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Effects of *Arabidopsis thaliana* cell walls on dehydrogenative polymers growth reaction using recombinant cationic cell-wall-peroxidase

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Abstract

Artificial lignin (dehydrogenative polymers: DHPs) has been developed to investigate lignin structures and properties. However, compared with native lignin in plant cell walls, DHPs have lower molecular weight and less content of β -O-4. The conditions required for dehydrogenative polymerization, particularly the reaction within the polysaccharide matrix, are believed to be crucial for promoting lignin growth reaction. In our previous article, xyloglucan esterified with ferulic acid was adsorbed on nematic order cellulose film. Sinapyl alcohol or coniferyl alcohol was polymerized on that film using recombinant cationic cell-wall-peroxidase. The molecular weight and content of β -O-4 of DHPs prepared on artificial cell walls were compared with syringyl-DHP-buffer prepared by polymerizing sinapyl alcohol and guaiacyl-DHP-buffer prepared by polymerizing coniferyl alcohol in buffer by using recombinant cationic cell-wall-peroxidase. DHPs prepared on artificial cell walls had lower molecular weight and less content of β -O-4 than syringyl-DHP-buffer and guaiacyl-DHP-buffer. These results suggested that lignin growth reaction in DHPs preparation on polysaccharide adsorbed on cellulose film may have been inhibited. However, it was considered that the suprastructure of artificial cell walls and plant cell walls is entirely different. This study investigated the effects of *Arabidopsis thaliana* cell walls on DHPs properties following dehydrogenative polymerization reaction with cell walls. Sinapyl alcohol or coniferyl alcohol was polymerized with *Arabidopsis thaliana* cell walls using recombinant cationic cell-wall-peroxidase. Syringyl-DHP-buffer and guaiacyl-DHP-buffer were prepared in the buffer and served as comparative controls. Sinapyl alcohol-DHPs prepared with *Arabidopsis thaliana* cell walls had lower molecular weight and less content of β -O-4 than syringyl-DHP-buffer. Conversely, coniferyl alcohol-DHPs prepared with *Arabidopsis thaliana* cell walls had slightly higher molecular weight and higher content of β -O-4 compared with guaiacyl-DHP-buffer. These results indicated that the effects of plant cell walls on lignin growth reaction differed between syringyl and guaiacyl monomers. Sinapyl-alcohol-DHPs are strongly adsorbed onto xylan, suggesting that polymerization may be restricted. Conversely, coniferyl-alcohol-DHPs are weakly adsorbed onto xylan, allowing some degree of free movement. Consequently, end-wise polymerization proceeds more slowly than the occurring radical coupling in buffer, exhibiting a polymerization pattern similar to that observed in the dropwise method. Furthermore, steric constraints imposed by the polysaccharide matrix may have suppressed coupling at the C5-position, thereby promoting the formation of β -O-4 linkages.

Keywords Recombinant cationic cell-wall-peroxidase, Dehydrogenative polymers, β -O-4, *Arabidopsis thaliana* cell walls

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Introduction

Lignin is the primary component of the cell walls of vascular plants. The primary precursors of angiosperm lignin are coniferyl alcohol (CA) and sinapyl alcohol (SA); the former leads to the formation of guaiacyl lignin and the latter to the formation of syringyl lignin. Angiosperm lignin is a copolymer of SA and CA, with at least 5 different types of chemical bonds between monomer units [1]. Furthermore, lignin structure varies significantly depending on the tree species [2], plant part [3], and tree age [4]. Consequently, native lignin structures have not yet been completely elucidated.

Artificial lignin (dehydrogenative polymers: DHPs) has been developed to investigate the structure and properties of native lignin. However, DHPs have lower molecular weight and less content of β -O-4 compared with native lignin in plant cell walls [5–7]. The secondary cell wall of plants is constructed from cellulose microfibrils that are deposited in nano-sized bundles, onto which hemicellulose is adsorbed. Following this, lignin occupies the gaps between the cellulose and hemicellulose. Consequently, the polysaccharide matrix is considered crucial in the formation of lignin. Barakat et al. and Terashima et al. reported that DHPs prepared in buffer containing xylans [8] or pectins [9] had higher molecular weight and content of β -O-4 than DHPs prepared in buffer without hemicellulose. However these structural differences are yet slight. And these structures of DHPs differ considerably from those of native lignin derived from plant cell walls. The lignification within plant cell walls is thought to occur on a solid polysaccharide matrix composed of cellulose and hemicellulose. Consequently, it is posited that the dehydrogenation polymerization reaction occurring in a buffer solution containing dissolved polysaccharides is markedly different from formation of the native lignin in plant cell walls.

In recent years, studies using artificial cell wall models have been conducted to analyze the effects of the polysaccharide matrix on the structure of DHPs [10, 11]. The advantage of artificial cell walls is that they effectively isolate the DHPs and polysaccharide components, so that it is possible to determine how polysaccharide components affect the structure of the resultant lignin [12]. Lyu et al. conducted a study in which various hemicelluloses were adsorbed onto bacterial cellulose films, and CA was subsequently polymerized using horseradish peroxidase (HRP) to prepare artificial cell walls. This system was used to investigate between the chemical structure of DHPs and hemicellulose. Their findings indicated that the presence of xylan led to higher content of β -O-4 in DHPs compared with instances where cellulose or xyloglucan (XG) was used [13]. However, Lyu et al. primarily used HRP, which is largely incapable of oxidizing SA

or lignin polymers. Therefore, we focused on cationic cell-wall-peroxidase (CWPO-C). CWPO-C was identified in *Populus alba* L. (poplar) an enzyme capable of oxidizing not only CA but also SA and lignin polymers [14, 15]. As described in our previous study, XG esterified with ferulic acid (FA), was adsorbed onto nematic order cellulose film, and SA or CA was polymerized using recombinant CWPO-C (rCWPO-C). It was established that the DHPs formed on artificial cell walls had lower molecular weight and less content of β -O-4 than that of DHPs prepared in buffer [16]. These results suggested that lignin growth reaction might be restricted in DHPs prepared on polysaccharide adsorbed onto cellulose film. It is unknown how hemicellulose is deposited onto cellulose within plant cell walls. Consequently, it is thought that artificial cell walls cannot mimic the suprastructure of the polysaccharide matrix found in plant cell walls, and the differences may affect lignin growth reaction. To use the suprastructures of plant cell walls, plant material was ground in a mortar and, then filtered to recover plant cell walls. Lignin monomers were subsequently added to plant cell walls to prepare DHPs. This model system enables the analysis of the influence of plant cell wall suprastructures on DHPs growth reaction. DHPs prepared by this method were designated as DHP-cell wall (DHP-CW). Grabber et al. reported that DHP-CW prepared from maize cell walls exhibited higher content of β -O-4 than DHPs prepared in buffer [17]. However, DHP-CW was prepared using the originally present peroxidase in plant cell walls, whereas DHPs were prepared in buffer using HRP. Therefore, it was unclear whether the increase in content of β -O-4 was because of the cell walls or the enzyme activity. In addition, the molecular weight of DHP-CW has not been analyzed. In this study, a dehydrogenative polymerization reaction, followed by the development of DHPs, was performed using plant cell walls to examine the effects of *Arabidopsis thaliana* (*A. thaliana*) cell walls on the characteristics of the resulting DHPs. Plant cell walls were isolated from the main stem of *A. thaliana*, then SA or CA was polymerized with the cell walls using rCWPO-C. Subsequently, the DHPs prepared with plant cell walls were analyzed.

Materials and methods

SA and CA were synthesized using the method described in a previous study [18] and were purified through silica gel column chromatography. The preparation of rCWPO-C was conducted as per the procedures established in a previous study [19]. rCWPO-C exhibited a Reinheitszahl value of 2.1, with an enzyme activity of 481 U/mg with 2,6-dimethoxyphenol (2,6-DMP). All other reagents, of extra-pure grade, were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), Tokyo Chemical Industry

Co., Ltd (Tokyo, Japan), and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and were used without further purification.

Plant materials and growth conditions

A. thaliana (ecotype Columbia) plants were used in this study. Seeds were immersed in 0.5% (v/v) sodium hypochlorite for 5 min and then rinsed three times with sterile water. After imbibition at 4 °C for 1 day, the seeds were sown in plastic Petri dishes containing Murashige and Skoog (MS) medium supplemented with 3.0% (w/v) sucrose, pH 5.6, and 0.3% (w/v) gellan gum. The plates were incubated in CLE-303 cultivation chambers (TOMY SEIKO Co., Ltd., Tokyo, Japan) set at 22 ± 1 °C with a 16 h light (100 μmol photons m⁻² s⁻¹):8 h dark cycle. Three-week-old seedlings were transferred to pots containing vermiculite and perlite (1:1, v/v) and were subsequently irrigated with 0.1% Hyponex (Hyponex Japan, Osaka, Japan) every 4 days [20].

Poplar plants were used in this study, too. 80 mL of MS medium (3.0% (w/v) sucrose, 0.3% guar gum, pH 5.6) was poured into the plant box, allowed to solidify, and then poplar main stem containing apical meristem (2.0 cm length) was inserted into the center of the box. The box was placed in a plant incubator under long-day conditions (22 °C, 16 h light, 8 h dark) for cultivation [21].

Preparation of *A. thaliana* cell walls and their enzyme activity

The main stems of the 5.5-week-old *A. thaliana* plants were frozen in liquid nitrogen and then crushed using a mortar and pestle. A total of 90 mg crushed sample was collected in a 2.0 mL Falcon tube, and 1.5 mL of Tris–HCl buffer (50 mM, pH 7.5) was added. The mixture was then vortexed. The resulting suspension solution was then separated into a filtrate and a residue using Miracloth (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). To the residue, 100 μL of Tris–HCl buffer was added. After vortexing the suspension with plant cell walls, the enzymatic activity of *A. thaliana* cell walls was measured at 25 °C in Tris–HCl buffer (50 mM, pH 7.5) based on the oxidation of guaiacol and 2,6-DMP. Absorbance of the enzymatic oxidation products (tetraguaiacol formed from guaiacol and collagen formed from 2,6-DMP) was measured at wavelengths of 470 nm and 469 nm, respectively, using Shimadzu UV-1850 (Shimadzu Corporation, Kyoto, Japan).

Enzyme activity (*A* U/mL) was calculated using the following equation based on the generation of product formed within one min after the addition of hydrogen peroxide [19, 22, 23]:

$$A = \frac{(\Delta E1 - \Delta E2) v}{\epsilon c v0},$$

where $\Delta E1$ is the rate of increase in absorbance (per min) of the reaction solution, $\Delta E2$ is the rate of increase in the absorbance (per min) of the blank solution, v is the total volume of the reaction mixture, c is the enzyme concentration, and $v0$ is the volume of the added enzyme solution. ϵ is the absorptivity of the oxidation product and is equal to 26600 L/mol·cm⁻¹ for tetraguaiacol [23] and 49600 L/mol·cm⁻¹ for coerulignone [22].

Preparation of DHPs with *A. thaliana* cell walls

100 μL of either SA or CA (2.5 μmol), which was dissolved in a minimal volume of methanol and the buffer, and rCWPO-C (0.84 U with 2,6-DMP) were added to *A. thaliana* cell walls (5 mg), along with 4.0 μL of 100 mM H₂O₂, added every 30 min for a total of five times. The mixture was stirred at room temperature for 20 h. The solution was then centrifuged at 10,000 G for 15 min, and the supernatant was collected. The precipitate was washed twice with ultrapure water and six times with diethyl ether. After drying under nitrogen, 100 μL of 80% dioxane–water containing 40 mM LiCl was added, and the extracted DHPs in the sample were designated residue-DHP (res-DHP). A residue was then washed twice with water before being freeze-dried and added to an acetic acid buffer solution (1% w/v; 20 mM; 40 °C; pH 4.8) containing Celluclast from *Trichoderma reesei* (200 μL, Novozymes A/S, Bagsværd, Denmark) and Viscozyme L from *Aspergillus niger* (200 μL, Novozymes A/S, Bagsværd, Denmark) [17]. The mixture was then incubated at 40 °C for 40 h. The precipitate was recovered by centrifugation for 15 min at 14,000 G, washed three times with water, and the insoluble residue was treated with 200 μL of 80% dioxane–water containing 40 mM LiCl and extracted DHPs. This sample was designated DHP-CW (Fig. 1). Syringyl-DHP-buffer (S-DHP-buffer) and guaiacyl-DHP-buffer (G-DHP-buffer) were prepared under identical conditions without plant cell walls, then collected by centrifugation (5000 G for 10 min), washed twice with reverse osmosis (RO) water, and subsequently freeze-dried.

Preparation of sinapyl alcohol-DHPs with poplar cell walls

The main stems of 6-week-old poplar plants were frozen in liquid nitrogen and then crushed using a mortar and pestle. A total of 90 mg crushed sample was collected in a 2.0 mL Falcon tube, and 1.5 mL of Tris–HCl buffer (50 mM, pH 7.5) was added. The mixture was then vortexed. The resulting suspension solution was then separated into a filtrate and a residue using Miracloth. 100 μL of SA (2.5 μmol), which was dissolved

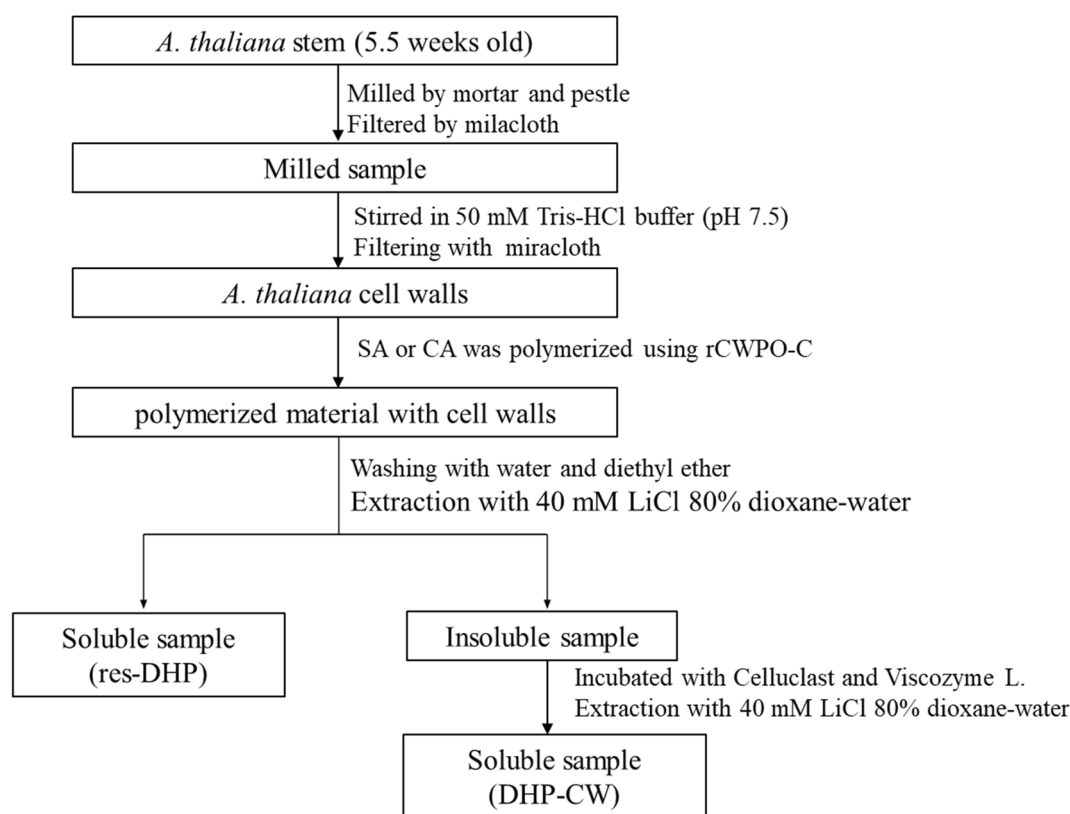


Fig. 1 Scheme of DHPs prepared with *A. thaliana* cell walls

in a minimal volume of methanol and the buffer, and rCWPO-C (0.84 U with 2,6-DMP) were added to poplar cell walls (5 mg), along with 4.0 μ L of 100 mM H_2O_2 , added every 30 min for a total of five times. The mixture was stirred at room temperature for 20 h. The solution was then centrifuged at 10,000 G for 15 min, and the supernatant was collected. The precipitate was washed twice with ultrapure water and six times with diethyl ether. After drying under nitrogen, 100 μ L of 80% dioxane–water containing 40 mM LiCl was added, and the extracted DHPs in the sample were designated residue-syringyl-DHP with poplar (res-S-DHP with poplar). A residue was then washed twice with water before being freeze-dried and added to an acetic acid buffer solution (1% w/v; 20 mM; 40 $^{\circ}$ C; pH 4.8) containing Celluclast from *Trichoderma reesei* (200 μ L) and Viscozyme L from *Aspergillus niger* (200 μ L) [17]. The mixture was then incubated at 40 $^{\circ}$ C for 40 h. The precipitate was recovered by centrifugation for 15 min at 14,000 G, washed three times with water, and the insoluble residue was treated with 200 μ L of 80% dioxane–water containing 40 mM LiCl and extracted DHPs. This sample was designated syringyl-DHP-CW poplar (S-DHP-CW poplar).

Gel permeation chromatography (GPC) analysis

res-DHP and DHP-CW were analyzed by GPC after filtration through 0.45- μ m membrane filters (Sartorius Stedim Lab Ltd., Stonehouse, United Kingdom). The analysis [24] was conducted using JASCO MD-4010 (JASCO Co., Tokyo, Japan). TSKgel α -3000 (TOSOH Inc., Tokyo, Japan) GPC column with an exclusion limit of 90,000 for PEG in water and 100,000 for polystyrene in THF. 80% dioxane–water (v/v) containing 40 mM LiCl served as the mobile phase with a flow rate of 0.5 mL/min and at 25 $^{\circ}$ C. The absorbance of the eluted samples was detected at 260, 270, and 432 nm.

Determination of acetyl bromide-soluble lignin (ABSL)

Amount of DHPs was analyzed by using the acetic bromide method [25]. Polymerized material with cell walls was washed with RO water and diethyl ether, then dried at 70 $^{\circ}$ C for 3 days. Polymerized material with cell walls (5.0 mg) was digested with 1.0 mL of 25% acetyl bromide at 70 $^{\circ}$ C for 3 h in a glass vial, and immediately cooled in ice. Then 2.0 mL of glacial acetic acid was added to complete the reaction. The diluted sample was mixed with 900 μ L of 2.0 M NaOH and 100 μ L of 7.5 M hydroxylamine hydrochloride, and the volume was adjusted to

5.0 mL with acetic acid. The absorbance at 280 nm of the mixture was recorded using Shimadzu UV-1850 (Shimadzu Corporation., Kyoto, Japan). The absorption coefficient was taken from the reference and set to 23.35 L/g cm⁻¹ [26]:

$$\text{Amount of DHPs (\%)} = \frac{100AsV}{\alpha WL},$$

where As is the absorbance of sample, V is the volume of the final solution (L), α is the molar extinction coefficient (L/g·cm⁻¹), W is the weight of the sample (g), and L is the cell thickness (cm).

Derivatization followed by reductive cleavage (DFRC)

The content of β -O-4 was analyzed by using DFRC according to Lu and Ralph [27]. Polymerized material with cell walls was washed with RO water and diethyl ether, then dried at 70 °C for 3 days. Polymerized material with cell walls (5.0 mg) was added to 3.0 mL acetyl bromide: acetic acid (20/80, v/v) at 70 °C for 30 min in a glass vial. After evaporation, the residue was resuspended in 3.0 mL acidic reduction solvent (dioxane/acetic acid/water, 5:4:1, v/v/v). Following the addition of 50 mg zinc dust, the mixture was transferred into a separating funnel with 10 mL dichloromethane and saturated ammonium chloride. The internal standard, 50 μ L of tetracosane (1.0 mg/mL), was added, and the aqueous phase was adjusted to between pH 2 and 3 using 3% HCl. After vigorous mixing, the organic layer was collected, and the extraction was repeated three times with 10 mL of dichloromethane. The organic layer was dehydrated with magnesium sulfate, redissolved in 1.5 mL dichloromethane, and acetylated overnight with 0.5 mL acetic anhydride and 0.5 mL pyridine. Once acetylation reagents were removed, the samples were dissolved in 50 μ L dichloromethane and subjected to gas chromatography with a flame ionization detector (GC-FID). GC-FID analysis was performed using a Shimadzu GC-2014 (Shimadzu Corporation, Kyoto, Japan) equipped with an InertCap column (0.25 mm $\times$ 30 m; GL Sciences Inc., Tokyo, Japan). The temperature was programmed at a rate of 3 °C/min from 140 to 240 °C and 30 °C/min from 240 to 310 °C. The final temperature (310 °C) was held for 12 min. The amounts of CA and SA were determined using calibration curves derived from acetylated pure standards.

Results

Enzyme activity and GPC chromatograms of sinapyl alcohol-DHPs with *A. thaliana* cell walls

Enzyme activity present in the cell wall residues prepared from *A. thaliana* was 9.0×10^{-3} U/mL with 2,6-DMP, and 1.1×10^{-2} U/mL with guaiacol. rCWPO-C, exogenously

added in this study for polymerization, was approximately 400 times higher than the intrinsic enzyme activity in the cell wall, at 3.8 U/mL with 2,6-DMP. Therefore, it was considered that the effect of enzymes present in the cell wall was negligible for the DHPs formation.

SA or CA was polymerized with *A. thaliana* cell walls using rCWPO-C. Polymerized material with cell walls was then washed with RO water and diethyl ether, respectively. The reason for washing polymerized material with cell walls with diethyl ether was to remove chlorophyll that was originally present in *A. thaliana* cell walls. Subsequently, 80% dioxane-water was added to extract DHPs formed on *A. thaliana* cell walls. DHPs extracted with 80% dioxane-water were designated as res-DHP. To recover DHPs strongly bound to cell walls that could not be extracted with 80% dioxane-water, the remaining residue after 80% dioxane-water extraction was incubated with Celluclast from *Trichoderma reesei* and Viscozyme L from *Aspergillus niger* and then washed with water. Subsequently, 80% dioxane-water was added to the treated sample to extract DHPs. Extracted DHPs were designated as DHP-CW.

To confirm that DHPs extracted from polymerized material with cell walls with 80% dioxane-water were not contaminated with the native lignin from *A. thaliana* cell walls, 80% dioxane-water extraction was also conducted on *A. thaliana* cell walls as a control. Lignin extracted from *A. thaliana* cell walls using 80% dioxane-water was designated as control 1. In the GPC analysis, lignin signal was barely visible in the elution chromatograms of control 1 (data not shown). Next, *A. thaliana* cell walls were incubated with Celluclast and Viscozyme L, and then lignin was recovered using 80% dioxane-water. Extracted lignin was designated as control 2. In the GPC analysis of control 2, there was no obvious elution peak in the GPC chromatograms (data not shown). Based on these results, it was determined that GPC chromatograms of DHPs recovered from polymerized material with cell walls using 80% dioxane-water contained almost no native lignin present in *A. thaliana* cell walls.

To confirm the effects of *A. thaliana* cell walls on the molecular weight of sinapyl alcohol-DHPs (SA-DHPs), res-syringyl-DHP (res-S-DHP) and syringyl-DHP-CW (S-DHP-CW) were analyzed by GPC. Chlorophyll was present in the GPC chromatograms of res-S-DHP and S-DHP-CW recovered in 80% dioxane-water. Therefore, the wavelength of chlorophyll elution peaks at 432 nm was also measured (Fig. 2, dotted line). Barone et al. reported that chlorophyll spectrum shows an absorbance ratio at 270 nm/ 432 nm (A_{270}/A_{432}) = 0.2 [28]. The elution peaks around Rt. 15.8 in the chromatograms of res-S-DHP and S-DHP-CW showed A_{270}/A_{432} = 0.2 (Fig. 2). Since the Abs. ratio of chlorophyll and the Abs.

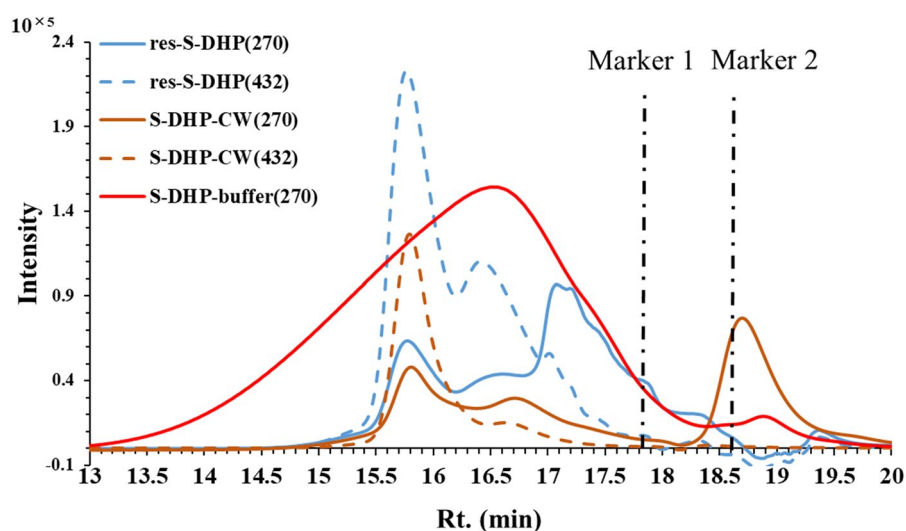


Fig. 2 Molecular weight distribution diagrams of res-S-DHP and S-DHP-CW. Blue line: res S-DHP at 270 nm, blue dotted line: res S-DHP at 432 nm, brown line: S-DHP-CW at 270 nm, brown dotted line: S-DHP-CW at 432 nm, red line: S-DHP-buffer at 270 nm. Markers 1 and 2 indicate retention times of 2-(3,5-dimethoxy-4-methylphenoxy)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl) propan-1-one and SA monomer, respectively (Fig. S1)

ratio of elution peaks near Rt. 15.8 were comparable, elution peaks around Rt. 15.8 in res-S-DHP and S-DHP-CW were mainly attributed to chlorophyll. A₂₇₀/A₄₃₂ of elution peaks around Rt. 16.5 for res-S-DHP was 0.4. It was estimated that approximately half of the elution peaks around Rt. 16.5 in res-S-DHP presented chlorophyll. So, the majority of DHPs in res-S-DHP and S-DHP-CW were present in elution peaks around Rt. 17. res-S-DHP and S-DHP-CW had lower molecular weight than S-DHP-buffer. These results showed that the molecular weight of SA-DHPs prepared with *A. thaliana* cell walls was lower than that of S-DHP-buffer.

GPC chromatograms of coniferyl alcohol-DHPs with *A. thaliana* cell walls

To investigate the effects of *A. thaliana* cell walls on the molecular weight of coniferyl alcohol-DHPs (CA-DHPs), res-guaiacyl-DHP (res-G-DHP) and guaiacyl-DHP-CW (G-DHP-CW) were analyzed by GPC. Figure 3 shows the GPC chromatograms of res-G-DHP (blue line) and G-DHP-CW (brown line). The shoulder peaks at around Rt. 15.8 in chromatograms of res-G-DHP and G-DHP-CW showed A₂₇₀/A₄₃₂ = 1.0. It was estimated that shoulder elution peaks appeared around Rt. 15.8, corresponding to res-G-DHP and G-DHP-CW were mainly attributed to DHPs. The elution peak of res-G-DHP appeared in a slightly higher molecular weight region than that of G-DHP-buffer. However, the maximum molecular weight of res-G-DHP was lower than that of G-DHP-buffer. G-DHP-CW had a higher maximum molecular weight, and the elution peak

top of G-DHP-CW also appeared in higher molecular weight region than G-DHP-buffer. Nevertheless, the maximum molecular weight of G-DHP-CW was lower than that of S-DHP-buffer (red line Fig. 3). These results indicated that CA-DHPs prepared with *A. thaliana* cell walls had slightly higher molecular weight than G-DHP-buffer.

Effects of *A. thaliana* cell walls on DHPs structure

To determine the amount of DHPs formed on *A. thaliana* cell walls, polymerized material with cell walls was analyzed using the acetyl bromide method. The amount of DHPs formed on *A. thaliana* cell walls was calculated by subtracting the lignin content of *A. thaliana* cell walls from that of polymerized material with cell walls. The results confirmed that a larger amount of CA-DHPs than SA-DHPs (Table 1).

To analyze the effects of *A. thaliana* cell walls on the chemical structure of DHPs, polymerized material with cell walls was subjected to DFRC analysis. The results were calculated by subtracting the content of β -O-4 in *A. thaliana* cell walls from that of polymerized material with cell walls. The results showed that SA-DHPs formed on the *A. thaliana* cell walls had less content of β -O-4 than that of S-DHP-buffer (Table 1). The content of β -O-4 in CA-DHPs formed on *A. thaliana* cell walls was threefold that of G-DHP-buffer. However, it was confirmed that CA-DHPs prepared with *A. thaliana* cell walls had less content of β -O-4 than that of native lignin in *A. thaliana* cell walls.

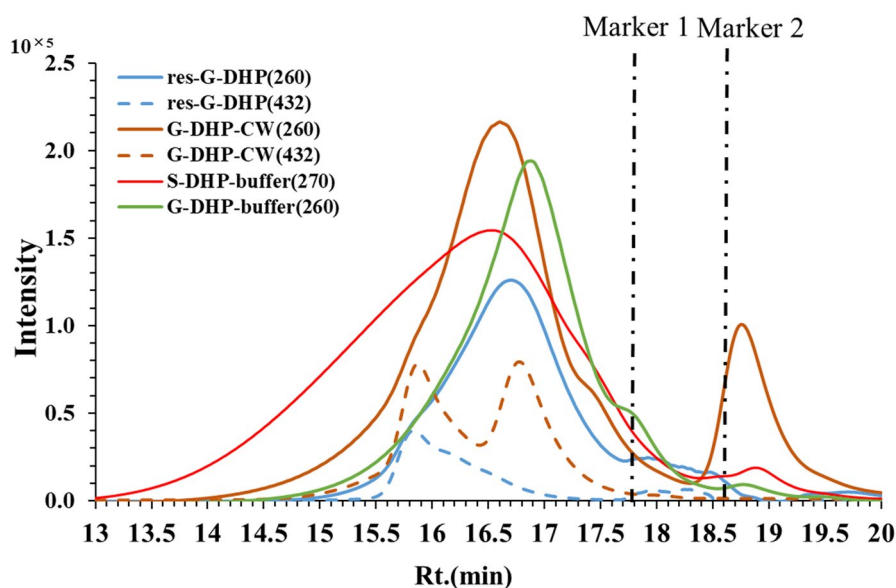


Fig. 3 Molecular weight distribution diagrams of res-G-DHP and G-DHP-CW. Blue line: res-G-DHP at 260 nm, blue dotted line: res-G-DHP at 432 nm, gray line: G-DHP-CW at 260 nm, gray dotted line: G-DHP-CW at 432 nm, red line: S-DHP-buffer at 270 nm, green line: G-DHP-buffer at 260 nm. Markers 1 and 2 indicate retention times of 2-(3,5-dimethoxy-4-methylphenoxy)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl) propan-1-one and SA monomer, respectively

Table 1 Amount of DHPs and content of β -O-4 in SA-DHPs and CA-DHPs

Sample	Lignin (%)	$\mu\text{mol S/g Lignin}$	$\mu\text{mol G/g Lignin}$
<i>A. thaliana</i> cell walls	17.3 $\pm$ 0.3	88 $\pm$ 5	356 $\pm$ 7
SA-DHPs with <i>A. thaliana</i> cell walls	3.8 $\pm$ 0.1	180 $\pm$ 13	
CA-DHPs with <i>A. thaliana</i> cell walls	8.8 $\pm$ 0.7		248 $\pm$ 33
S-DHP-buffer	100	280 $\pm$ 9	
G-DHP-buffer	100		75 $\pm$ 2

Data presented as mean $\pm$ standard deviation of three separate measurements taken from three independent samples

Discussion

The amount of CA-DHPs formed on *A. thaliana* cell walls was higher than that of SA-DHPs (Table 1).

SA-DHPs prepared with *A. thaliana* cell walls had lower molecular weight and less content of β -O-4 than S-DHP-buffer. These findings indicate that the polymerization of SA-DHPs was difficult to proceed in the presence of the *A. thaliana* cell wall matrix. Unlike poplar, *A. thaliana* lacks a distinct layered structure in its secondary cell walls (S1–S3 layers) and exhibits lower lignin deposition [29]. This difference suggests that the plant cell wall's suprastructure differs considerably between herbaceous and woody plants. In this study, we also prepared res-S-DHP with poplar and S-DHP-CW poplar. As shown in Fig. S2, res-S-DHP with poplar and S-DHP-CW poplar had lower molecular weight than S-DHP-buffer. Thus, inhibition of SA-DHPs polymerization by plant cell walls occurred irrespective

of whether the cell walls originated from herbaceous or woody species. In contrast, CA-DHPs formed on *A. thaliana* cell walls had slightly higher molecular weight and higher content of β -O-4 than G-DHP-buffer. These results showed that the effects of plant cell walls on DHPs growth reaction differed between syringyl and guaiacyl monomers. Solid-state nuclear magnetic resonance (NMR) analyses by Kang et al. demonstrated electrostatic interactions between xylan and methoxyl groups of lignin in *A. thaliana* and maize stems. Moreover, syringyl lignin possesses a greater number of methoxyl groups than guaiacyl lignin, leading to stronger electrostatic interactions with xylan [30]. Kirui et al. demonstrated spatial proximity between acetyl group of xylan and C5-position of guaiacyl-lignin by using solid-state NMR [31]. Although the direct evidence remains limited, our results can be interpreted as follows, based on the findings reported by Kang et al.

and Kirui et al. When SA serves as the substrate, SA and SA-oligomers, each with two methoxyl groups per monomer, exhibit strong electrostatic interactions with xylan. The strong adsorption of SA and SA-oligomers restricts their mobility on the plant cell walls matrix, suppressing radical coupling reactions freely. Consequently, polymerization is significantly restricted. Washing with water and diethyl ether removed, unreacted SA and low molecular weight oligomers are removed, resulting in a lower amount of SA-DHPs on plant cell walls, and SA-DHPs prepared with plant cell walls had lower molecular weight and less content of β -O-4 than S-DHP-buffer that polymerized under the condition of unconstrained environment. When CA is used as the substrate, CA and CA-oligomers exhibit weaker electrostatic interactions with xylan. Their looser adsorption permits a certain free movement and leads to two consequences: (i) a slower radical coupling rate of adsorbed CA than radical coupling freely during CA radical coupling in buffer, promoting an end-wise polymerization pattern similar to the dropwise method; and (ii) steric constraints imposed by spatial proximity between C5-position and xylan may act as steric barrier to limit coupling at the C5-position, thereby favoring the formation of β -O-4 linkages. As a result, CA-DHPs exhibited greater deposition on the cell walls, and CA-DHPs prepared with *A. thaliana* cell walls had higher molecular weight and higher content of β -O-4 than G-DHP-buffer.

In our previous study, XG esterified with FA was adsorbed on nematic order cellulose film, and subsequent polymerization of SA or CA was carried out on the film. The resulting DHPs formed on artificial cell walls had lower molecular weight and less content of β -O-4 than DHPs prepared in buffer [16]. Although polymerization occurred on the polysaccharide matrix, the reaction failed to recapitulate the lignin polymerization process characteristic of plant cell walls. Our model utilized a simplified system in which only rCWPO-C was exogenously added to plant cell walls. However, it is proposed that various laccases and Class III peroxidases are related to lignin formation in plants [32, 33]. These oxidative enzymes may strictly regulate the rate of substrate oxidation and, consequently, control the local substrate concentration during lignification. In addition, the concentration and spatiotemporal localization of rCWPO-C in our system may differ substantially from the plant cell wall polysaccharide matrix, which could also affect the rate of substrate oxidation. Lignin growth reaction in plants may not be fully recapitulated in our model because the rate of substrate oxidation and substrate local concentration could not be strictly controlled.

Conclusions

We determined that SA-DHPs prepared with *A. thaliana* cell walls had lower molecular weight and less content of β -O-4 than S-DHP-buffer. Otherwise, CA-DHPs prepared with *A. thaliana* cell walls had slightly higher molecular weight and higher content of β -O-4 than G-DHP-buffer. These results suggested that the effects of DHPs growth reaction on *A. thaliana* cell walls differed between syringyl and guaiacyl monomers.

Abbreviations

2,6-DMP	2,6-Dimethoxyphenol
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
A270/ 432	Absorbance ratio at 270 nm/432 nm
ABSL	Acetyl bromide-soluble lignin
CA	Coniferyl alcohol
CA-DHPs	Coniferyl alcohol-DHPs
CWPO-C	Cationic cell-wall-peroxidase
DFRC	Derivatization followed by reductive cleavage
DHP-CW	DHP-cell wall
DHPs	Dehydrogenative polymers
FA	Ferulic acid
G-DHP-CW	Guaiacyl-DHP-cell wall
G-DHP-buffer	Guaiacyl-DHP-buffer
GC-FID	Gas chromatography with flame ionization detector
GPC	Gel permeation chromatography
HRP	Horseradish peroxidase
MS	Murashige and Skoog
NMR	Nuclear magnetic resonance
Poplar	<i>Populus alba</i> L.
rCWPO-C	Recombinant cationic cell-wall-peroxidase
res-DHP	Residue-DHP
res-G-DHP	Residue-guaiacyl-DHP
res-S-DHP	Residue-syringyl-DHP
res-S-DHP with poplar	Residue-syringyl-DHP with poplar
RO	Reverse osmosis
S-DHP-CW	Syringyl-DHP-cell wall
S-DHP-CW with poplar	Syringyl-DHP-cell wall poplar
S-DHP-buffer	Syringyl-DHP-buffer
SA	Sinapyl alcohol
SA-DHPs	Sinapyl alcohol-DHPs
XG	Xyloglucan

Supplementary Information

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Supplementary material 1.

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Author contributions

YT was the principal investigator of this project. YT and NM designed the experiments. NM performed the experiments and analyzed the data. KF, KS, and AP participated in the analyses and discussions of the results. NM wrote the manuscript. YT and KS revised the manuscript. All authors read and approved the final manuscript.

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Data availability

Data generated or analyzed during this study are included in this article and its supplementary information files. Certain data are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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