Plant Physiol. (1997) 114: 871–879

Structural Characterization of Modified Lignin in Transgenic Tobacco Plants in Which the Activity of 4-Coumarate:Coenzyme A Ligase Is Depressed

Shinya Kajita*, Shojiro Hishiyama, Yoichi Tomimura, Yoshihiro Katayama, and Shunji Omori

Research Center, Mitsubishi Paper Mills Limited, 46 Wadai, Tsukuba, Ibaraki 300–42, Japan (S.K., S.O.); Forestry and Forest Products Research Institute, P.O. Box 16, Tsukuba, Ibaraki 305, Japan (S.H., Y.T.); and Graduate School of Bio-Application & System Engineering, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan (Y.K.)

Transgenic tobacco (*Nicotiana tabacum* L.) plants in which the activity of 4-coumarate:coenzyme A ligase is very low contain a novel lignin in their xylem. Details of changes in hydroxycinnamic acids bound to cell walls and in the structure of the novel lignin were identified by base hydrolysis, alkaline nitrobenzene oxidation, pyrolysis-gas chromatography, and ^{13}C-nuclear magnetic resonance analysis. In the brownish tissue of the transgenic plants, the levels of three hydroxycinnamic acids, *p*-coumaric, ferulic, and sinapic, which were bound to cell walls, were apparently increased as a result of down-regulation of the expression of the gene for 4-coumarate:coenzyme A ligase. Some of these hydroxycinnamic acids were linked to cell walls via ester and ether linkages. The accumulation of hydroxycinnamic acids also induced an increase in the level of condensed units in the novel lignin of the brownish tissue. Our data indicate that the behavior of some of the incorporated hydroxycinnamic acids resembles lignin monomers in the brownish tissue, and their accumulation results in dramatic changes in the biosynthesis of lignin in transgenic plants.

The deposition of lignin in vascular cell walls is a key step in the differentiation of these cells into the functional elements that participate in the transport of water and in the structural support of plants. The biosynthesis of lignin has been studied extensively and the major enzymes involved in the synthesis of precursors to lignin have been identified (Higuchi, 1985; Lewis and Yamamoto, 1990). In the past decade many genes for enzymes related to the biosynthesis of lignin have been cloned from a variety of plant species, and the expression of these genes at various stages of lignification has been characterized (Sederoff et al., 1994; Whetten and Sederoff, 1995). In several recent studies transgenic plants carrying chimeric genes for the enzymes involved in lignin biosynthesis were generated and the altered lignins in these transformants were analyzed (Elkind et al., 1990; Dwivedi et al., 1994; Halpin et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Hibino et al., 1995; Mcintyre et al., 1996). The analysis of lignins in these transgenic plants has provided useful information about the role of each enzyme in lignin biosynthesis. The characterization of the structures of the lignin in such transgenic plants is also important for the development of new plants with lignins suitable for pulp-making and with fibers of enhanced digestibility (Halpin et al., 1994; Bernard-Vailhe et al., 1996a, 1996b).

In a recent study we produced transgenic tobacco plants that carried chimeric genes for 4CL, and in which the activity of 4CL was depressed (Kajita et al., 1996). The low 4CL activity in these transgenic plants was due to the down-regulation of the endogenous gene(s) for 4CL. In transgenic plants with low 4CL activity the xylem tissue in stems was brown rather than the yellowish-white of the xylem tissue of control plants. Cell walls in the brownish tissue did not stain normally with phloroglucinol-HCl or in the Mäule reaction. These reactions are standard methods for the detection of lignin. The brownish cell walls in several transgenic plants that had depressed 4CL activity and that carried the chimeric sense-gene construct for 4CL had a similar response in the staining analysis. Moreover, levels of benzaldehydes detected after alkaline nitrobenzene oxidation of the CWR of plants with brownish xylem tissue were, without exception, lower than those of control plants (Kajita et al., 1996). It seemed likely, then, that the lignin in the brownish tissue was rich in condensed units.

To confirm the structure of the lignin in the brownish tissue and to evaluate the mechanism responsible for the change in lignin structure, we used offspring from a typical transgenic plant that had been transformed with the chimeric sense gene for 4CL, as well as offspring from a control transgenic plant. We investigated the detailed chemical structures of lignins in the transgenic plants by ^{13}C-NMR and Py-GC, as well as by traditional oxidative and base hydrolytic methods for the qualitative and quantitative analysis of lignins. We also monitored levels of H-units in the lignins of the transgenic plants, which we had failed to examine in our previous study (Kajita et al., 1996). Our results indicate that many derivatives of hydroxycinnamic acid are linked via ester and/or ether bonds

* Corresponding author; e-mail kajita_s@ccm.mpm.co.jp; fax 81-298-64-4310.

Abbreviations: 4CL, 4-coumarate:CoA ligase; CWR, cell wall residue; G-unit, guaiacyl unit; H-unit, *p*-hydroxyphenyl unit; MTL, milled tobacco lignin; ppm, parts per million; Py-GC, pyrolysis-GC; S-unit, syringyl unit.

to the cell wall fraction, and that the novel lignin has elevated levels of condensed units as a result of the increase in levels of accumulated hydroxycinnamic acids.

MATERIALS AND METHODS

We used offspring derived from two different transgenic tobacco (*Nicotiana tabacum* L.) plants (Kajita et al., 1996), one (designated S4 in the previous report) that had been transformed with the binary vector pSNT4CL, which included a chimeric gene for 4CL in the T-DNA region, and one (control; designated C1 in the previous report) that had been transformed with pBI121DG, which included only a gene construct for resistance to kanamycin in the same region. The S4 plant and its offspring had brownish xylem tissue that included novel lignin, and depressed 4CL activity as a result of the down-regulation of the endogenous gene for 4CL.

The second generations (T_1) produced from selfed seeds of the S4 and C1 plants were selected on kanamycin-containing medium (300 mg L^{-1}), and kanamycin-resistant plants (20 plants per mother plant) were chosen for experiments. The existence of the relevant gene construct in each offspring was confirmed by PCR with the total DNA from each plant. Offspring from each plant were used in further experiments after cultivation for 5 months.

Preparation of CWR

The brownish regions of the xylem tissue from stems of S4 offspring were carefully separated from the noncolored tissue and combined. The sample of the brownish tissue was dried and ground until it could pass through 100-mesh (150 μm in diameter). The milled sample was extracted successively with a mixture of toluene and ethanol (2:1, v/v) for 10 h, with ethanol for 10 h, and with hot water for 6 h in a Soxhlet extractor. The extract-free powder (CWR) was dried again under a vacuum for 3 weeks and used for analysis. The reference sample from control tissue was prepared in the same way as that from offspring of the C1 plant.

Determination of Lignin Content

The lignin content of the CWR was determined by the Klason method and the acetyl bromide procedure. Two parallel experiments were performed using each method. For determination by the Klason method, the CWR (500 mg) was treated with 72% sulfuric acid at 20°C for 4 h. The sample was then diluted with water to a sulfuric acid concentration of 3% and boiled for 4 h. After filtration through a glass filter, washing with hot water, and heating in an oven at 105°C overnight, the acid-insoluble residue (Klason lignin) was weighed. For determination by the acetyl bromide procedure, the CWR (5 mg) was digested with 2.5 mL of a mixture of acetyl bromide and acetic acid (25:75, w/w) plus 0.1 mL of 70% perchloric acid at 70°C for 30 min. The solution was then cooled and transferred to a volumetric flask (50 mL) that contained 10 mL of 2 N sodium hydroxide and 12 mL of acetic acid. The solution was made up to 50 mL with acetic acid. A blank sample was prepared from the same solution without the CWR. The UV absorption spectrum of the solution was recorded against the blank solution. The amount of lignin was determined from the A_{280} and a specific absorption coefficient of 20 L g^{-1} cm^{-1} (Iiyama and Wallis, 1990).

Analysis by Alkaline Nitrobenzene Oxidation

The CWR (20 mg) was subjected to analysis by alkaline nitrobenzene oxidation as described previously (Kajita et al., 1996). The products after oxidation of the sample were silylated and analyzed by GC (model G1800A, Hewlett-Packard) on a capillary column (DB-1, J&W Scientific, Folsom, CA) with an electron ionization detector. The column temperature was 200°C initially and after 5 min was increased to a final temperature of 270°C at a rate of 5°C min^{-1}. The products were identified from their mass spectra and quantitated by reference to a calibration curve prepared from the results of analysis of authentic compounds. Each sample was analyzed in three parallel experiments.

Analysis of Alkali-Labile Hydroxycinnamic Acids

Levels of hydroxycinnamic acids linked to cell walls via ester and/or ether linkages were measured in duplicate for each sample by hydrolysis and subsequent analysis by GC.

The CWR (300 mg) was treated with 20 mL of 1 N sodium hydroxide at 20°C for 24 h or 7 mL of 4 N sodium hydroxide at 170°C for 2 h. After removal by filtration of the residue the solution was acidified with 1 N hydrochloric acid and saturated with sodium chloride. The solution was then extracted three times with diethylether and the ether-soluble fraction was recovered. This fraction was dehydrated with anhydrous sodium sulfate overnight. The solution was evaporated to dryness and the residue was dissolved in 1 mL of pyridine. The silylated sample was analyzed by GC on a capillary column (model HP-5, Hewlett-Packard). The column temperature was 100°C initially and after 5 min was increased to a final temperature of 270°C at a rate of 10°C min^{-1}. Identification of the products was performed as described above.

Py-GC

The analysis was performed as described by Izumi et al. (1995). The CWR (200 μg) was pyrolyzed in a Curie-point pyrolyzer (JHP-3, Japan Analytical Industry Co., Ltd., Tokyo, Japan) at 500°C for 4 s. The products of pyrolysis were transferred to a gas chromatograph (model GC-14A, Shimazu Co., Kyoto, Japan) with a stainless-steel capillary column (Ultra Alloy-8 h-1, Hitachi Co., Tokyo, Japan). The temperature of the column was initially kept at 50°C for 1 min and was then increased to a final temperature of 280°C at a rate of 5°C min^{-1}. The levels of G- and S-type products were estimated from the response factors for each product, as reported by Izumi et al. (1995).

Preparation of MTL and ^{13}C-NMR Spectroscopy

MTLs were isolated from the brownish tissue and control tissue using a procedure described by Björkman (1956),

with slight modifications. The CWR (10 g of each sample) was milled in a ball-mill apparatus with toluene for 96 h. The lignin was extracted with a mixture of dioxane and water (96:4, v/v) for 1 week. After extraction, the solution was concentrated, and then this concentrated solution was added dropwise to 20 times its volume of diethylether. The resultant precipitate was dissolved in acetic acid (90%) and the solution was added to 20 times its volume of water. The precipitated MTL was recovered by centrifugation and dried under a vacuum for 3 d. For further purification, MTL was dissolved in a mixture of dichloroethane and ethanol (2:1, v/v) and the solution was added dropwise to 20 times its volume of diethylether. The precipitated MTL was washed three times with diethylether and dried under a vacuum for 1 week.

The ^{13}C-NMR spectrum of MTL was recorded at 303K from 100 mg of the sample of MTL that had been dissolved in DMSO-d_6 with a spectrometer (62.89 MHz; model AC-250P, Bruker Japan Co., Ltd., Tsukuba, Japan). A pulse-repetition time of 2.0 s with a flipping angle of 45° was used, with 60,000 scans. Trimethylchlorosilane (1% of solvent) was used as the internal standard and all chemical shifts are given downfield from the signal due to trimethylchlorosilane.

RESULTS

Quantitation of Lignin

Prior to all experiments we measured the density of the CWR from the offspring of the S4 and C1 plants. The density of CWR from the brownish tissue was 1.45 ± 0.09 g cm^{-3} and that of the control sample was 1.23 ± 0.13 g cm^{-3}.

The lignin content of the CWR of the brownish tissue in offspring of the S4 plant and that of the control tissue in offspring of the C1 plant were measured by the Klason and acetyl bromide methods (Table I). Using the Klason method we determined that the amount of lignin in the brownish tissue was 19.9 ± 0.8% (w/w) of the CWR and that in the control tissue it was 22.8 ± 1.1%. By contrast, the amount in the brownish tissue was determined to be 20.3 ± 0.1%, whereas that in the control was 23.4 ± 0.2% by the acetyl bromide method.

Analysis by Alkaline Nitrobenzene Oxidation

Analysis by nitrobenzene oxidation was performed to determine the monomeric composition of lignin in each sample (Table I). In such oxidation analysis, *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde are derived from H-, G-, and S-units, respectively, in the noncondensed fraction of lignin. The three benzaldehydes were detected in both the brownish and the control samples from transgenic tobacco plants. However, the levels of the respective aldehydes were very different from each other. The level of *p*-hydroxybenzaldehyde per unit of lignin from the treated sample of the brownish tissue was about three times higher than that from the control sample (Table I). This result suggests that in the brownish tissue, the level of H-units in the noncondensed lignin fraction was increased by the reduction in the level of expression of the gene for 4CL. By contrast, the levels of the other two aldehydes were dramatically reduced in the brownish tissue. The ratio of the amount of syringaldehyde to that of vanillin (the S/V ratio) in the brownish tissue and in the control tissue was 0.6 and 1.0, respectively. Moreover, the total level of aldehydes detected in the analysis of the brownish tissue was about 40% of that of the control tissue.

Analysis of Alkali-Labile Cinnamic Acids

Hydroxycinnamic acid derivatives, linked via ester and/or ether linkages to cell walls, were released by alkaline hydrolysis and analyzed by GC (Table II). During hydrolysis at 20°C, ester-linked hydroxycinnamic acids should be removed from cell walls, whereas treatment with 4 N NaOH at 170°C should release both ester- and ether-linked hydroxycinnamic acids (Lam et al., 1992). In the control tissue ferulic acid and a trace of *p*-coumaric acid were detected after alkaline treatment at 20°C. However, no sinapic acid was detected in the analysis of the CWR of the control sample. By contrast, these three hydroxycinnamic acids were recovered from the CWR of the brownish tissue after the same treatment. The level of each hydroxycinnamic acid in the brownish tissue was much higher than that in the control tissue. A high level of hydroxycinnamic acids was also detected in the brownish tissue after hydrolysis at 170°C. These data indicate that levels of ester- and ether-linked hydroxycinnamic acids were considerably elevated in the brownish tissue. The respective levels of vanillic and syringic acids detected after hydrolysis at 170°C of the brownish and the control samples were not very different. However, the level of *p*-hydroxybenzoic acid from the brownish sample was slightly higher than that from the control (results not shown).

Characterization of Lignin by Py-GC

Characterization of the chemical structure of lignin in each type of tissue was also performed by Py-GC. Figure 1 and Table III show the chromatograms of the products of

Table I. *Chemical analysis of lignins in cell wall residue prepared from offspring of each transgenic plant*

Origin	Lignin Content		Benzaldehydes				S/V Ratio
	Klason	Ac-Br	H	V	S	Total value	
	% CWR		*μmol g^{-1} Klason lignin*				
C1	22.8 ± 1.1	23.4 ± 0.2	90.9 ± 4.3	934.3 ± 9.0	979.2 ± 2.2	2004	1.05
S4	19.9 ± 0.8	20.3 ± 0.1	288.0 ± 3.4	311.7 ± 9.4	186.9 ± 1.6	785	0.60

Table II. *Levels of cinnamic acid derivatives linked to cell walls in offspring from each transgenic plant*

CA, *p*-Coumaric acid; FA, ferulic acid; SA, sinapic acid.

Origin	1 N NaOH at 20°C			4 N NaOH at 170°C			Residue[a]
	CA	FA	SA	CA	FA	SA	
	nmol g^{-1} CWR						*% (w/w)*
C1	Trace	221 ± 3	ND[b]	Trace	620 ± 30	Trace	67.7 ± 0.8
S4	1276 ± 15	2783 ± 52	500 ± 5	1999 ± 92	5316 ± 160	1115 ± 11	62.6 ± 1.4

[a] Residue after hydrolysis of the sample at 20°C. [b] ND, Not detected.

pyrolysis and the amounts of the products from each tissue, respectively. We identified 3 products that were derived from H-units, 11 from G-units, and 9 from S-units of noncondensed lignins.

We noted some differences between the pyrograms of the brownish and control tissues. First, high levels of 4-vinylphenol and 4-vinylguaiacol were detected among the products of pyrolysis of CWR from the brownish tissue. These compounds could have originated from *p*-coumaric acid and ferulic acid, respectively, bound to lignin via ester and/or ether bonds (Scheijen and Boon, 1991; Kuroda et al., 1995). Other possible sources of these products are derivatives of hydroxycinnamic acid in fatty acids that are bound to cell walls (e.g. cutin or suberin; Tegelaar et al., 1989). Second, the levels of *trans*-isoeugenol and *trans*-4-propenylsyringol in the pyrogram of the brownish tissue

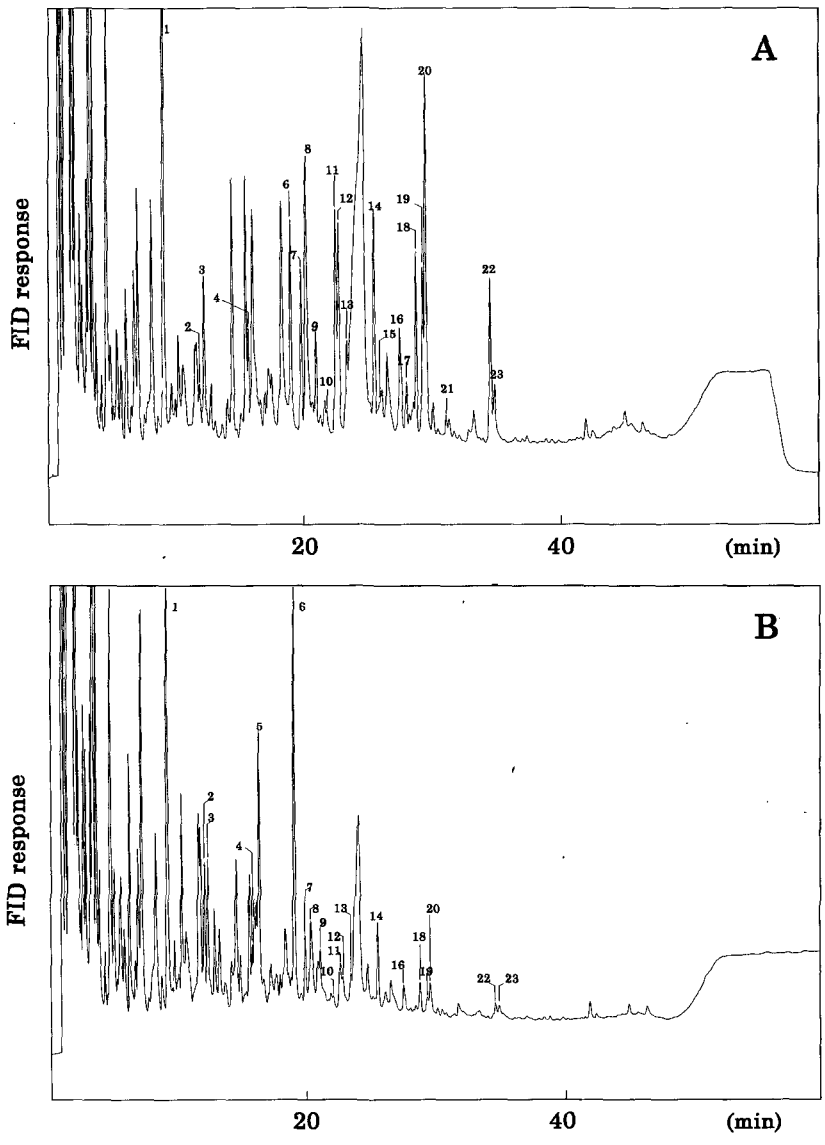

Figure 1. Pyrograms obtained by Py-GC analysis of control (A) and brownish (B) tissues from the offspring of the C1 and S4 plants, respectively. The numbering of peaks corresponds to the numbering of the chemical compounds in Table III. FID, Flame ionization detector.

Table III. *Results of PyGC of CWR from the offspring of the C1 and S4 plants*

Peak No.[a]	Type	Product[b]	Amount		Relative Value[c]
			C1	S4	
			$\mu V\ s^{-1}$		% (w/w)
1	H	Phenol	970,304	620,449	n.d.[d]
2	H	4-Methylphenol	88,017	171,038	n.d.
5	H	4-Vinylphenol	Trace	334,459	n.d.
			$\mu mol\ g^{-1}$ lignin		
3	G	Guaiacol	42	44	105
4	G	4-Methylguaiacol	21	13	62
6	G	4-Vinylguaiacol	80	141	176
8	G	Eugenol	101	49	49
9	G	Vanillin	39	41	105
10	G	*cis*-Isoeugenol	12	6	50
12	G	*trans*-Isoeugenol	62	16	26
13	G	Acetoguaiacone	37	17	46
15	G	Propioguaiacone	14	Trace	n.d.
19	G	Coniferaldehyde	43	6	14
20	G	*trans*-Coniferyl alcohol	125	14	11
7	S	Syringol	45	26	58
11	S	4-Methylsyringol	57	20	35
14	S	4-Vinylsyringol	64	28	44
16	S	Syringaldehyde	51	12	24
17	S	*cis*-4-Propenylsyringol	16	Trace	n.d.
18	S	*trans*-4-Propenyl-syringol	44	9	20
21	S	Propiosyringone	7	Trace	n.d.
22	S	Sinapaldehyde	66	8	12
23	S	*trans*-Sinapyl alcohol	99	24	24

[a] We identified 23 peaks of products that were markers of lignin after pyrolysis of cell wall residues. [b] Products of G- and S-types were quantitated with the response factors reported by Izumi et al. (1995). [c] Amount of product from the brownish tissue relative to that from the control. [d] n.d., Not determined.

were relatively low compared with levels in the control. Some of these products might have been derived from *trans*-coniferyl alcohol and *trans*-sinapyl alcohol residues, respectively, in lignin (Faix et al., 1992). Third, the levels of cinnamyl alcohols and aldehydes detected among the products of pyrolysis of the brownish tissue were also low relative to those of the control. These products might have been partially derived from lignin units with β-O-4-type bonds (Van der Hage et al., 1993). Finally, the sum of the products of pyrolysis of the brownish tissue was lower than that of the control. In particular, the level of S-type products from the brownish tissue was more severely reduced than levels of H- and G-type products.

Isolation of MTL from the Xylem Tissue of Transgenic Tobacco

MTL was isolated as described by Björkman (1956), with slight modifications. In our first attempt at the isolation of MTL, we failed to obtain purified MTL from the brownish tissue by the standard procedure. This failure was due to the existence of large amounts of fatty acid derivatives in the CWR of the brownish tissue (S. Kajita, S. Hishiyama, Y. Tomimura, Y. Katayama, S. Omori, unpublished results). Thus, as a first step in the isolation of MTL we removed fatty acids by extraction with diethylether.

The purified MTL was isolated by our modified version of Björkman's method from the control and the brownish tissues and accounted for 9.64 and 6.03% (w/w) of the Klason lignin in each tissue, respectively. Contamination of the MTL by small amounts of protein (the nitrogen in both samples of MTL was approximately 0.4% [w/w]) was revealed by analysis with a nitrogen analyzer.

^{13}C-NMR Analysis of MTL

The purified MTLs were analyzed by ^{13}C-NMR spectroscopy (Fig. 2). Data in the literature for isolated lignin, obtained with the same solvent (DMSO-d_6) at a similar temperature, were used for assignment of signals (Himmelsbach and Barton, 1980; Scalbert et al., 1986; Robert, 1992; Pan et al., 1994; Tai et al., 1995; Bernard-Vailhe et al., 1996).

The NMR spectrum of the MTL from the control tissue (Fig. 2A) indicated that the lignin in wild-type tobacco was composed of H-, G-, and S-units, as is widely the case in angiosperms. This result was confirmed by signals for C3/C5 in S-units (153 ppm, no. 4), C2/C6 in H-units (129 ppm, no. 9), C6 in G-units (119 ppm, no. 11), C5 in G-units, C3/C5 in H-units (115 ppm, no. 12), C2 in G-units (111 ppm, no. 13), and C2/C6 in S-units (106 and 104 ppm, nos. 14 and 15). In addition to these peaks, signals derived from

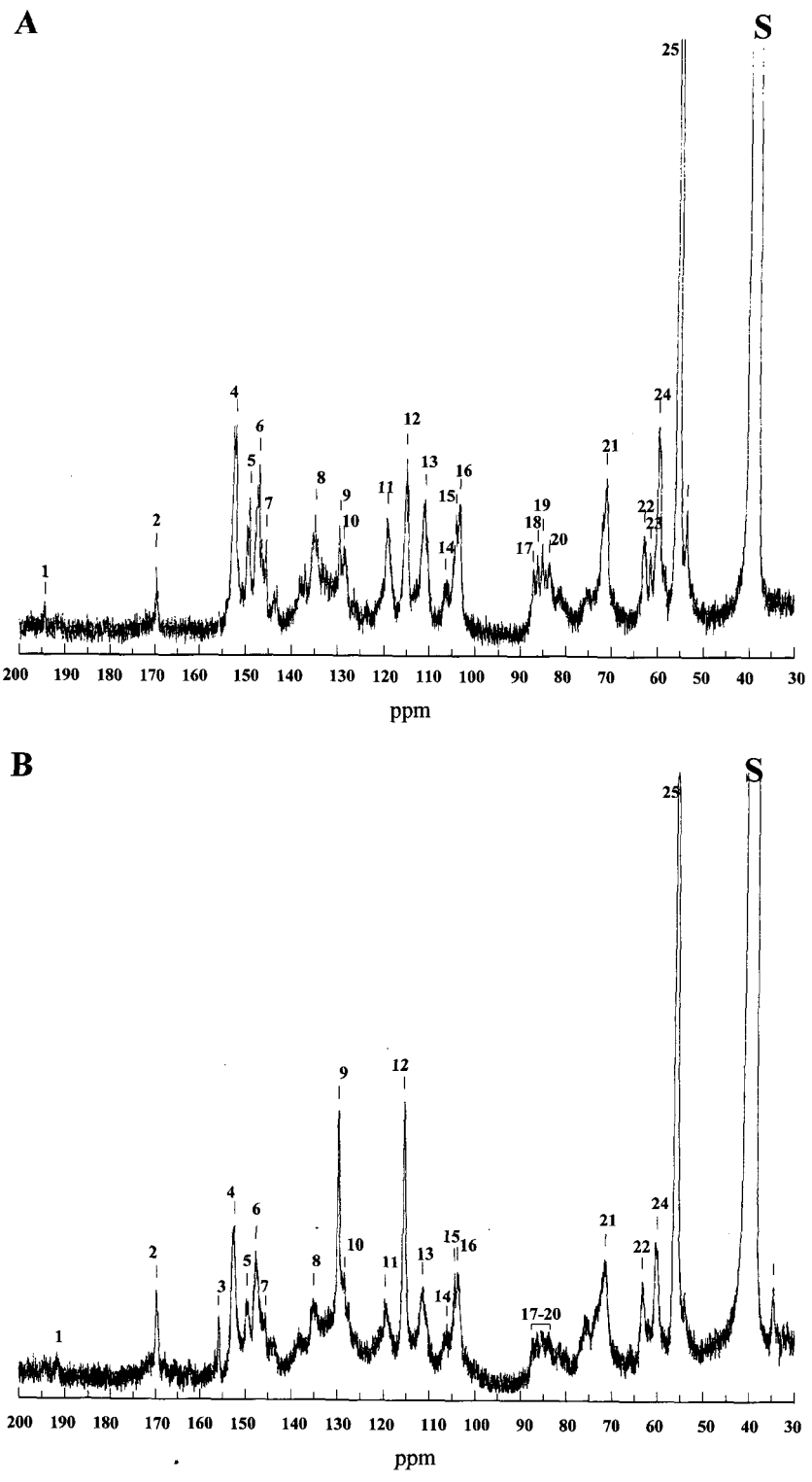

Figure 2. ^{13}C-NMR spectra of MTLs from the control (A) and brownish (B) tissues derived from the offspring of the C1 and S4 plants, respectively. See text for full details. S, Solvent.

interunit linkages for C2 in a β-β linkage (103 ppm, no. 16), Cα in a β-5 linkage (87 ppm, no. 17), Cβ in a β-O-4 linkage (83–86 ppm, nos. 18–20), Cα and Cβ in a β-O-4 linkage (72 ppm, no. 21), Cγ in β-5 and β-O-4 linkages (63 ppm, no. 22), and Cγ in β-O-4 linkage (60–61 ppm, nos. 23 and 24) were observed in the spectrum of the MTL from the control. The strongest peak was located at 56 ppm (no. 25), representing the carbon atoms of the methoxy groups in the G- and S-units. A signal corresponding to cinnamyl aldehydes (no. 1) was also observed in the spectrum of the control MTL.

Relative to the signals in the spectrum of the control MTL, the signal (no. 9) for C2/C6 in H-units and the signal

(no. 12) for C5 in G- and C3/C5 in H-units were apparently stronger in that of MTL from the brownish tissue (Fig. 2B). Moreover, the signal (no. 2) for Cγ in esterified hydroxycinnamic acids was also relatively strong in the spectrum of the brownish tissue. These signals could have been derived from esterified *p*-coumaric and ferulic acids that accumulated in the MTL of the brownish tissue, as indicated by the results of hydrolysis and Py-GC. Furthermore, the intensities of the signals for noncondensed interunit linkages, derived mainly from β-O-4-type bonds (nos. 18–20), were relatively low in the spectrum of the brownish tissue. The signal for cinnamyl aldehyde units (no. 1) was also weak in the spectrum of the MTL from the brownish tissue compared with that from the control.

DISCUSSION

We reported previously that brownish and normal-colored xylem tissues were presented in transgenic plants with the chimeric gene for 4CL (Kajita et al., 1996). The brownish tissue that included a novel lignin was observed specifically in transgenic plants with very low 4CL activity, not in control plants or in plants with a chimeric gene in which the activity of 4CL was normal (Kajita et al., 1996). This result indicates that alterations in lignin biosynthesis in the brownish tissues were strongly dependent on the down-regulation of 4CL in the transgenic plants. In a recent study we showed that down-regulation of 4CL occurred specifically in the brownish tissue and not in the normal-colored tissue in the plants with low 4CL activity by immunohistochemical analysis with antiserum raised against 4CL (S. Kajita, Y. Mashino, N. Nishikubo, Y. Katayama, and S. Omori, unpublished results). Thus, it seems appropriate that the chemical analysis of lignins in transgenic plants with very low 4CL activity should be performed using only the brownish tissue from such plants. To evaluate the relationship between down-regulation of 4CL and alterations in lignin, in this study we used CWR from the brownish tissues of offspring derived from the S4 plant, in which the activity of 4CL was depressed, as well as CWR from control plants.

Our structural analysis revealed significant differences between the lignin in the brownish tissue and that in the control tissue. It appeared that in the brownish tissue of the transgenic plants derivatives of hydroxycinnamic acid, *p*-coumaric, ferulic, and sinapic acids accumulated as a result of the inhibition of 4CL activity, and it seems likely that these derivatives were incorporated into the cell wall fraction. The results of hydrolytic analyses also indicated that the composition of hydroxycinnamic acids linked to cell walls via ester and/or ether linkages was dramatically altered in the brownish tissue. Moreover, the NMR spectra of MTLs also indicated that these hydroxycinnamic acids were linked to a novel lignin fraction in the brownish tissues. It appeared that hydroxycinnamic acids were incorporated into cell walls, where they might play important roles in cross-linking between different polysaccharides and/or between polysaccharides and lignin, as observed in other plant species (Ishii, 1991; Lam et al., 1992; Ishii and Tobita, 1993; Ralph et al., 1995). Thus, the abnormal accumulation of hydroxycinnamic acids might lead to alterations in linkages between components of the cell wall. The observed increase of density of the CWR from the brownish tissue seems to support this hypothesis.

The results of alkaline nitrobenzene oxidation and Py-GC indicated that high levels of condensed units were present in the brownish tissue, since the levels of products after each treatment were relatively low, as was also suggested in a previous report (Kajita et al., 1996). The yields of products in both types of analysis were strongly dependent on the number of condensed units in lignin (Yasuda and Sakakibara, 1975; Kuroda et al., 1994). Furthermore, the low yield of the MTL from the brownish tissue also indicated a high level of condensed units in the lignin of the brownish tissue. The yield of milled lignin from compression wood, which included large amounts of condensed lignin, was reported by Yasuda and Sakakibara (1975) to be lower than that from normal wood. In the NMR spectrum of condensed lignin, the intensity of the signals due to β-O-4 linkages is low, as was also observed in the spectrum of the novel lignin from the brownish tissue. Increased levels of the condensed lignin in the brownish tissue might be due to the H- and/or G-units, which can form condensed linkages at C3 and/or C5 positions in aromatic rings more easily than S-units. Accumulated *p*-coumaric and ferulic acids in the brownish tissue might contribute to the formation of condensed lignin, since free hydroxycinnamic acids and glycosidic esters of hydroxycinnamic acids might be incorporated into lignin macromolecules through copolymerization with lignin monomers (Ralph et al., 1992; Ralph and Helm, 1993; Iiyama et al., 1994).

If hydroxycinnamic acids that accumulated in the brownish tissue were incorporated mainly into lignin via ester bonds, as has been observed in other plants, the hydroxycinnamic acids should be recoverable as benzaldehydes after alkaline nitrobenzene oxidation, and signals due to carbon atoms in hydroxycinnamic acids are very simple to detect in NMR spectra (Shimada et al., 1971; Himmelsbach and Barton, 1980; Scalbert et al., 1986; Fernandez et al., 1990; Ralph et al., 1994). However, the yield of benzaldehydes from the brownish tissue was very low compared with that from the control tissue in the analysis by alkaline nitrobenzene oxidation (Table I). Furthermore, signals for C4 (159 ppm) and Cα (145 ppm) of *p*-coumaric acid could not be assigned in the NMR spectrum of the novel lignin from the brownish tissue (Fig. 2). These data suggest that some of the accumulated hydroxycinnamic acids had been incorporated into the novel lignin with various linkages and that they contributed to the formation of the condensed novel lignin in the brownish tissue.

Recently, Lee and Douglas (1996) reported that in wild-type tobacco plants the rate of conversion of sinapic acid to sinapoyl-CoA was quite low compared with that of *p*-coumaric or ferulic acids to the corresponding CoA esters. They also reported that recombinant proteins produced in *Eschericha coli* from isolated cDNAs for tobacco 4CL failed to recognize sinapic acid as a substrate. More-

over, Kutsuki et al. (1982) suggested that sinapoyl-CoA or sinapaldehyde might not be synthesized from sinapic acid in several angiosperms that have S-type lignins, since the sinapate:CoA ligase activity was not detected in these plants. In a novel biosynthetic pathway for lignin monomers suggested by Higuchi (1985) and Ye et al. (1994), sinapoyl-CoA is also synthesized from 5-hydroxyferuloyl-CoA. Our studies revealed that the level of S-units in the novel lignin of the brownish tissue was quite low relative to that in the control, indicating that suppression of the synthesis of S-type monomers had occurred in the brownish tissue. Our data and observations in previous studies suggest that the activation with CoA of hydroxycinnamic acids is necessary for the effective biosynthesis of S-type monomers.

Our present data were obtained from the offspring of only two independent transgenic plants. Thus, it is possible that changes in lignin structure in the brownish tissue were specific to the present cases and that down-regulation of 4CL might not be related to alterations in lignin in other transgenic lines. However, brownish tissue that included novel lignin was observed specifically in transgenic plants with very low 4CL activity without exception (Kajita et al., 1996). Thus, it seems likely that the change in lignin structure in the brownish tissue was indeed due to down-regulation of 4CL in the tissue. Our present data remain to be confirmed with multiple transgenic lines derived from a number of independent transformants.

ACKNOWLEDGMENTS

Dr. M. Wälchli (Bruker Japan Co., Ltd.), M. Susaki-Kenmotsu, and Dr. K. Endo (Research Center, Mitsubishi Paper Mills Ltd.) are gratefully acknowledged for recording the NMR spectra of the lignins. The authors also thank Prof. K. Kuroda and Dr. A. Izumi (University of Tsukuba, Japan) for their contribution to the analysis by Py-GC.

Received December 20, 1996; accepted March 24, 1997.

LITERATURE CITED

Atanassova R, Favet N, Martz F, Chabbert B, Tollier M-T, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. Plant J **8:** 465–477

Bernard-Vailhé M-A, Cornu A, Robert D, Maillot M-P, Besle J-M (1996a) Cell wall degradability of transgenic tobacco stems in relation to their chemical extraction and lignin quality. J Agric Food Chem **44:** 1164–1169

Bernard-Vailhé M-A, Migné C, Cornu A, Maillot M-P, Grenet E, Besle J-M (1996b) Effect on modification of the *O*-methyltransferase activity on cell wall composition, ultrastructure and digestibility of transgenic tobacco. J Sci Food Agric **72:** 385–391

Björkman A (1956) Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. Sevensk Papperstidn **59:** 477–485

Doorsselaere JV, Baucher M, Chognot E, Chabbert B, Tollier M-T, Peti-Conil M, Leple J-C, Pilate G, Cornu D, Monties B, and others (1995) A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid *O*-methyltransferase activity. Plant J **8:** 855–864

Dwivedi UN, Campbell WH, Yu J, Datla RSS, Bugos RC, Chiang VL, Podila GK (1994) Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. Plant Mol Biol **26:** 61–71

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA **87:** 9057–9061

Faix O, Bremer J, Meier D, Fortmann I, Scheijen MA, Boon JJ (1992) Characterization of tobacco lignin by analytical pyrolysis and Fourier-transformed-infrared spectroscopy. J Anal Appl Pyrolysis **22:** 239–259

Fernandez N, Morck R, Johnsrud SC, Kringstad KP (1990) Carbon-13 NMR study on lignin from Bagasse. Holzforschung **44:** 35–38

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 Biotech Biochem **59:** 929–931

Higuchi T (1985) Biosynthesis of lignin. *In* T Higuchi, eds, Biosynthesis and Biodegradation of Wood Components. Academic Press, New York, pp 141–160

Himmelsbach DS, Barton FE (1980) ^{13}C Nuclear magnetic resonance of grass lignins. J Agric Chem **28:** 1203–1208

Iiyama K, Lam T B-T, Stone BA (1994) Covalent cross-links in the cell wall. Plant Physiol **104:** 315–320

Iiyama K, Wallis AFA (1990) Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. J Sci Food Agric **51:** 145–161

Ishii T (1991) Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-wall. Carbohydr Res **219:** 15–22

Ishii T, Tobita T (1993) Structure characterization of feruloyl oligosaccharides from spinach-leaf cell walls. Carbohyr Res **248:** 179–190

Izumi A, Kuroda K, Ohi H, Yamaguchi A (1995) Structural analysis of lignin by pyrolysis-gas chromatography. III. Comparative studies of pyrolysis-gas chromatography and nitrobenzene oxidation for the determination method of lignin composition in hardwood. Jpn Tappi J **49:** 1339–1346

Kajita S, Katayama Y, Omori S (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate:coenzyme A ligase. Plant Cell Physiol **37:** 957–965

Kuroda K, Suzuki A, Kato M, Imai K (1995) Analysis of rice (*Oryza sativa* L.) lignin by pyrolysis-gas chromatography. J Anal Appl Pyrolysis **34:** 1–12

Kuroda K, Yamaguchi A, Sakai K (1994) Analysis of sugi wood and its lignin preparations by pyrolysis-gas chromatography. Mokuzai Gakkaishi **40:** 987–995

Kutsuki H, Shimada M, Higuchi T (1982) Distribution and roles of *p*-hydroxycinnamate:CoA ligase in lignin biosynthesis. Phytochem **21:** 267–271

Lam TB-T, Iiyama K, Stone BA (1992) Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes. Phytochem **31:** 1179–1183

Lee D, Douglas CJ (1996) Two divergent members of a tobacco 4-coumarate:coenzyme A ligase (4CL) gene family; cDNA structure, gene inheritance and expression, and properties of recombinant proteins. Plant Physiol **112:** 193–205

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol **41:** 455–496

Mcintyre CL, Bettenay HM, Manners JM (1996) Strategies for the suppression of peroxidase gene expression in tobacco. II. *In vivo* suppression of peroxidase activity in transgenic tobacco using ribozyme and antisense constructs. Transgenic Res **5:** 263–270

Ni W, Paiva NL, Dixon RA (1994) Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. Transgenic Res **3:** 120–126

Pan X, Lachenal D, Neirinck V, Robert D (1994) Structure and reactivity of spruce mechanical pulp lignins: ^{13}C-NMR spectral studies of isolated lignins. J Wood Chem Tech **14:** 483–506

Ralph J, Grabber JH, Hatfield RD (1995) Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr Res **275:** 167–178

Ralph J, Hatfield RD, Quideau S, Helm RF, Grabber JH, Jung H-JG (1994) Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. J Am Chem Soc **16:** 9448–9456

Ralph J, Helm RF (1993) Lignin/hydroxycinnamic acids/polysaccharide complexes: Synthetic models for regiochemical characterization. *In* Jung HG, Buxton DR, Hatfield RD, Ralph J, eds, Forage Cell Wall Structure and Digestibility. ASA-CSSA-SSSA, Madison, WI, pp 201–246

Ralph J, Helm RF, Quideau S, Hatfield RD (1992) Lignin-feruloyl ester cross-links in Grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. J Chem Soc Perkin Trans **1:** 2961–2969

Robert D (1992) Carbon-13 nuclear magnetic resonance spectrometry. *In* SY Lin and DW Dence, eds, Methods in Lignin Chemistry. Springer-Verlag, Berlin, pp 250–273

Scalbert A, Monties B, Guittet E, Lallemand JY (1986) Comparison of wheat straw lignin preparations I: chemical and spectroscopic characterizations. Holzforschung **40:** 119–127

Scheijen MA, Boon JJ (1991) Micro-analytical investigations on lignin in enzyme-digested tobacco lamina and midrib using pyrolysis-mass spectrometry and Curie-point pyrolysis-gas-chromatography/mass spectrometry. J Anal Appl Pyrolysis **19:** 153–173

Sederoff R, Campbell M, O'Malley D, Whetten R (1994) Genetic regulation of lignin biosynthesis and the potential modification of wood by genetic engineering in loblolly pine. *In* BE Ellis et al., eds, Recent Adv Phyto Chem: Genetic Engineering of Plant Secondary Metabolism. Plenum Press, New York, pp 313–355

Shimada M, Fukuzuka T, Higuchi T (1971) Ester linkages of *p*-coumaric acid in bamboo and grass lignins. Tappi **54:** 72–78

Tai D, Terazawa M, Chef C-L, Chug H (1995) Biodegradation of birch wood by a white-rot fungus, *Phanerochaete chrysosporium*. *In* M Terazawa, CA McLeod, Y Tamai, eds, Tree Sap. Hokkaido University Press, Sapporo, Japan, pp 131–139

Tegelaar EW, Leeuw JW, Holloway PJ (1989) Some mechanisms of flash pyrolysis of naturally occurring higher plant polyesters. J Anal Appl Pyrolysis **15:** 289–295

Van der Hage ERE, Mulder MM, Boon JJ (1993) Structural characterization of lignin polymers by temperature-resolved in-source pyrolysis-mass spectrometry and Curie-point pyrolysis-gas chromatography/mass spectrometry. J Anal Appl Pyrolysis **25:** 149–183

Whetten R and Sederoff R (1995) Lignin biosynthesis. Plant Cell **7:** 1001–1013

Yasuda S, Sakakibara A (1975) The chemical composition of lignin from compression wood. Mokuzai Gakkaishi **21:** 363–369

Ye Z-H, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell **6:** 1427–1439