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Coniferyl aldehyde dimers in dehydrogenative polymerization: model of abnormal lignin formation in cinnamyl alcohol dehydrogenase-deficient plants

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Abstract The enzymatically dehydrogenative polymerization of coniferyl aldehyde and coniferyl alcohol was studied to understand lignins in cinnamyl alcohol dehydrogenase (CAD)-downregulated plants. The sample dimers were prepared by polymerization under three reaction systems (coniferyl alcohol, coniferyl aldehyde, and their combination) with horseradish peroxidase/H₂O₂ under the conditions of limited reaction time. In addition, the residual amount of substrate in each reaction was determined at specified time intervals. In the reaction system of coniferyl aldehyde, the 5-5-type dimer was formed in preference to β - β and β -5 dimers; in the reaction system of coniferyl alcohol the β -5 dimer was preferentially formed. Furthermore, it was revealed when quantifying dimers among reaction systems that the total dimer formation capability of coniferyl alcohol clearly surpassed that of coniferyl aldehyde. However, the dimers cross-coupled with coniferyl alcohol and coniferyl aldehyde were formed in amounts not accounted for by the difference seen in dimer formation abilities with the two substrates.

Key words Coniferyl alcohol DHP · Coniferyl aldehyde DHP · Coniferyl aldehyde dimer · Coniferyl alcohol – coniferyl aldehyde DHP · Model of abnormal lignin

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Introduction

It is common knowledge that cinnamyl alcohol dehydrogenase (CAD) down-regulates tobacco and poplar, forming red-colored xylems, without a significant reduction in lignin content.^{1–6} A CAD-downregulated loblolly pine with a similar red-colored xylem had been discovered in natural woods, and its abnormal lignin has recently been partially characterized.^{7,8}

Our previous investigation^{9,10} revealed that coniferyl aldehyde and mixtures of coniferyl aldehyde and coniferyl alcohol gave wine-red dehydrogenation polymers (DHPs) with horseradish peroxidase. Their color was similar to that of lignified tissues of transgenic plants with a downregulated CAD and to that of the xylem of a mutant loblolly pine deficient in CAD. Their color might be ascribed to the extensively conjugated *p*-hydroxycinnamyl aldehyde structures. The extensively conjugated β - β , β -5, and 5-5 linkages of coniferyl aldehydes might be formed during dehydrogenation of coniferyl aldehyde. It was thought that such linkages might occur in the abnormal lignins of transgenic plants with a downregulated CAD and a mutant loblolly pine deficient in CAD.

Our further investigation¹¹ showed that the amount of dehydrodivanillin produced by nitrobenzene oxidation of the DHPs gradually increased with the increasing content of coniferyl aldehyde during enzymatic condensation, whereas the amount of vanillin decreased concomitantly compared with that in the controls. The amount of thioacidolysis products derived from coniferyl aldehyde DHP was markedly less than that derived from coniferyl alcohol DHP.⁹ These results suggest that coniferyl aldehyde is predominantly linked as β - β , β -5, and 5-5 substructures, differing from the case of coniferyl alcohol.

This paper describes the characterization and quantification of dimers in the reaction mixtures of coniferyl aldehyde with coniferyl alcohol by horseradish peroxidase/H₂O₂. The study was undertaken to achieve a better understanding of the abnormal lignins.

Materials and methods

Instruments

The nuclear magnetic resonance (NMR) spectra were obtained with a Varian Unity Inova 400 FT-NMR spectrometer (400 MHz). Total ion chromatograms and mass spectra were recorded on a Shimadzu GCMS-QP 5000 gas chromatograph-mass spectrometer (EI, 70 eV, capillary column DB-1; J&W Scientific) that was 30 m \times 0.25 mm (i.d.), with film of 1 μ m; carrier gas He; column temperatures of 180°–280°C, increased at +5°C/min).

Reagents

Coniferyl alcohol was synthesized via two steps from vanillin and monoethyl malonate, which was obtained by HCl treatment of potassium monoethyl malonate (Tokyo Chemical Industry): condensation in the presence of pyridine and a catalytic amount of piperidine at 80°C, and reduction of the product with diisobutylaluminum hydride (1.0 M in toluene solution; Kanto Chemicals) in toluene at 0°C, respectively.¹² Coniferyl aldehyde was synthesized by oxidation of isoeugenol methoxymethyl ether to coniferyl aldehyde methoxymethyl ether with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and subsequent demethoxymethylation of the latter compound with H₂SO₄ to coniferyl aldehyde.¹³ Other reagents and horseradish peroxidase (Tokyo Chemical Industry) were commercially available.

Preparation, isolation, and identification of dimers in the reaction mixture of coniferyl alcohol and coniferyl aldehyde

Coniferyl alcohol (2.0 mmol) and coniferyl aldehyde (2.0 mmol) were dissolved in warm water (712 ml) in a flask. The solution was then cooled in an icebath. Horseradish peroxidase (0.14 mg, 188 purpurogallin number/mg) and 1% H₂O₂ (6.76 ml) were added dropwise to the flask at 0°C with stirring for 30 min. After continued stirring for 60 min, the reaction was stopped by addition of NaCl, and the reaction mixture was extracted with ethyl acetate. The combined ethyl acetate layer was washed with NaCl solution, dried over anhydrous sodium sulfate, and concentrated in vacuo. The products were isolated by silica gel column chromatography and thin-layer chromatography; the purified dimeric products were identified by NMR and mass spectra.

Quantification of the dimers produced and residual coniferyl alcohol and coniferyl aldehyde in reaction mixtures

Dehydrogenative polymerization of coniferyl alcohol and coniferyl aldehyde

A mixture of coniferyl alcohol (9.0 mg, 50 μ mol) and coniferyl aldehyde (8.9 mg, 50 μ mol) was dissolved in warm

water (17 ml), and the solution was cooled. Horseradish peroxidase (1 ml of 3.4 μ g/ml solution) and 0.01% H₂O₂ (17 ml) solutions were added to the solution dropwise for 30 min. The reaction mixture (3 ml) was then pipetted into test tubes at 30, 70, 110, and 150 min, respectively. Two normal HCl (0.5 ml) was added to the respective test tubes to stop the enzyme reaction. Then tetracosane (60 μ g) and hinokinin (20 μ g) were added to the solution as internal standards, and the reaction mixture was extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate, and the solution was concentrated in vacuo. The remaining coniferyl alcohol and coniferyl aldehyde and the produced dimers were silylated with hexamethyldisilazane/trimethylchlorosilane/pyridine (2:1:10) (TMSI-H) (GL Science) and quantified by gas chromatograph-mass spectrometer (GC-MS).

Dehydrogenative polymerization of coniferyl alcohol

Coniferyl alcohol (18.0 mg, 0.1 mmol) was treated as described in the above section. The remaining coniferyl alcohol and the dimers produced in the reaction mixture were silylated and quantified by GC-MS with tetracosane (300 μ g) and hinokinin (40 μ g) as internal standards.

Dehydrogenative polymerization of coniferyl aldehyde

Coniferyl aldehyde (17.8 mg, 0.1 mmol) was treated as described in the above section. The remaining coniferyl aldehyde and the dimers produced in the reaction mixture were silylated and quantified by GC-MS with tetracosane (300 μ g) and hinokinin (40 μ g) as internal standards.

Results and discussion

Characterization of dimers formed by dehydrogenative polymerization of coniferyl alcohol and coniferyl aldehyde

Figure 1 shows the chemical structures of dimers isolated from the reaction mixture of coniferyl alcohol and coniferyl aldehyde. A total ion chromatogram of the reaction mixture in dehydrogenative polymerization of coniferyl alcohol and coniferyl aldehyde is shown in Fig. 2. Fragmentation patterns of trimethylsilane (TMS) derivatives of these dimers are shown in Table 1. Retention times of β - β (5-TMS in Table 1) and β -5 (6-TMS) dimers of coniferyl aldehydes in these analytical conditions were completely identical to those of dimers isolated independently.

Four dimers identified as the enzymatic dehydrogenation of coniferyl alcohols were as follows: β -O-4 dimers (3, 4); β -5 dimer (10); and β - β dimer (11). These dimers had been characterized in previous reports.^{14,15} Three dimers identified as the enzymatic dehydrogenation of coniferyl alcohol and coniferyl aldehyde were as follows: β -O-4 dimers (1, 2) and β -5 dimer (9). These dimers were identified as the coniferyl aldehyde linked to the β -position of

coniferyl alcohol, which had been identified as a hydrolysis product of wood lignin, and dehydrogenative products.¹⁶⁻¹⁸ Four dimers identified in the enzymatic dehydrogenation of coniferyl aldehydes were as follows: β - β dimer (5), β -5 dimer (6), β -*O*-4 dimer (7), and 5-5 dimer (8). Enzymatic dehydrogenation of coniferyl aldehyde had been studied in detail.¹⁹ However, this is the first report to our knowledge on the isolation 5-5 dimer of coniferyl aldehydes.

5-5 Dimer of coniferyl aldehydes (8): ¹H-NMR(CDCl₃): δ 4.01 (6H, s, OCH₃), 6.64 (2H, dd, J = 7.7, 15.8, H- β , H- β'), 7.13 (2H, d, J = 1.8, H-aromatic), 7.20 (2H, d, J = 1.8, H-aromatic), 7.43 (2H, d, J = 15.8, H- α , H- α'), 9.67 (2H, d, J = 7.7, H- γ , H- γ').

These spectral data of the dimers give useful information for structural determination of a CAD-deficient pine lignin and a CAD-downregulated tobacco lignin.²⁰

Residual monomers and dimers formed during enzymatic dehydrogenation of coniferyl alcohol and coniferyl aldehyde

Amounts of dimers per milliliter of the reaction mixture were determined successively during the enzymatic dehydrogenation period. The results are shown in Tables 2, 3, and 4 for the dehydrogenation systems of a mixture of coniferyl alcohol and coniferyl aldehyde (Table 2), coniferyl alcohol alone (Table 3), and coniferyl aldehyde alone (Table 4). The β - β (5-TMS) and β -5 (6-TMS) dimers of coniferyl aldehydes with the same retention time in the total ion chromatogram are shown as the combined amount of two dimers.

The amount of coniferyl alcohol dimers (alc-alc type) was the largest, followed by coniferyl alcohol-coniferyl al-

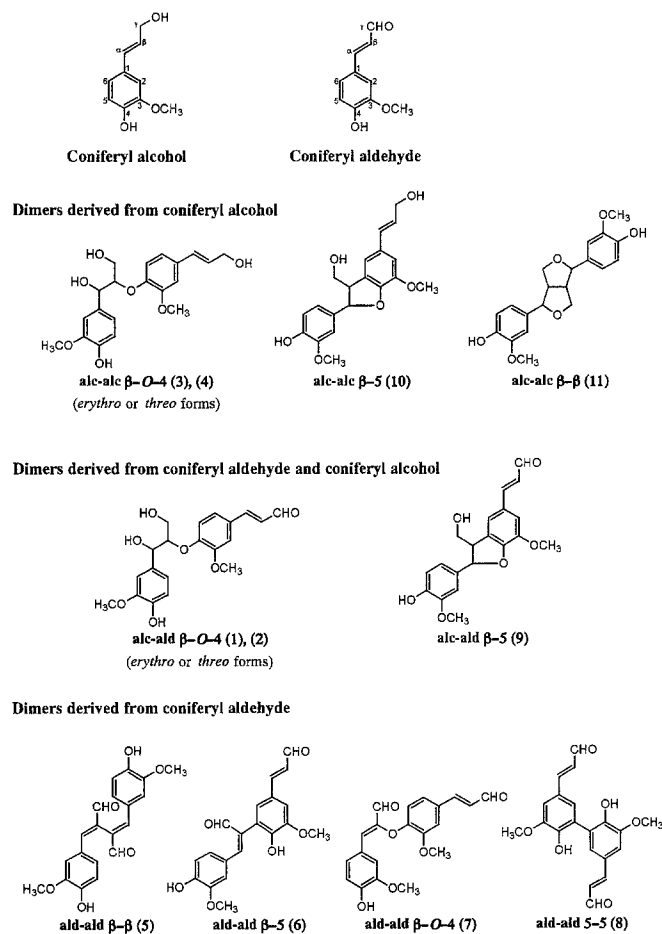


Fig. 1. Dimers identified from the dehydrogenation products of a mixture of coniferyl aldehyde and coniferyl alcohol

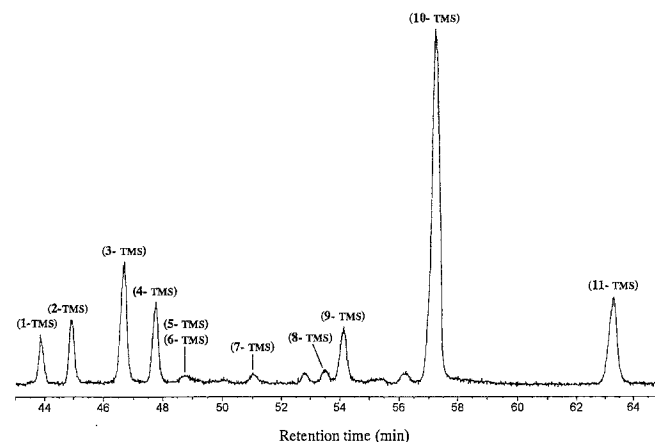


Fig. 2. Total ion chromatogram of the dimers in dehydrogenation products formed from coniferyl alcohol and coniferyl aldehyde. The numbers in parentheses on the peaks correspond to those in Fig. 1.

Table 1. Fragmentation patterns of trimethylsilane (TMS) derivatives of dimers formed by dehydrogenation of coniferyl alcohol and coniferyl aldehyde

Compound	Dimer type	Molecular ion and prominent fragments m/z (relative intensity)
1-TMS	alc-ald β - <i>O</i> -4	590(2), 323(3), 297(100), 235(4), 223(4), 209(6), 194(4), 177(4), 147(4), 103(8), 73(93)
2-TMS	alc-ald β - <i>O</i> -4	590(2), 323(2), 297(100), 235(3), 223(4), 209(9), 194(3), 177(3), 147(5), 103(9), 73(88)
3-TMS	alc-alc β - <i>O</i> -4	664(3), 324(10), 298(16), 297(72), 235(2), 209(12), 147(4), 103(9), 73(100)
4-TMS	alc-alc β - <i>O</i> -4	664(2), 324(15), 298(19), 297(100), 235(3), 209(12), 147(4), 103(8), 73(95)
5-TMS	ald-ald β - β	498(16), 469(3), 302(2), 274(7), 243(6), 209(7), 181(4), 73(100)
6-TMS	ald-ald β -5	498(10), 469(3), 454(2), 408(3), 384(3), 303(9), 209(7), 166(3), 73(100)
7-TMS	ald-ald β - <i>O</i> -4	426(11), 369(5), 338(2), 265(4), 248(5), 207(21), 179(4), 73(100)
8-TMS	ald-ald 5-5	498(14), 483(4), 450(1), 409(3), 393(3), 349(2), 207(7), 73(100)
9-TMS	alc-ald β -5	500(5), 410(28), 380(7), 207(7), 103(13), 73(100)
10-TMS	alc-alc β -5	574(8), 484(32), 454(5), 396(1), 394(2), 209(7), 103(7), 73(100)
11-TMS	alc-alc β - β	502(28), 235(42), 223(67), 209(38), 194(23), 179(13), 131(14), 73(100)

Table 2. Yield of dimers formed from a reaction system of coniferyl alcohol and coniferyl aldehyde with reaction time

Reaction time (min)	alc-alc type			alc-ald type		ald-ald type		
	β -O-4 (3), (4)	β - β (11)	β -5 (10)	β -O-4 (1), (2)	β -5 (9)	β -O-4 (7)	β - β + β -5 (5), (6)	5-5 (8)
30	40.7 (24.9)	31.7 (19.4)	51.2 (31.4)	20.9 (12.8)	12.8 (7.8)	1.72 (1.1)	2.65 (1.6)	1.47 (0.9)
70	33.0 (18.2)	29.0 (16.0)	82.9 (45.7)	14.7 (8.1)	12.5 (6.9)	3.96 (2.2)	3.35 (1.8)	2.12 (1.2)
110	29.3 (18.8)	26.2 (16.8)	59.9 (38.5)	14.8 (9.5)	18.6 (12.0)	2.12 (1.4)	3.38 (2.2)	1.28 (0.8)
150	32.0 (18.3)	30.0 (17.2)	79.4 (45.4)	13.8 (7.9)	12.6 (7.2)	3.13 (1.8)	2.92 (1.7)	0.99 (0.6)

Results are given in nanomoles per milliliter and percent relative content (in parentheses)

The number in parentheses in the subheadings refer to the TMS numbers in Table 1

alc-alc, coniferyl alcohol – coniferyl alcohol; alc-ald, coniferyl alcohol – coniferyl aldehyde; ald-ald, coniferyl aldehyde – coniferyl aldehyde

Table 3. Yield of dimers formed from the reaction system of coniferyl alcohol with reaction time

Reaction time (min)	alc-alc type		
	β -O-4 (3), (4)	β - β (11)	β -5 (10)
30	109 (30.9)	82.9 (23.5)	161 (45.6)
70	106 (28.2)	80.6 (21.5)	189 (50.3)
110	106 (29.3)	81.5 (22.5)	174 (48.1)
150	61 (24.6)	50.7 (20.5)	136 (54.9)

Results are given as nanomoles per milliliter and percent relative content (in parentheses)

Table 4. Yield of dimers formed from the reaction system of coniferyl aldehyde with reaction time

Reaction time (min)	ald-ald type		
	β -O-4 (7)	β - β + β -5 (5), (6)	5-5 (8)
30	10.9 (42.7)	6.7 (26.3)	7.9 (31.0)
70	15.9 (35.4)	10.7 (23.8)	18.3 (40.8)
110	20.8 (35.4)	11.2 (19.1)	26.7 (45.5)
150	22.0 (44.3)	11.6 (23.3)	16.1 (32.4)

Results are given as nanomoles per milliliter and percent relative content (in parentheses)

dehyde dimers (alc-ald type) and coniferyl aldehyde dimers (ald-ald type), as shown in Table 2. Also, the amount of coniferyl alcohol dimers in the dehydrogenation system of coniferyl alcohol alone was higher than that of coniferyl aldehyde dimers in the system of coniferyl aldehyde alone (Tables 3, 4).

In the dehydrogenation system of coniferyl alcohol and coniferyl aldehyde, the β -5 dimer of coniferyl alcohols (10-TMS in Table 1) was formed in the largest amount, followed by β -O-4 (3-TMS, 4-TMS) and β - β (11-TMS) dimers of coniferyl alcohols. The total amount of β -O-4 dimers (1-TMS, 2-TMS) was larger than that of β -5 dimer (9-TMS) in coniferyl alcohol-coniferyl aldehyde dimers. The amount of β -O-4 dimer (7-TMS) was almost equal to the total amount of β - β (5-TMS) and β -5 (6-TMS) dimers, followed by a small amount of the 5-5 dimer (8-TMS) in coniferyl aldehyde dimers (Table 2).

The dimers of coniferyl alcohol were produced in the same order in the dehydrogenation system of coniferyl alcohol alone and the mixed system of coniferyl alcohol and coniferyl aldehyde. However, the system of coniferyl aldehyde

Table 5. Amounts of residual coniferyl alcohol and coniferyl aldehyde and their rates during dehydrogenative polymerization of a mixture of coniferyl alcohol and coniferyl aldehyde

Reaction time (min)	Residual substrate (nmol/ml) (%)	
	Coniferyl alcohol	Coniferyl aldehyde
30	8.66 (0.61)	423 (30)
70	7.92 (0.55)	224 (16)
110	4.98 (0.35)	202 (14)
150	4.76 (0.33)	177 (12)

Table 6. Amounts of residual coniferyl alcohol and its rate during dehydrogenative polymerization of coniferyl alcohol

Reaction time (min)	Remaining substrate (coniferyl alcohol) (nmol/ml) (%)
30	2.44 (0.09)
70	2.56 (0.09)
110	3.19 (0.11)
150	2.65 (0.09)

Table 7. Amounts of residual coniferyl aldehyde and its rate during dehydrogenative polymerization of coniferyl aldehyde

Reaction time (min)	Remaining substrate (coniferyl aldehyde) (nmol/ml) (%)
30	1573 (55)
70	1562 (55)
110	892 (31)
150	409 (14)

alone produced a 5-5 dimer in larger amounts, differing from the case of the mixed system (Table 4).

The amounts of residual substrate per milliliter of reaction mixture were determined successively during the enzymatic dehydrogenation period. The results are shown in Tables 5, 6, and 7, which represent the dehydrogenation system of coniferyl alcohol and coniferyl aldehyde (Table 5), the dehydrogenation system of coniferyl alcohol alone (Table 6), and the dehydrogenation system of coniferyl aldehyde alone (Table 7). The amount of residual coniferyl alcohol was markedly less than that of coniferyl aldehyde in the dehydrogenation system of coniferyl alcohol and coniferyl aldehyde. Furthermore, the gap was significant

between the two systems of coniferyl alcohol alone and coniferyl aldehyde alone. The amount of monomers remaining in the reaction mixture decreased successively during the dehydrogenation process in the systems of coniferyl alcohol alone and coniferyl aldehyde alone. The results of the quantitative analysis on the residual substrates and the dimers formed suggested that there was a difference in the reactivity among substrates in the dehydrogenative polymerization with peroxidase.

Reactivity of coniferyl alcohol and coniferyl aldehyde during dehydrogenative polymerization with peroxidase/H₂O₂

The present investigation confirmed the formation of dimers (1) or (2) and dimer (9), with β -O-4 and β -5 linkages of coniferyl alcohol and coniferyl aldehyde. However, the dimers of coniferyl alcohol linked to the β -position of coniferyl aldehyde were scarcely detected. In Tables 2–4, the relative content of linkage at the β -position of coniferyl aldehyde was lower than that of coniferyl alcohol, whereas the relative content of linkage at the 5-position of coniferyl aldehyde was higher than that of coniferyl alcohol.

During the dehydrogenative polymerization reaction of coniferyl alcohol the 5-5 dimer was not detected, and the corresponding 5-5 dimer (8-TMS) was predominantly formed during the dehydrogenation reaction of coniferyl aldehyde alone. The result suggested that coniferyl aldehyde was predominantly polymerized via 5-5 linkage to give a condensed structure, as the formation of abnormal lignin was discussed in previous papers.^{10,11} Also, it must be remembered that biphenyl and biphenyl ether bonds were reported to be rich in abnormal lignin in a CAD-deficient pine mutant.²¹

When a mixture of coniferyl alcohol and coniferyl aldehyde was dehydrogenated with peroxidase/H₂O₂, predominantly dimers of coniferyl alcohol were formed. Correspondingly, the consumption speed of coniferyl alcohol was high, whereas that of coniferyl aldehyde was significantly lower. It was thus indicated that the reactivity of coniferyl alcohol during dehydrogenative polymerization was significantly higher than that of coniferyl aldehyde. Moreover, it was suggested that the electron density of the aromatic ring was sparse, as electron withdrawing of the carbonyl group at the γ -position in coniferyl aldehyde caused less removal of the hydrogen atom, different from coniferyl alcohol.

A comparison of substrates used during the two dehydrogenation reactions with coniferyl aldehyde alone (Table 7) and coniferyl alcohol plus coniferyl aldehyde (Table 5) showed that the former reaction left a considerable amount of substrate until the middle phase of the reaction. The polymerization did not proceed utilizing only coniferyl alcohol in the case of the dehydrogenation reaction of coniferyl alcohol and coniferyl aldehyde. The polymerization of coniferyl aldehyde might be accelerated in the presence of coniferyl alcohol.

The results of the present investigation indicates that when coniferyl alcohol is used as a substrate in the presence of coniferyl aldehyde it is polymerized predominantly in CAD-downregulated plants. Yahiaoui et al.⁵ reported that the downregulated CAD less tobacco with 8% to the normal one produced a lignin content that was not markedly decreased, although the amount of reaction product and the ratio of syringyl to guaiacyl type products in thioacidolysis decreased; moreover, the extractability of the lignin by alkali was considerably increased. These alterations were more significant in comparison with that of tobacco with 11% CAD activity. These results suggest that the carbon-carbon bonds and cinnamyl aldehyde content in lignin were increased. Furthermore, regulation of the CAD gene during lignin biosynthesis should be less than about 10% of the activity of CAD with normal lignification. This level of CAD activity might induce incorporation of cinnamyl alcohols with cinnamyl aldehydes into lignin.

The present investigation on the different reactivity of coniferyl alcohol and coniferyl aldehyde in dehydrogenative polymerization could help reveal the mechanism of abnormal lignin formation in CAD-deficient plants.

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References

- Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier M-T, Schuch W (1994) Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *Plant J* 6:339–350
- Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T (1995) Increase of cinnamaldehyde groups in lignin of transgenic tobacco plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. *Biosci Biotechnol Biochem* 59:929–931
- Baucher M, Chabbert B, Pilate G, Doorsselaere JV, Tollier M-T, Petit-Conil M, Cornu D, Monties B, Montagu MV, Inzé D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. *Plant Physiol* 112:1479–1490
- Yahiaoui N, Marque C, Corbière H, Boudet AM (1998) Comparative efficiency of different constructs for down regulation of tobacco cinnamyl alcohol dehydrogenase. *Phytochemistry* 49: 295–306
- Yahiaoui N, Marque C, Myton KE, Negrel J, Boudet AM (1998) Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta* 204:8–15
- Bernard Vailhé MA, Besle JM, Maillot MP, Cornu A, Halpin C, Knight M (1998) Effect of down-regulation of cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. *J Sci Food Agric* 76:505–514
- MacKay JJ, O'Malley DM, Presnell T, Booker FL, Campbell MM, Liu W, Whetten RW, Sederoff RR (1997) Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proc Natl Acad Sci USA* 94: 8255–8260
- Ralph J, MacKay JJ, Hatfield RD, O'Malley DM, Whetten RD, Sederoff RR (1997) Abnormal lignin in a loblolly pine mutant. *Science* 277:235–239

9. Higuchi T, Ito T, Umezawa T, Hibino T, Shibata D (1994) Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: wine-red lignin from coniferyl aldehyde. *J Biotechnol* 37:151–158
10. Ito T, Kawai S, Ohashi H, Higuchi T (1996) Chemical and structural studies on abnormal lignin of transgenic plants with antisense cinnamyl alcohol dehydrogenase (CAD) gene. In: TAPPI proceedings of pulping conference, Nashville, TN, pp 111–115
11. Ito T, Kawai S, Ohashi H, Higuchi T (1999) Behavior of cinnamyl aldehyde structures for chemical degradation. In: Proceedings of the 10th ISWPC, Yokohama, pp 84–87
12. Quideau S, Ralph J (1992) Facile large-scale synthesis of coniferyl, sinapyl, and *p*-coumaryl alcohol. *J Agric Food Chem* 40:1108–1110
13. Nakamura Y, Higuchi T (1976) A new synthesis of coniferyl aldehyde and alcohol. *Wood Res* 59/60:101–105
14. Katayama Y, Fukuzumi T (1978) Enzymatic synthesis of three lignin-related dimers by an improved peroxidase-hydrogen peroxide system (in Japanese). *Mokuzai Gakkaishi* 24:664–667
15. Terashima N, Atalla RH (1995) Formation and structure of lignified plant cell wall: factors controlling lignin structure during its formation. In: Proceedings of the 8th ISWPC, Helsinki, pp 69–76
16. Aoyama M, Sakakibara A (1976) Isolation of a new phenylcoumaran compound from hydrolysis products of hardwood lignin. *Mokuzai Gakkaishi* 22:591–592
17. Nimz H (1967) Über zwei aldehydische dilignole aus fichtenlignin. *Chem Ber* 100:2633–2639
18. Freudenberg K, Lehmann B (1960) Aldehydische Zwischenprodukte der Ligninbildung. *Chem Ber* 93:1354–1366
19. Connors WJ, Chen C-L, Pew JC (1970) Enzymic dehydrogenation of the lignin model coniferaldehyde. *J Org Chem* 35:1920–1924
20. Ralph J, Kim H, Lu F, Ralph SA, Landucci LL, Ito T, Kawai S, Ohashi H, Higuchi T (1999) Incorporation of hydroxycinnamaldehydes into lignins. In: Proceedings of the 10th ISWPC, Yokoyama, pp 58–63
21. Lapiere C, Pollet B, MacKay JJ, Sederoff RR (2000) Lignin structure in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *J Agric Food Chem* 48:2326–2331