Cell Wall Degradability of Transgenic Tobacco Stems in Relation to Their Chemical Extraction and Lignin Quality

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The down regulation of cinnamyl alcohol dehydrogenase (CAD) activity in tobacco stems resulted in improved degradability without changes in cell wall composition. In this study, some changes were evidenced in cell wall solubilization by NaOH, cellulase, and DMSO treatments. Transgenic plants had an altered cell wall structure, the components of which were more accessible to alkali extraction. In plants at the same stage of maturity, cell wall degradability was linked to the yield of acid-precipitated fraction of the NaOH-soluble extract. Aldehydes that should have been incorporated in the cell wall were possibly detected in the different extracts. Guaiacyl and syringyl monomers were distributed differently in the controls and the antisense saponified residues and acid-precipitated fractions. It is suggested that polymeric lignin structure differs. These findings may help in the understanding of the improvement of cell wall digestibility.

Keywords: *Nicotiana tabacum; cinnamyl alcohol dehydrogenase; lignin structure; degradability*

INTRODUCTION

Lignins and bound phenolic compounds have been widely reported to limit forage cell wall digestibility (Jung, 1989). Their role depends on their concentrations and also probably on their structure and organization (Jung and Allen, 1995) Cinnamyl alcohol dehydrogenase (CAD) catalyzes the final reduction step in the synthesis of cinnamyl alcohol precursors for polymerization to lignin. The down regulation of CAD activity in bm1 maize (Kuc and Nelson, 1964; Gee *et al.*, 1968), in bm6 sorghum (Bucholtz *et al.*, 1980; Pillonel *et al.*, 1991), and in chemically inhibited poplar (Grand *et al.*, 1985) influences the quantity and quality of the lignin formed. In bm1 maize and bm6 sorghum not only was there less esterified *p*-coumaric acid and less lignin but also the lignin contained smaller amounts of uncondensed guaiacyl and syringyl units (Kuc and Nelson, 1964; Gee *et al.*, 1968; Barrière *et al.*, 1994; Chabbert *et al.*, 1994). These features suggest a more condensed structure, which was confirmed by ^{1}H NMR study (Gordon and Griffith, 1973). In addition, the early studies on bm1 maize had shown that these mutants had a more alkali extractable lignin (Gordon and Neudoerffer, 1973). Furthermore, Bucholtz *et al.* (1980) proposed that bm6 mutation induces an accumulation of aldehyde groups into the polymer. Pillonel *et al.* (1991) found that CAD reduction in bm6 sorghum was apparently accompanied by incorporation of cinnamaldehydes into lignin.

These structural alterations were associated with an improvement in nutritional value (Cymbaluk *et al.*, 1973; Barrière *et al.*, 1994). Various studies on brown midrib sorghum have shown that in addition to decreased lignin content, alterations in lignin chemical structure, particularly decreased *p*-coumaric acid content, may also contribute to increased extent of degradation (Gerhart *et al.*, 1994). These observations suggested that the content and quality of lignin could be genetically altered (Watson, 1991) by modifying the gene encoding cinnamyl alcohol dehydrogenase.

Antisense RNA strategy has been used to modify CAD activity in tobacco (Halpin *et al.*, 1992). Compared to the controls, the transgenic tobaccos exhibiting a brownish color phenotypically similar to that of brown midrib mutants had a similar lignin content, but the lignin was more susceptible to chemical extractions (Halpin *et al.*, 1994). In addition, syringyl and guaiacyl aldehydes were detected using pyrolysis mass spectrometry but not by thioacidolysis.

In an other study (Bernard-Vailhé, 1995) an improvement in the *in situ* cell wall degradation of the transformed tobacco plants was observed. Paradoxically in those plants, cell wall composition and lignin content remained unchanged. The aim of this study was to evidence structural modifications in cell wall that may explain improvements in degradability. The modified tobacco plants represented a source of information concerning the role of structural features of cell walls in limiting carbohydrate degradability. Lignin reactivity was further investigated through its alkali extractibility, followed by enzyme degradation and DMSO extraction. This cell wall fractionation was undertaken to detect changes in cell wall organization or cell wall polymer structure. Alkali treatment was used since it was reasonable to assume that aldehydes might be present in the absence of the CAD reducing system. We investigated whether differences in degradation could be explained by modifications of lignin properties.

MATERIALS AND METHODS

Plant Material. Three lines (lines 40, 48, and 50) of control (C) and transgenic (AS) tobacco plants (*Nicotiana tabacum* cv. Samson) were prepared by Zeneca Seeds as part of the EU Eclair Oplige program. The CAD was inhibited using the antisense strategy (Halpin *et al.*, 1992). Line 40 antisense plants showed 20%, line 48 only 14%, and line 50 only 7% of control plant CAD activity. Five batches designated 40, 40-8, 40-14, 48, and 50 were studied. The plants were harvested

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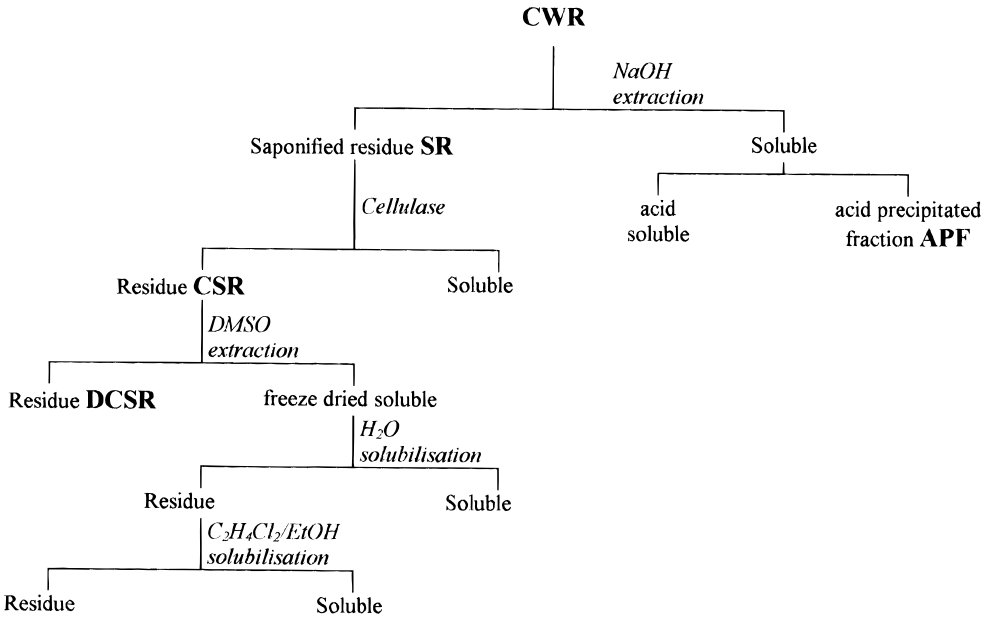

Figure 1. Scheme of the sequential treatment of the cell walls (line 50).

at 16 weeks of age except for batches 40-8 and 40-14, which were composed of 8- and 14-week-old plants. Cell wall residues (CWR) of normal and transgenic tobacco stems were prepared as described by Bernard-Vailhé (1995).

Chemical and Biological Treatments. The scheme of the successive treatments is shown in Figure 1. CWR (1 g) was shaken twice with 1 M NaOH (30 mL) under N_2 at 35 °C for 2 h, in triplicate for line 50, in duplicate for line 40, and once for line 48, because of the amounts of each sample available. Saponified residues (SR) were recovered after centrifugation (1300*g*, 5 min). UV spectra were recorded on alkali filtrates to make sure all UV-absorbing material was removed from the cell walls. After the second hydrolysis, the SR was recovered on a sintered crucible (porosity 2), washed with water, and dried at 60 °C for 24 h. The alkali filtrates and water washings were collected, acidified with 6 M HCl to pH 2, left for 16 h at 4 °C and centrifuged (10000*g*, 20 min). The acid supernatants were collected. The pellets were solubilized in NaOH, dialyzed (membrane cutoff 6000−8000 MW), and freeze-dried to yield a purified acid-precipitated fraction (APF).

The study of the structure was thereafter carried out on line 50, which had the lowest CAD activity and showed the greatest improvement in dry matter degradation (Bernard-Vailhé, 1995). SR was treated with the cellulase R10 Onozuka according to the method of Rexen (1977). The cellulase-treated residues (CSR) were extracted with DMSO (20 mL/g) under N_2 at 40 °C for 30 min and centrifuged (1300*g*, 5 min). This DMSO-cellulase-saponified residue (DCSR) was washed with water on a sintered crucible (porosity 2) and dried at 60 °C for 24 h. The supernatants and filtrates were dialyzed (membrane cutoff 6000−8000 MW) and freeze-dried.

Chemical Analysis. Lignin content (AcOBr) was measured in triplicate according to the acetyl bromide method of Iiyama and Wallis (1988). Uncondensed monomers of lignin were determined according to the alkaline nitrobenzene oxidation method of Venverloo (1971) as modified by Mosoni *et al.* (1994). Twenty milligrams of CWR was hydrolyzed with 5 mL of 2 N NaOH and 0.5 mL nitrobenzene for 3 h at 160 °C. After extraction with dichloromethane, the nitrobenzene oxidation products were dissolved in methanol. They were then analyzed by HPLC (column Lichrosorb C_{18}, 25 cm × 4.6 mm, $\varnothing$ = 5 μm, PhaseSep) and eluted with a H_2O−MeOH−H_3PO_4 gradient using two solutions (940:60:2 and 0:1000:2 mL L^{-1}) at 0.6 mL/min flow rate. The acid supernatants were extracted with ethyl acetate and analyzed by HPLC, and the silylated derivatives were analyzed by GC−MS. Carbohydrate composition was determined according to the method of Blakeney *et al.* (1983).

Lignins for ^{13}C NMR study were prepared according to the method of Björkman (1956) modified as follows: About 10 g of CWR was ultraground in a vibratory mill using porcelain balls for 10 days at 4 °C. The fine particles were extracted twice with dioxane−water (9/1 v/v) for 24 h and centrifuged. The pooled supernatants were concentrated under vacuum and precipitated in a solution of 5% (w/v) Na_2SO_4 to eliminate water-soluble compounds. After 16 h at 4 °C, the pellets were recovered after centrifugation. They were washed with water, dissolved in acetic acid, and precipitated in distilled diethyl ether. Liquid state ^{13}C NMR spectra (^{13}C = 62.7 Mhz) were obtained at 323 K from a WM250 Brücker spectrometer with samples in DMSO-d_6 solution. For quantitative analysis, the inverse gated decoupled spectra were recorded with 90° flip angle and 10 s pulse delay with an average of 10 000 transients. The integral of the six aromatic carbons served as internal reference for quantitative estimation (precision ±10%).

UV spectra were recorded for solutions against the corresponding solvent on a Shimadzu 600 spectrophotometer at wavelengths between 200 and 600 nm. IR spectra were recorded for KBr disks on a FTIR Nicolet 55XS spectrometer.

The APFs were stained with acid phloroglucinol to evidence cinnamaldehydes.

RESULTS

Yield of Extractions. Table 1 shows the yields in SR and APF of each batch after alkaline hydrolysis. The difference observed between control and antisense saponified residue contents is significant only in batches 40-14 and 50. After dialysis, the yields of APF were significantly ($P < 0.05$) greater for antisense stems than for C, except for batch 40. The greatest increase in APF was observed for line 50, which also presented the best improvement in degradability (Bernard-Vailhé, 1995).

Tobacco line 50, which had the lowest CAD activity, the greatest improvement in degradability, and the greatest yield in APF, was subjected to a sequential extraction (Table 2). After cellulase treatment, the recovery of the residue was lower for the control (30.5%) than for the antisense (36.8%). Thus, the SR of the control was more degradable than the corresponding fraction of the antisense. The DMSO-insoluble residues (DCSR) were recovered at the same yield from control and antisense (about 87%). However, the recovery after freeze-drying of the dialyzed DMSO extract was slightly

Table 1. Yields of Saponified Residues (SR) and Acid-Precipitated Fractions (APF) of Control (C) and Antisense (AS) Tobacco Lines

	tobacco line 40						tobacco line 48		tobacco line 50	
sample	C40	AS40	C40-8	AS40-8	C40-14	AS40-14	C48	AS48	C50	AS50
SR	62.6 ± 0.7	63.4 ± 0.0	70.5 ± 1.0	72.2 ± 1.0	70.6 ± 0.2[a]	67.4 ± 0.2[b]	70.6	69.2	67.5 ± 2.5[a]	62.3 ± 1.2[b]
APF	23.0 ± 0.4	24.1 ± 0.8	11.5 ± 1.6[a]	13.6 ± 0.3[b]	12.4 ± 0.0[a]	18.2 ± 0.1[b]	17.5	20.2	18.9 ± 1.4[a]	26.5 ± 0.4[b]

[a] Percent of cell wall content. Data not sharing the same superscript within a couple of C and AS samples are significantly different ($P < 0.05$).

Table 2. Composition[a] of the Extracted Fractions for Control (C) and Antisense (AS) Line 50

			nitrobenzene oxidation products[c]				carbohydrate content[b]		
	yield[a]	lignin content[b]	total	guaiacyl	syringyl	S/G	total	xylose	glucose
CWR									
C	72.3	23.5 ± 1.3	22.5 ± 0.0	11.6 ± 0.3	10.4 ± 0.3	0.73	63.0 ± 2.3	15.7 ± 0.1	42.8 ± 1.4
AS	72.6	22.4 ± 1.0	23.4 ± 0.1	12.9 ± 0.8	10.3 ± 0.5	0.67	66.4 ± 1.4	18.0 ± 0.3	43.4 ± 0.6
SR									
C	67.9	22.2 ± 0.2	29.0 ± 0.1	18.8 ± 3.6	9.9 ± 2.7	0.44	67.4 ± 3.5	7.5 ± 0.5	57.0 ± 0.9
AS	62.3	15.9 ± 0.5	34.8 ± 1.3	21.3 ± 3.6	13.3 ± 1.0	0.52	68.8 ± 3.0	7.8 ± 0.2	56.4 ± 0.2
CSR									
C	30.5	29.8	5.5	2.1	0.80	0.32			
AS	36.8	29.7	4.5	1.9	0.71	0.31			
DCSR									
C	87.1	30.5	4.9	0.72	2.1	2.47			
AS	87.0	38.7	3.2	0.70	1.9	2.23			
APF									
C	18.9	7.8 ± 0.8	36.5 ± 1.5	17.6 ± 0.8	18.3 ± 2.4	0.87	67.2 ± 1.1	48.4 ± 1.9	17.4 ± 0.3
AS	26.5	13.2 ± 0.5	22.7 ± 0.3	12.8 ± 1.2	9.6 ± 1.1	0.63	48.3 ± 1.8	36.6 ± 1.1	9.9 ± 0.2

[a] % previous fraction except for the CWR (% of the dry matter) and for the APF (% of the CWR). [b] % of the residue. [c] % of the lignin content.

greater for antisense (9.1% of CSR) than for control (7.4% of CSR).

Chemical Composition of the Residues of Line 50. Compositions of the cell walls of control and antisense tobacco were similar.

The carbohydrate contents of the control and antisense SR were similar and represented about 68% of the residue. This fraction essentially contained a high proportion of glucose together with xylose.

The main difference between control and antisense SR was in the lignin content and composition. Lignin content of the antisense SR was lower (16%) than that of the control (22%). In addition, the composition of the lignin was slightly different. Nitrobenzene oxidation yield was about one-third of lignin content in both SR, but in the control it represented a greater proportion of the initial content (83%) than in the antisense (66%). The guaiacyl units remaining in the SR of the control represented most of the initial content. In antisense, guaiacyl and syringyl units were recovered at the same extent in the APF. Whichever the substrate (C or AS), syringyl units were more extensively extracted by NaOH than guaiacyl units.

After enzymatic treatment, the lignin contents in the residues were similar in control and antisense plants. Lignin monomeric composition remained unchanged after DMSO extraction. The yield of nitrobenzene oxidation products in CSR and DCSR dramatically decreased to about 5% lignin.

Chemical Composition of the Solubilized Fractions of Line 50. *Acid-Precipitated Fraction.* The carbohydrate fraction of the APF essentially contained a high proportion of xylose together with glucose and only traces of arabinose. Alkali treatment released a similar yield of carbohydrates from both control and AS (22 and 17%, respectively).

As expected from the study of the SR, lignin contents of control and antisense APF were different. Compared to the control APF (7.8%), the lignin content of the antisense APF was nearly doubled (13.2% APF).

In the control APF, nitrobenzene oxidation products accounted for more than one-third of lignin content (37%), but only 23% in the AS APF. Nevertheless, nitrobenzene oxidation products in the APF of the antisense represented a greater proportion (15%) of the initial content than those of the control (9%). This difference was clearly evidenced for the guaiacyl unit content: 9% of initial content for control and 15% for antisense. Variations in the S/G ratio suggested that guaiacyl and syringyl units were divided homogeneously between the SR and the APF of the antisense. On the FTIR spectra, the bands characteristic of guaiacyl [1250, 1464, 1455 cm^{-1} (shoulder)] and syringyl (1214 and 1330 cm^{-1}) units were more pronounced in the APF of the antisense than in the control. APF of the antisense reacted positively with acid phloroglucinol, whereas the APF of the control remained almost colorless.

Acid Supernatants. The total recovery of lignin and uncondensed monomers in SR plus APF of the antisense plant was slightly lower than in control plants. Some phenolic compounds initially detected as lignins by the acetyl bromide method remained in the acid supernatants. Their presence in the acid supernatant was suggested by the higher absorbance at 280 nm for the antisense than for the control. When compared to a ferulic acid standard, it represented only 1.4% of the initial lignin content for the control and 9.7% for the antisense. In addition, the UV spectrum of the antisense showed a strong absorption at 340 nm, which could be related to the absorbance of the conjugated carbonyl group of aromatic aldehydes. Vanillin and syringaldehyde were isolated by HPLC from the ethyl acetate extracts of the acid supernatants and further identified by HPLC retention times, UV spectra, and GC−MS of the silylated derivatives. Vanillin and syringaldehyde contents in the acid supernatant of the antisense (0.330 and 0.312 g kg^{-1}, respectively) were 5 and 10 times greater than in the corresponding fraction of the control.

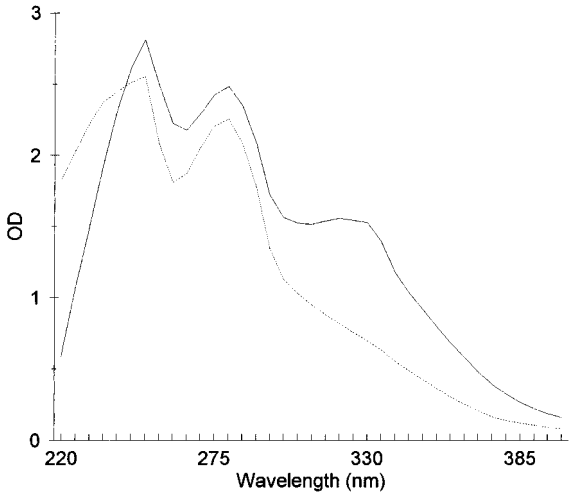

Figure 2. UV spectra of the DMSO-extracted fractions of line 50: (dashed line) control; (solid line) antisense.

DMSO Extraction. The UV spectrum of control DMSO extracts exhibited two maxima near 250 and 280 nm. In addition, the corresponding antisense fraction exhibited a plateau between 300 and 340 nm (Figure 2). Control DMSO freeze-dried extract was almost totally solubilized with water. The FTIR spectrum was that of a xylan plus bands characteristic of guaiacyl and syringyl units (1120, 1220, and 1500−1600 cm^{-1}). In contrast, the DMSO freeze-dried extract of the antisense was only very slightly solubilized by water but partly solubilized by dichloroethane/ethanol. The UV spectrum of this latter soluble extract showed a peak near 340 nm.

NMR Spectra. During the preparation of the Björkman lignins, the brownish color of the antisense CWR disappeared. The NMR spectra of Björkman lignins were similar for control and antisense plants and were typical of dicot lignins (Figure 3). They both indicate the presence of guaiacyl (mainly signals 10, 11, 18, 28, 29, 31, and 40) and syringyl (signals 9, 33, 34, 37, and 38) units. Some slight differences were observed between antisense and control, such as a lower S/G ratio for AS (0.69) than for C (0.73). The condensed aromatic quaternary carbons per unit were in larger proportion compared to tertiary aromatic carbons in AS (0.66) than in control (0.42). This probably resulted from large proportions of 5−5′ and β-5 linkages in tobacco. The signals corresponding to cinnamaldehydes (2), benzaldehyde (3), and aliphatic carbons (4) were slightly but not significantly higher in the AS Björkman lignins than in control lignins. Two signals $\times 1$ at 196 ppm corresponding to an aldehyde group and $\times 2$ at 108.4 ppm, observed in AS lignins, could not be clearly identified.

DISCUSSION

Neither the nonmodified lignin content nor the slight decrease in the S/G ratio explained the improvement in *in situ* dry matter disappearances observed in the transgenic tobacco plants (Bernard-Vailhé, 1995). As the cell wall composition remained unchanged by CAD inhibition, it may be that changes in cell wall organization or cell wall polymer structure had occurred and were responsible for the increased cell wall degradability.

Compared to the control, the cell walls of the antisense were more susceptible to NaOH extraction, with batch 40 presenting the lowest increase. Moreover, large differences in yields of APF were observed between control and antisense of 40-8 and 40-14, especially for the latter. Halpin *et al.* (1994), however, found that lignins even in antisense plants of batch 40 were more extensively extracted by alkali and also by thioglycolic acid than in controls.

Cell wall *in situ* disappearances (48 h) of lines 40, 40-14, 48, and 50 were 20.1, 13.8, 19.8, and 12.1%, respectively, for control and 19.0, 19.7, 23.2, and 19.0%, respectively, for antisense stems (Bernard-Vailhé, 1995). Improvements in cell wall degradation followed the same trend as increases in the yield of APF, suggesting that structural features responsible for the greater alkali solubility are a cause of the improvements in degradability. Observations of the three tobacco lines suggest that the lignin modification begins to appear when CAD activity is reduced by about 80% and support the previous work of Halpin *et al.* (1994). The correlation observed between the APF yield and cell wall degradability suggests that the study of the alkali-soluble wall polymers could help to detect structural factors that play a role in limiting cell wall degradability.

The quantity and quality of the lignin solubilized by alkali showed considerable variation between control and antisense plants. In agreement with the observations on normal and bm1 maize (Kuc and Nelson, 1964), the APF of the antisense plant was more colored (reddish) than the corresponding fraction of the control. This color probably originated from the lignified tissues of the AS stem. Kuc and Nelson (1964) found that in bm1 maize the color could not be separated from the lignin by any common solvent. Recents work on DHP synthesis (Higuchi *et al.*, 1994; Tollier *et al.*, 1994) suggested that the reddish color of transgenic plants with the antisense CAD gene could result from a possible accumulation of cinnamaldehydes in lignins. Previous observations on bm6 sorghum (Bucholtz *et al.*, 1980; Pillonel *et al.*, 1991) had shown that the depression of CAD activity was accompanied by the incorporation of cinnamaldehydes into lignins. The presence of cinnamaldehyde compounds was clearly detected in the APF of the AS by acid phloroglucinol staining. Treatment with acid phloroglucinol is classically used as a test for cinnamaldehyde residues in lignin (Wardrop, 1971) but cinnamyl alcohols and related benzaldehydes may also give a positive reaction (Clifford *et al.*, 1974). FTIR study of the APF fractions and NMR study of Björkman lignins failed to clearly evidence the accumulation of aldehydes in the transgenic plants. No cinnamaldehyde was identified among thioacidolysis products of CAD transgenic poplar (Tollier *et al.*, 1994), and only traces of cinnamaldehydes were identified among thioacidolysis products of both control and antisense tobacco cell walls (Halpin *et al.*, 1992). One hypothesis is that aldehydes are incorporated into lignin structures not cleaved by thioacidolysis (Lapierre *et al.*, 1986). Substantial amounts of C_6-C_1 aldehydes (vanillin and syringaldehyde) were recovered as free aldehydes in the acid supernatant fraction of the antisense. In the absence of CAD activity, metabolism of cinnamaldehydes may have been shifted toward biogenesis of C_6-C_1 compounds. C_6-C_1 compounds could result from the oxidative cleavage of the α,β-conjugated double bond of the corresponding cinnamaldehydes, according to the model of Puech (1984). The role of CAD gene inhibition in the production of C_6-C_1 monomers requires further investigation.

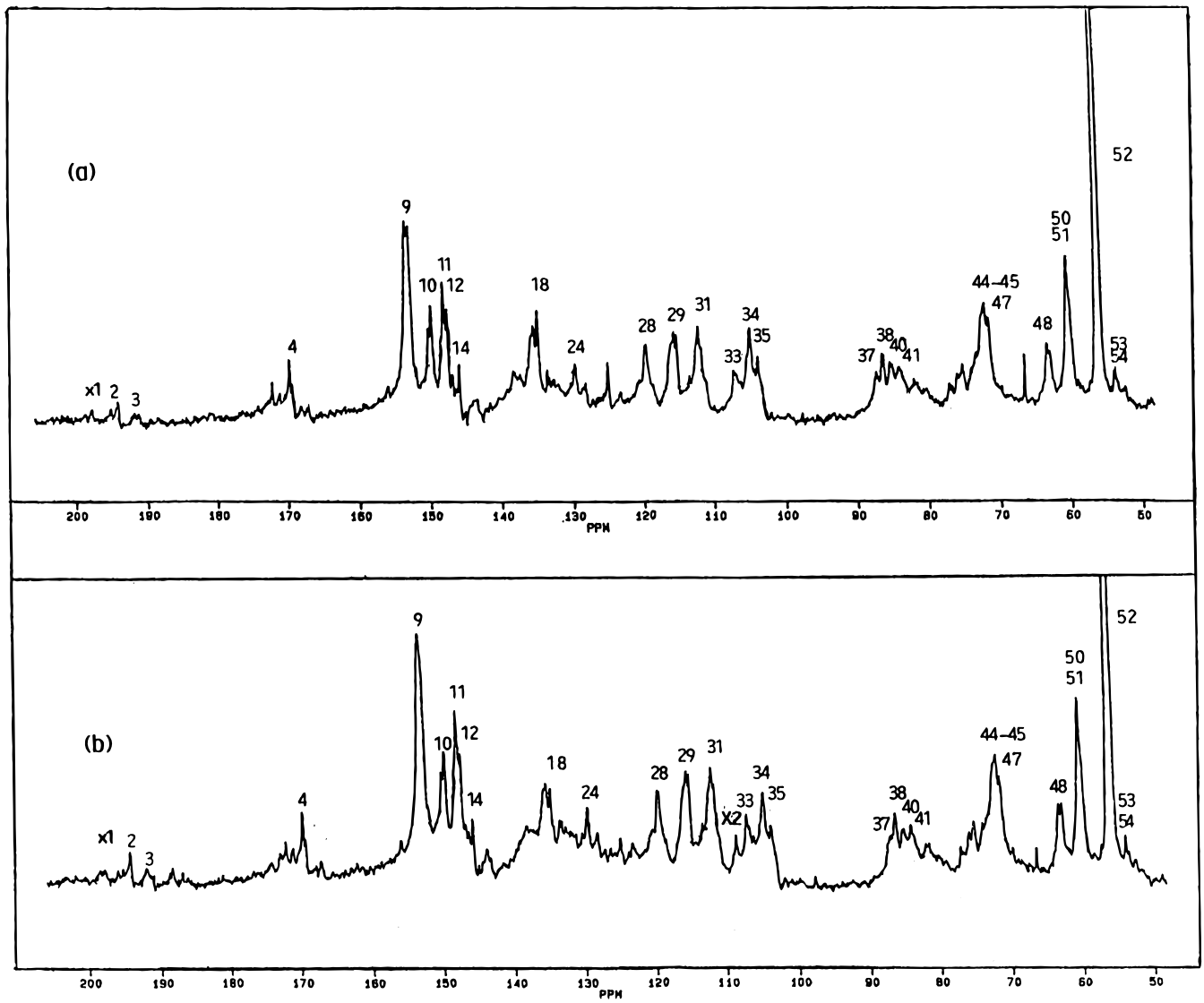

Figure 3. ^{13}C NMR spectra of control (a) and antisense (b) Björkman lignins of line 50.

The nitrobenzene oxidation products showed that syringyl units were more sensitive to NaOH extraction in the control plants than in the antisense plants. On the basis of this result, alkali lignin of control and antisense tobacco stems appeared to be different with regard to the uncondensed structures, while the initial content of nitrobenzene oxidation products appeared to be similar. The solubility of triticale straw lignins in alkali has been related to their relatively high amount of free phenolic groups in the polymer (Lapierre *et al.*, 1989). In our case if the genetic mutation had affected the frequency of these groups, it would be in too slight a proportion to be detected.

It may be that small increases in aldehyde incorporation, while hard to detect, could alter lignin structure sufficiently to account for the improvement in alkali solubilization. PYMS spectra of the same CWR of control and AS tobacco line (Halpin *et al.*, 1994) showed that inclusion of aldehyde monomers in place of alcohols in lignin influences the possibility for condensation into resinols and other dimeric structures, producing a polymer with altered intermolecular bonding. Increased incorporation of aldehydes may not be the only modification to lignin structure in CAD antisense plant. It is probable that other changes have occurred, such as changes in the molecular weight of lignins or the number of linkages between lignins and polysaccharides.

Unpurified alkali lignin of normal and bm1 maize lignin contained 23 and 30% of a hemicellulose, which on hydrolysis yielded xylose as the only detectable sugar (Kuc and Nelson, 1964). Both APF fractions analyzed in this study contained xylose and glucose, probably resulting from the hydrolysis of xyloglucans, but no difference in polysaccharide content was observed for control and antisense.

Modifications of cell wall degradability after CAD inhibition affect some part of the cell walls, which was removed after the alkaline and enzymatic treatments. Some differences were still present since a part of the coloration remained in the residues. This coloration was solubilized by DMSO, and the extracts of control and antisense were different near 340 nm. Morrison (1973) showed that DMSO solubilized a mixture of LCC from cell walls of raygrass. The presence of xylan together with aromatic signals in FTIR spectra of the DMSO-extracted fractions suggested a lignin−carbohydrate complex. Unlike the control, the DMSO extract of the antisense was more soluble in solvent than in water. This suggested a higher lignin to carbohydrate ratio.

The genetic mutation thus had an effect on cell wall structure by producing small amounts of aldehydes and certainly other cell wall changes. Features of lignins other than simply their quantity are involved in the improvement in degradability. However, the variations

occurring in lignin structures in relation to cell wall degradability deserve further study.

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