mg/100 mL), 3,4-methylenedioxycinnamic acid (4-coumarate-CoA ligase

inhibitor) (10 mg /100 mL) constitute the experimental group. The culture solution of the other two ginkgo shoots consisted of unlabeled UDP-glucose (100mg/100mL), unlabeled coniferin (100 mg/100 mL), and 3,4-methylenedioxycinnamic acid (10 mg /100 mL), which served as the control group. The shoots were cultivated in an artificial climate chamber at 25 °C for 30 d. The samples were kept in the light time and the dark time for 15 h and 9 h every day, respectively. When the precursor solution was completely absorbed, distilled water was added to the ginkgo plant.

Determination of ^{13}C and ^{2}H Abundance

The cut ginkgo shoots that were administered with both ^{13}C-enriched coniferin and ^{2}H-enriched UDP-glucose were debarked and cut into pieces 2.0 cm long, which were then cut into 100-µm-thick sections on a sliding microtome from cambium to pith, according to Terashima *et al.* (1979), as shown in Fig. 4.

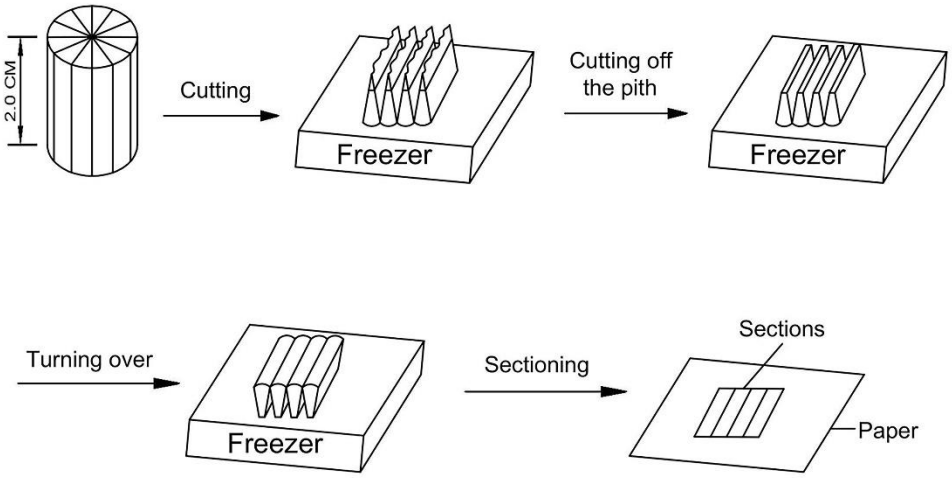

Fig. 4. Preparation of the tangential sections *via* a freezing microtome

The air-dried xylem sections were milled (40 mesh to 60 mesh) and extracted with ethanol-benzene (1/2, v/v), followed by hot water. The C isotope value $\delta^{13}C$ (Vienna Pee Dee Belemnite (VPDB)) and the H isotope value δD (VSMOW, Vienna Standard Mean Ocean Water) of 1 mg of the extractive-free wood meal samples were measured *via* an elemental analyzer (FLASH2000, Thermo Fisher Scientific GmbH, Dreieich, Germany) combined with an isotope ratio mass spectrometer (Delta V, Thermo Fisher Scientific GmbH, Dreieich, Germany), respectively. The $^{13}C/^{12}C$ and $^{13}C\alpha/^{12}C\alpha$ values in the sample were calculated using Eqs. 1 and 2,

$$^{13}C/^{12}C = 1.105765\% \times (1 + \delta^{13}C \div 1000) \quad (1)$$

$$^{13}C\alpha/^{12}C\alpha = 1.0762\% + (^{13}C/^{12}C - 1.0762\%) \div 0.3 \times 9 \quad (2)$$

where $^{13}C/^{12}C$ is the ratio of ^{13}C to ^{12}C in the sample, $\delta^{13}C$ is the C isotope value of the sample $\delta^{13}C$ (VPDB) (‰), 1.105765% is the ^{13}C isotope abundance of the standard (Vienna Pee Dee Belemnite), $^{13}C\alpha/^{12}C\alpha$ is the ^{13}C and ^{12}C isotopic ratios of Cα in the lignin structural units of the sample, 1.0762% is the ^{13}C isotope values in intact ginkgo wood, 0.3 is the lignin content in a ginkgo plants, and 9 is the ratio of the number of total carbons to Cα in the lignin structures.

The D/H and D6/H6 values in the samples were calculated using Eqs. 3 and 4,

$$D/H = 0.015575\% \times (1+ \delta D \div 1000) \quad (3)$$

$$D6/H6 = 0.013505\% + (D/H - 0.013505\%) \div 0.50 \times 5 \quad (4)$$

where D/H is the ratio of D to H in the sample, δD is the H isotope value of the sample δD (VSMOW) (‰), 0.015575% is the D isotope abundance of the standard (Vienna Standard Mean Ocean Water), D6/H6 is the isotope ratio of D to H in cellulose at position 6 in the sample, 0.013505% is the D isotopic value in intact ginkgo plants, 0.50 is the cellulose content in a ginkgo plant, and 5 is the ratio of the number of total hydrogens to H6 in the cellulose structures.

Preparation of the Enzymatically Degraded Lignin-Carbohydrate Complexes (EDLCCs)

Extractive-free wood meal (20 mesh) of the newly-formed xylems (300 µm to 900 µm from the cambium) from the cut ginkgo shoots were milled to a 80 mesh to 100 mesh size *via* a Wiley mill and then dried over phosphorus pentoxide for 7 d in a vacuum desiccator. Then, after 72 h of water-cooled vibration ball milling, the LCCs were extracted using methodology described by Björkman and Anders (1957). The LCC yield was 13.8% from the isotope-enriched ginkgo wood meal and 14.1% from the unlabeled ginkgo wood meal.

Five hundred milligrams of cellulase (Onozuka RS, Yakult Co., Japan) and 500 mg of hemicellulase (from *Aspergillus niger*, Sigma-Alrich, St. Louis, MO) were dissolved in 50 ml of 0.05 M acetic acid/sodium acetate buffer (a pH of 4.6) and filtrated with a G4 glass filter. The enzyme solution was stored at 5 °C. Then, The LCCs (600 mg) were mixed with 16 mL of the enzyme solution and 24 mL of 0.05 M acetic acid/sodium acetate buffer. Three drops of toluene were added as a protective agent. The mixture was cultured in a water baths shaker at 50 °C for 48 h. The EDLCCs were collected *via* centrifugation, washed four times with water, and then freeze-dried.

Extraction of Hemicellulose from the Newly-formed Xylem and Determination of ^{2}H Abundance

Ginkgo wood meal labeled with ^{2}H/^{13}C was extracted with a mixture of benzene and ethanol in a volume ratio of 2:1. Then, the dried sample was extracted with 8% NaOH solution at a solid-liquid ratio of 1:20 for 12 h at 80 °C. After the extraction, the mixture was filtrated. The pH value of the filtrate was adjusted to 5.5 with the diluted HCl solution, and 95% ethanol solution with 3 times the volume of filtrate was added, and centrifuged. The precipitate was washed with 70% ethanol solution. The extracted hemicellulose was obtained by freeze-drying, and its ^{2}H abundance was determined.

High-resolution Cross Polarization/Magic Angle Spinning (CP/MAS) ^{13}C-Nuclear Magnetic Resonance (NMR) Determination

An Avance III 600-MHz solid-state NMR spectrometer with a solid probe (Bruker, Billerica, MA) was used. The experimental conditions were as follows: a temperature of 25 °C, a 3 ms contact time, a 0.05 s reception time, a pulse width of 35 kHz, and a pulse delay of 2 s. Each sample was accumulated approximately 5000 times.

^{13}C- and ^{1}H-NMR Spectra of the EDLCCs

First, 80 mg of the product was placed in a φ5-mm NMR tube, and 0.5 mL of DMSO-d_6 solvent was added to dissolve it. All NMR spectra were recorded on a Bruker Avance III 500-MHz spectrometer equipped with a φ5 mm broad band fluorine observation (BBFO) probe at a temperature of 25 °C. The ^{13}C-NMR spectrum was recorded with the following conditions: a pulse delay of 2.6 s and a reception time of 0.94 s, while data points were collected at 32 kbit and after the accumulation of 7168 scans. The ^{1}H-NMR spectrum was recorded with following conditions: a pulse delay of 4.3 s, 500 scans, and an acquisition time of 0.74 s, while the data points were collected at 32 kbit.

RESULTS AND DISCUSSION

Analysis of the Abundance and Distribution of ^{13}C and ^{2}H in the Newly-formed Xylem

Figure 5 shows the distribution of ^{13}C (derived from coniferin-[α-^{13}C]) in the newly-formed ginkgo tree xylem. It can be found from Fig. 5 that the ^{13}C/^{12}C value of Cα of the lignin side chain was higher in the area 300 to 1300 μm away from the cambium, indicating that the lignification was more active in this area. Terashima and Seguchi labeled the newly-formed xylem of pine with coniferin-(side chain α-^{13}C) and found the increase of ^{13}C/^{12}C was high in the region of 200 to 700 μm distant from the cambium (Terashima *et al*. 1991). The fact that the formation of lignin was most active in that region has been also shown by microautoradiography of differentiating xylem of pine (Terashima *et al*. 1988; Donaldson 2001). In the present research, the maximum ^{13}C/^{12}C value of Cα of the lignin side chain was 3.43%, which indicated that the α-^{13}C abundance was 3.2 times higher than the natural abundance (^{13}C/^{12}C: 1.05765%).

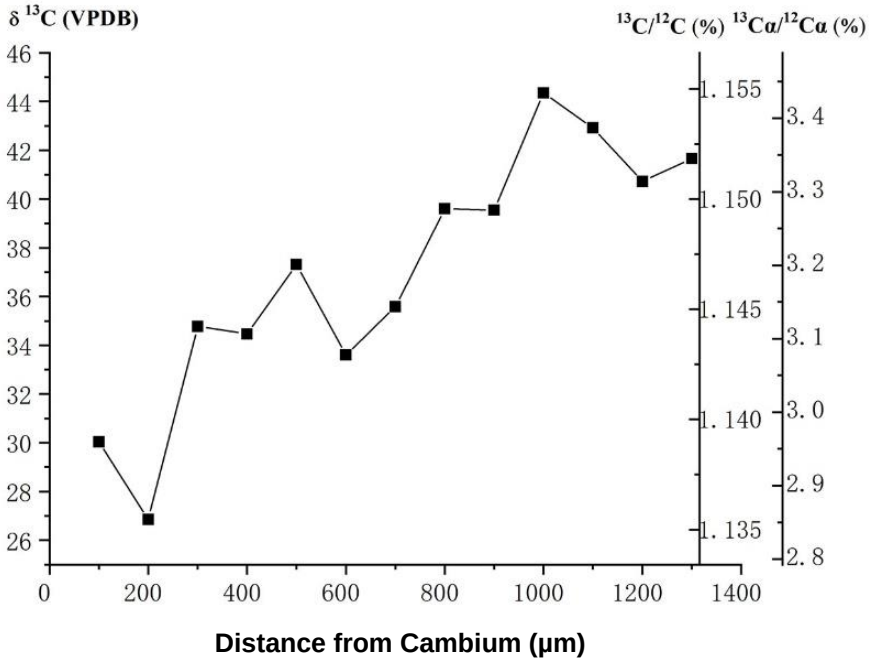

Fig. 5. The ^{13}C abundance in the newly-formed ginkgo xylem labeled by ^{2}H-^{13}C dual isotopes

The result of hemicellulose abundance determination showed that the δD(VSMOW) value was -83.257‰ determined by mass spectroscopy, which was close to natural abundance of plant composition, while the δD(VSMOW) value of the labeled ginkgo newly-formed xylem reached 266.523 to 290.671‰, as shown in Fig. 6. Therefore, the authors suggest that deuterium in the culture solution was mainly deposited in cellulose and the deposition of D in hemicellulose can be ignored. It is suggested that there was no pathway for conversion of UDP glucose-[6-$^{2}H_2$] to glucuronoxylan and glucomannan. Almost all of the deuterium isotope was involved in cellulose units.

It can be found from the Fig. 6 that the value of D/H was higher in the region 100 to 900 μm distance from the cambium, indicating that D was mainly deposited on the cellulose in this region. In the investigation of depositing stages of polysaccharide, Imai and Terashima labeled xylan in newly-formed xylem with *myo*-inositol-(2-^{3}H), and found that xylan mainly deposited in the following two stages: (1) start of S_1 formation in the region of 100 to 200 μm distance from the cambium; (2) start of S_3 formation in the region of 400 to 500 μm distance from the cambium. These regions were revealed by microautoradiography of the differentiating xylem (Imai and Terashima 1992). The maximum D6/H6 value of the cellulose main chain H6 was 0.0868%, which revealed that the enrichment of D6 was 6.43 times higher than the natural abundance (D6/H6: 0.0135%).

According to the analysis of the abundance of ^{13}C and ^{2}H, the lignins and celluloses were successfully labeled in the ginkgo tree and the precursors were effectively metabolized in the plants and polymerized into macromolecules. The results were consistent with previous studies (Xie *et al*. 1991; Xiang *et al*. 2013). In order to increase the abundance of ^{13}C and ^{2}H in the EDLCC, 300 μm to 900 μm microtome sections, which were enriched with both ^{13}C and ^{2}H, were used in the isolation process.

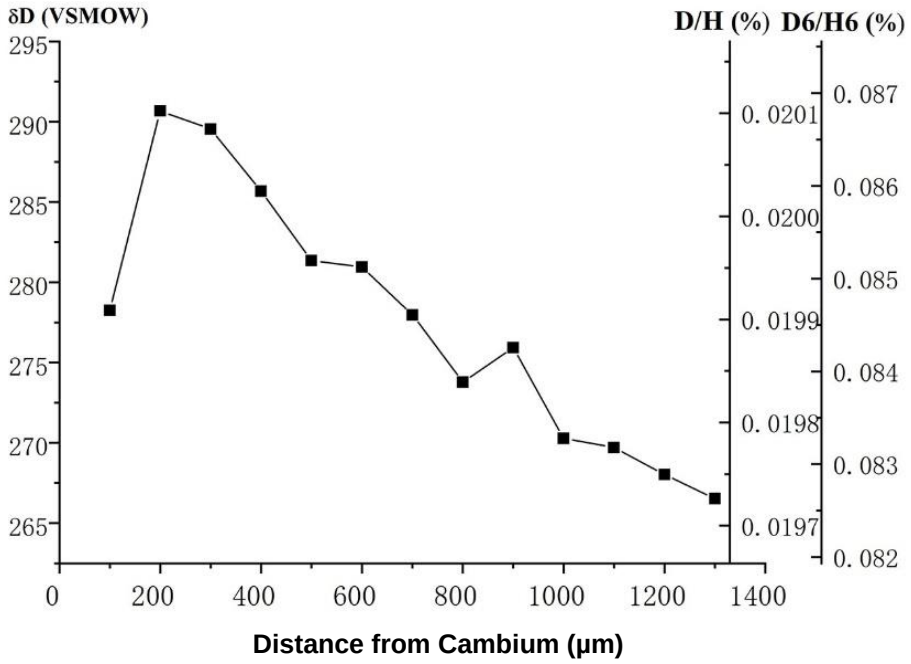

Fig. 6. The ^{2}H abundance in the newly-formed ginkgo xylem labeled by ^{2}H-^{13}C dual isotopes

Solid-state CP/MAS ^{13}C-NMR Analysis of the 6-^{2}H/α-^{13}C Labeled Ginkgo Wood

Solid-state magnetic resonance detection is used to study the structure of polymer compounds, *e.g.*, the raw materials in paper (Bardet *et al*. 2002; Evstigneyev *et al*. 2018; Xue *et al*. 2019). Coniferin participates in the lignin biosynthesis process and does not interfere with the normal lignification of plants (Gu *et al*. 2002b). To distinguish the isotope-labeled signals of the C atoms found in the lignin side chain from non-labeled ones, CP/MAS ^{13}C NMR differential spectroscopy was applied.

The CP/MAS ^{13}C-NMR results of the newly-formed ginkgo xylem are shown in Fig. 7. The signal assignments are shown in Table 1. The signals in the aliphatic region of the newly-formed xylem showed major enhancement, which indicated that the coniferin-^{13}Cα could be converted into lignin moieties in ginkgo plants. The signals at 134.2 ppm (No. 3') and 132.3 ppm (No. 4') were from the -Cα=C- in the coniferyl alcohol structure according to Lüdemann and Nimz (1973). The signals at 105 ppm (No. 5') could arise from the C-α in the guaiacylpropane unit with a ketal linkage to carbohydrates, according to Jacques *et al*. (1974) and Xie *et al*. (2000). The No. 6' (88.3 ppm) and No. 7' (85.1 ppm) signals were drastically enhanced in the differential spectra, due to C-α enrichment, which could be assigned to C-α in phenylcoumaran and C-α in pinoresinol in lignin, respectively. Signal No. 8' (75.8 ppm) could be assigned to C-α in ferulic acid (lignins) with hydroxyl groups from carbohydrates according to Sipilä and Brunow (1991) and Xie *et al*. (2000). Carnachan *et al.* (2000) found that ferulic acid was bound to carbohydrates in the primary cell walls of 41 species of gymnosperms by using UV-fluorescence microscopy and gas chromatograph, and the contents of ferulic acid were 90 μg/g in ginkgo xylem treated with sodium hydroxide. Terashima *et al*. (2004) further found that the cross-linking of polysaccharides in ginkgo xylem was realized by free radical dimerization of ferulic acid polysaccharide esters. The signal at 73.2 ppm (No. 9') was strong and broad, and it was related to the C-α found in β-O-4 structural units. This strong signal indicated that the β-O-4 structure was the dominant structure found in the ginkgo wood protolignin. This result was similar to the observations by Lewis *et al*. (1988) on ferulic acid-[α-^{13}C]-labeled *Leucaena leucocephala* lignins. Signal No. 11' (63.1 ppm) arose from the C-α and C-β in the β-1 structure found in the ginkgo lignin.

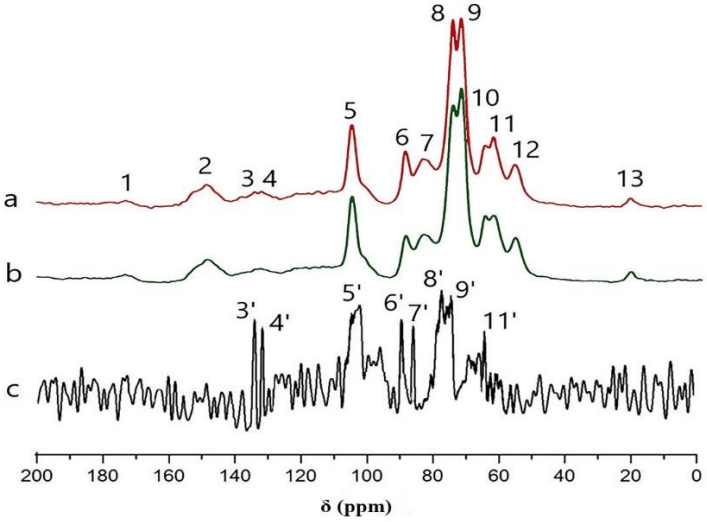

Fig. 7. Solid-state CP/MAS ^{13}C-NMR spectra of ginkgo wood meals: a - Newly-formed gingko xylem labeled with 6-^{2}H/α-^{13}C; b - Intact newly-formed ginkgo xylem; and c - Differential spectrum obtained by subtracting spectrum (b) from spectrum (a)

Table 1. CP/MAS ^{13}C-NMR Signal Analysis of Newly-Formed Ginkgo Xylem

Signal	Chemical Shifts (δ, ppm)			Assignments
	a	b	c	
1	173.1	173.3		C-6 in uronic acid and esters, C-γ in cinnamic acid and esters
2	149.8	148.7		C-3, C-4 in guaiacyl
3, 3’	134.2	134.4	134.2	C-α in coniferyl alcohols
4, 4’	132.3	132.6	132.3	C-α in coniferyl alcohols
5, 5’	105.5	105.0	105.3	C-1 in celluloses, C-α in lignin bonded to carbohydrate with ketal linkages
6, 6’	89.1	88.5	88.3	C-α in β-5
7, 7’	83.7	84.6	85.1	C-α in β-β, C-4 in celluloses,
8, 8’	74.8	74.3	75.8	C-α with ester linkage to carbohydrates, C-2,3,5 in celluloses and hemicelluloses
9, 9’	72.3	72.1	73.2	C-α in β-O-4
10	65.1	65.0		C-γ and C-β in β-1
11,11’	62.1	62.2	63.1	C-6 in celluloses, C-γ in β-O-4, C-α, and C-β in β-1
12	56.0	56.1		-OCH_3
13	21.2	21.3		-CH_3

Analysis of ^{13}C-NMR Spectra of α-^{13}C/6-^{2}H-Enriched EDLCCs

After the LCCs were hydrolyzed with cellulases and hemicellulases, most of the carbohydrates were hydrolyzed, which made information of the carbonyl region (160 ppm to 200 ppm) and the aliphatic region (0 ppm to 110 ppm) available. The remaining components after hydrolysis were primarily lignin macromolecules and carbohydrates covalently linked to the lignins. The ^{13}C-NMR spectra of the α-^{13}C/6-^{2}H EDLCCs and unlabeled EDLCCs are shown in Fig. 8, and the tentative assignments of the signals are shown in Table 2.

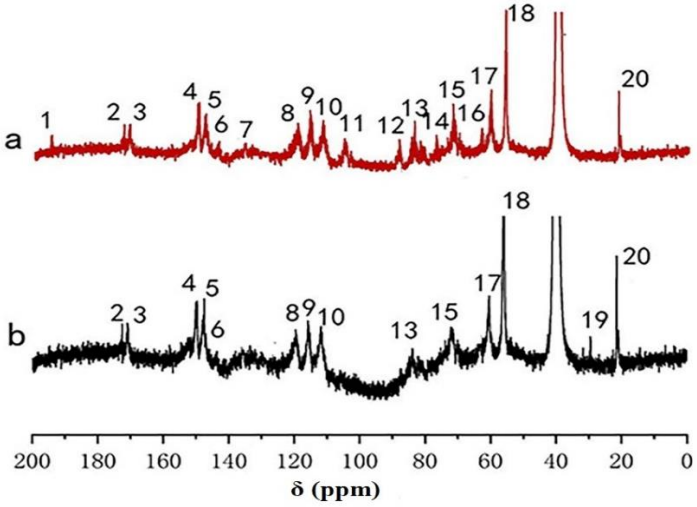

Fig. 8. The ^{13}C-NMR spectra of ginkgo EDLCCs: a - α-^{13}C/6-^{2}H labeled ginkgo EDLCCs; and b - unlabeled ginkgo EDLCCs

At δ194.1 ppm (No. 1), the labeled ginkgo EDLCCs were substantially enhanced compared to the unlabeled ginkgo EDLCCs, which was related to the ^{13}C labeling of the α-CO in the vanillin found in the labeled ginkgo. The signal at 105.1 ppm (No. 11) was

enhanced by α-^{13}C labeling and was assigned to the ketal linkage between the C-α of the lignin side chains and the carbohydrates The enhanced signal at 85.6 ppm (No. 12) was the C-α found in phenylcoumaran. The signal at 82.1 ppm (No. 13) was obvious in the ^{13}C-enriched EDLCCs and was assigned to the C-α with an ether linkage to carbohydrates (Taneda *et al*. 1987; Xie *et al*. 2000) and the C-α signal found in pinoresinol. The signal at 76.1 ppm (No. 14) was the C-α with an ester linkage to carbohydrates. Signal No. 15 (72.2 ppm) was the C-α in the β-O-4 substructure.

From the above data, it was concluded that the lignins and carbohydrates found in the ginkgo LCCs are primarily connected by C-α ether linkages, C-α acetal bonds, and C-α ester bonds.

Table 2. Chemical Shifts and Assignments of the ^{13}C-NMR signals from the EDLCCs

Signal	Chemical Shifts (ppm)		Assignments
	a	b	
1	194.1	-	α-CO in vanillin, and γ-CHO in cinnamaldehyde
2	172.2	172.4	Cinnamic acid, and acetyl
3	170.2	170.3	Cinnamic acid, and acetyl
4	150.2	150.3	C-4 in guaiacyl with α ether, and C-α in cinnamaldehyde
5	148.2	148.5	C-4 in guaiacyl, and C-3 in guaiacyl
6	144.2	144.3	C-4 in phenylcoumaran
7	135.1	-	C-α in coniferyl alcohols
8	119.1	119.3	C-6 in guaiacyl
9	115.2	115.4	C-6 in phenylcoumaran, and C-5 in guaiacyl
10	111.3	111.4	C-2 in guaiacyl
11	105.1	-	C-α ketal linkage with carbohydrates, and C-1 in cellulose
12	85.6	-	C-α in phenylcoumaran
13	82.1	82.3	C-α with ether linkage with carbohydrates, C-α in pinoresinol, C-β in β-aryl ethers, and C-4 in celluloses
14	76.1	-	C-α linked to carbohydrate ester bonds, and C-2,3,5 in celluloses and hemicelluloses
15	72.2	72.3	C-α in β-aryl ethers, and C-2, 3, 5 in celluloses
16	62.1	-	C-α and C-β in β-1
17	60.1	60.2	C-γ in β-aryl ethers, and C-6 in celluloses
18	55.3	55.4	-OCH_3
19	-	28.2	Unknown
20	20.1	20.0	-CH_3

Analysis of the ^{1}H-NMR Spectra of the α-^{13}C/6-^{2}H-enriched EDLCCs

After enzymatic hydrolysis *via* cellulases and hemicellulases, many of the β-1,4-glycosidic bonds between the glucose units in the cellulose macromolecules were broken; this made the chemical structure information between the lignin and cellulose units available. In the ^{1}H-NMR differential spectrum (Fig. 9), cellulose 6-H was successfully labeled with ^{2}H, and signals for the glucose units appeared. This showed that the α-^{13}C/^{2}H-labeled EDLCCs contained ^{2}H-labeled glucose units and that there was a chemical connection between the lignins and celluloses.

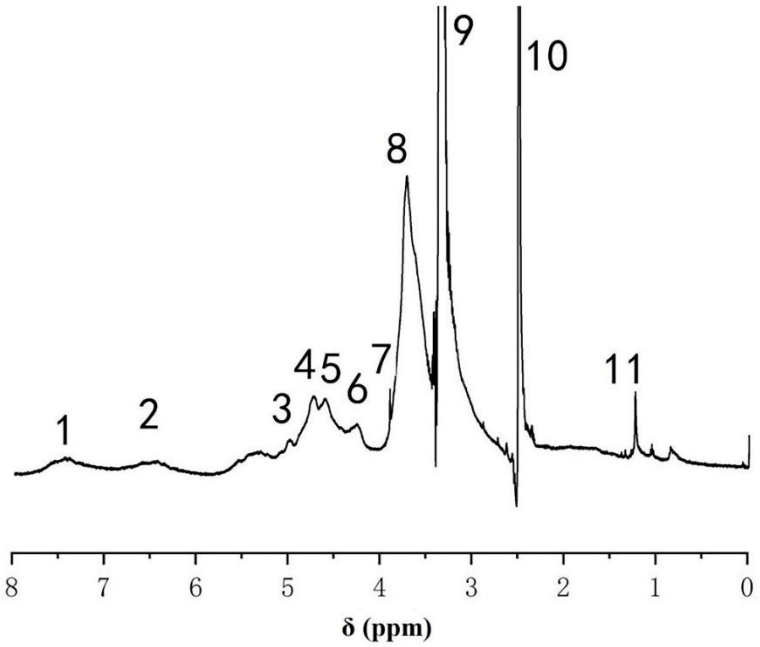

Fig. 9. The ^{1}H-NMR differential spectrum of the EDLCCs from ginkgo wood obtained by subtracting spectrum α-^{13}C/6-^{2}H-labled EDLCC from the unlabeled spectrum

The vibration signals No. 4 (4.73 ppm) and No. 5 (4.65 ppm) were assigned to the 6-H and 6-H' found in benzyl 6-cellulose, according to the result of Nishida *et al*. (1984). This indicated the existence of an ether bond between the α-position of lignins and the 6-position of celluloses. The resonance signals at No. 6 (4.25 ppm) and No. 7 (3.91 ppm) were caused by the 6-H and 6-H' found in esterified cellulose (Deus *et al*. 1991; Hikichi *et al*. 1995). This result revealed that ester linkages were formed by hydroxyl groups on the 6-position of celluloses and the carboxyl group on γ-position of ferulic acid (lignins), also based the results of ^{13}C-NMR determination of the milled wood lignin (MWL) from ginkgo xylem with ^{13}C-enrichment of γ-position of lignin (unpublished). Since the strong signal at 3.75 ppm (No. 8) was from the 6-H and 6-H' of the unsubstituted celluloses (Nishida *et al*. 1984), the hydroxyl groups on most cellulose molecular units in the ginkgo plant were unsubstituted. The assignments of the signals are listed in Table 3, and the structure of the cellulose-lignin complexes and related lignin substructures are shown in Fig. 10.

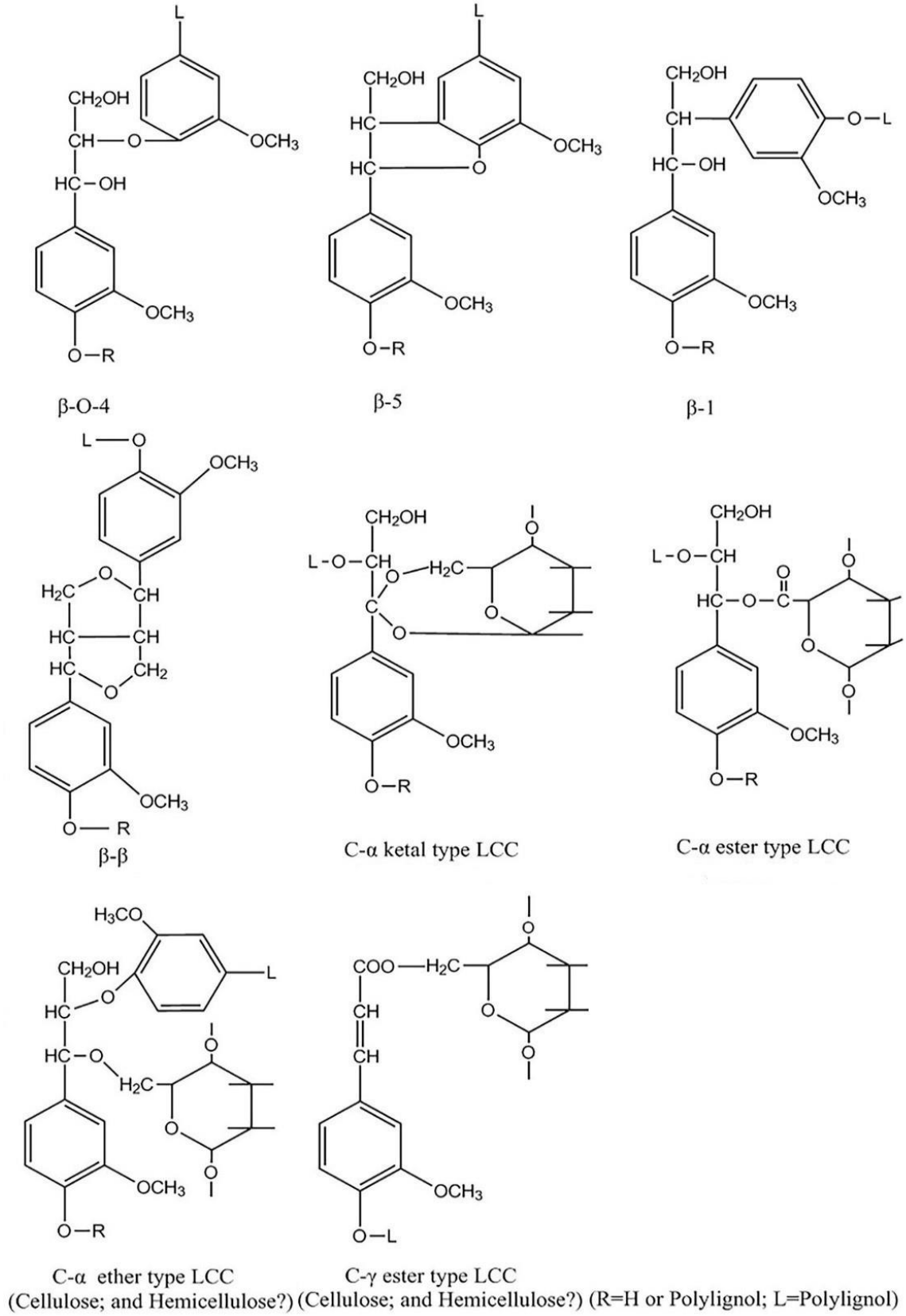

Fig. 10. Lignin structural units and their connections with celluloses and hemicelluloses

Table 3. Assignment of the Signals of the ^{1}H-NMR Differential Spectrum of EDLCCs Obtained by Subtracting Spectrum α-^{13}C/6-^{2}H-labeled EDLCC from the Unlabeled EDLCC Spectrum

Signal	Chemical Shifts (ppm)	Assignments
1	7.42	H of the guaiacyl units of lignin structure
2	6.48	α-H in phenylpropane units of lignin
3	5.00	α-H and β-H in β-5, β-O-4, β-1 of lignin
4	4.73	Benzyl cellulose 6-H
5	4.65	Benzyl cellulose 6-H ’
6	4.25	Ester-bonded cellulose 6-H
7	3.91	Ester-bonded cellulose 6-H’
8	3.75	Cellulose 6-H,H’
9	3.31	Water signal in DMSO-d_6 solvent
10	2.50	DMSO-d_6 solvent peak
11	1.25	Highly obscured aliphatic H

CONCLUSIONS

1. Through the analyses of the abundance and distribution of ^{13}C and ^{2}H in the newly-formed xylem of ginkgo shoots, it was found that the deposition of ^{13}C on lignin was more active at 300 to 1300 μm distance from the cambium and the deposition of D on cellulose was more active at 100 to 900 μm distance from the cambium. The 6-^{2}H/α-^{13}C dual-labeled ginkgo wood was analyzed *via* solid-state CP/MAS ^{13}C NMR. The lignin structural units were primarily connected *via* the β-O-4, β-5, β-1, and β-β structures found in ginkgo plants with a minor coniferyl alcohol structure. Moreover, the C-α in the lignin side chains were connected to carbohydrates through ketal linkages and ester linkages.

2. There were ether linkages and ketal linkages between the C-α found in the lignin side chains and carbohydrates, as shown by ^{13}C-NMR of the ^{13}C-enriched enzymatically degraded LCC. The ^{1}H-NMR differential spectrum of the α-^{13}C/6-^{2}H dual-labeled and unlabeled EDLCC ginkgo wood samples showed a benzyl ether bond between the C-6 position of celluloses and the side chain α-carbon of lignins. The results also revealed that ester linkages were formed by hydroxyl groups on the 6-position of cellulose and the γ-position of ferulic acid of lignin.

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