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Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamaldehydes into the lignin of transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced expression of cinnamyl alcohol dehydrogenase

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Abstract. Xylem from stems of genetically manipulated tobacco plants which had had cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) activity down-regulated to a greater or lesser degree (clones 37 and 49, respectively) by the insertion of antisense CAD cDNA had similar, or slightly higher, lignin contents than xylem from wild-type plants. Fourier-transform infrared (FT-IR) microspectroscopy indicated that down-regulation of CAD had resulted in the incorporation of moieties with conjugated carbonyl groups into lignin and that the overall extent of cross-linking, particularly of guaiacyl (4-hydroxy-3-methoxyphenyl) rings, in the lignin had altered. The FT-Raman spectra of manipulated xylem exhibited maxima consistent with the presence of elevated levels of aldehydic groups conjugated to a carbon-carbon double bond and a guaiacyl ring. These maxima were particularly intense in the spectra of xylem from clone 37, the xylem of which exhibits a uniform red coloration, and their absolute frequencies matched those of coniferaldehyde. Furthermore, xylem from clone 37 was found to have a higher content of carbonyl groups than that of clone 49 or the wild-type (clone 37: clone 49: wild-type; 2.4:1.6:1.0) as measured by a degradative chemical method. This is the first report of the combined use of FT-IR and FT-Raman spectroscopies to study lignin structure in situ. These analyses provide strong evidence for the incorporation of cinnamaldehyde groups into the lignin of transgenic plants with down-regulated CAD expression. In addition, these non-destructive analyses also suggest that the plants transformed with antisense CAD, in particular clone 37, may contain lignin that is less condensed (cross-linked) than that of the wild-type.

Key words: Lignin-Cell walls – Cinnamyl alcohol dehydrogenase – Infrared – Raman

Introduction

Lignin is a poly-phenylpropanoid polymer found in all higher plants. It is the second most abundant natural polymer after cellulose and has been estimated to comprise 15–20% of global biomass (Cui and Dolphin 1991). Lignin has a negative impact on the use of plant material in a number of non-food industries and generally must be removed. For example, the presence of residual lignin results in pulp and paper with inferior performance characteristics (Thomas 1970) and poor brightness stability (yellowing with age; Gellerstedt and Pettersson 1975). Lignin also reduces the digestibility of forages by ruminants (Jung and Deetz 1993).

As lignin removal is expensive in terms of energy and reagents and can produce toxic by-products, novel and environmentally benign methods are constantly being sought to remove lignin (McDougall et al. 1993) or reduce the amount of lignin present in plants.

As traditional plant-breeding methods are unlikely to produce economically viable cultivars with reduced, or altered, lignin contents in the short-to-medium term, approaches using genetic manipulation of the enzymes in the lignin biosynthetic pathway (Fig. 1) have been favoured (Whetten and Sederoff 1991; Dean and Eriksson 1992; Sederoff et al. 1994; Boudet et al. 1995; Campbell and Sederoff 1996).

Phenylalanine ammonia lyase (Elkind et al. 1990), O-methyl transferase (Dwivedi et al. 1993: Ni et al. 1994; Atanassova et al. 1995; van Doorsselaere et al. 1995), cinnamoyl CoA reductase (Goffner et al. 1994) and cinnamyl alcohol dehydrogenase (CAD; Halpin et al. 1994) have been targeted for antisense-based gene silencing. In this paper, we examined the effects of down-regulation of CAD expression on the structure of lignin in

Abbreviations: CAD = cinnamyl alcohol dehydrogenase; FT-IR = Fourier-transform infrared; DRIFT = diffuse reflectance Fourier-transform infrared

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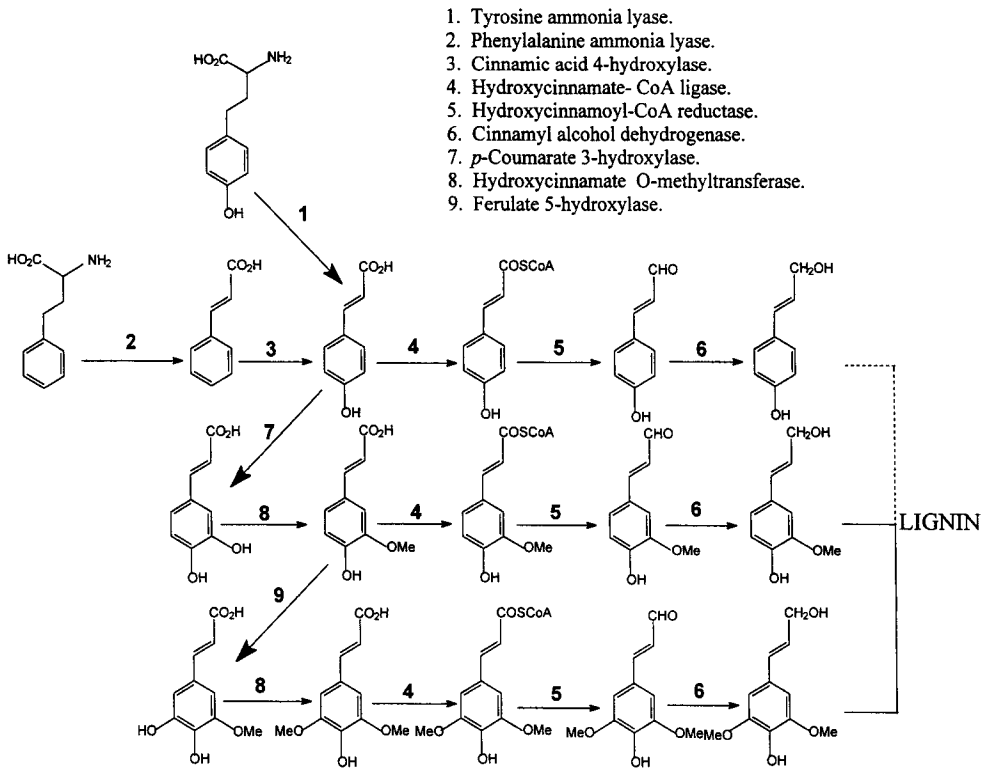

Fig. 1. A simplified schematic of the lignin biosynthetic pathway. *Me*, methyl

transgenic tobacco plants as observed by Fourier-transform infrared (FT-IR) and Raman spectroscopies.

Materials and methods

Sample preparation. Transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun) plants were prepared using Zeneca Seeds (Jealott's Hill, Bracknell, UK) construct (pTCAD19) in an antisense orientation. Two transformed cell lines with down-regulated CAD activity were selected: clone 49 (single copy of the transgene) and clone 37 (multiple copies of the transgene). Assays for CAD activity, using the method of Halpin et al. 1992, showed that Clone 49 had a residual CAD activity of 25% of that of the control and showed no phenotypical differences compared with the wild-type plants while clone 37, which had a residual CAD activity of 9% of that of the control, displayed a strong red colouration of the isolated xylem. Plants of the F1 generation were harvested just before flowering and no differences in growth or development were noted between transgenic and wild-type plants. The stems were divided into three equal parts corresponding to apical, intermediate (red colouration zone for clone 37) and basal parts. Portions of the xylem of each section were separated from the cortex and pith, cut into strips, sequentially extracted with 20, 40, 60 and 80% aqueous ethanol then 100% ethanol (previously dried over molecular sieves) to dehydrate the xylem and extract free (non-covalently bound) phenolic compounds which may interfere with subsequent spectral interpretations. The xylem strips were then freeze-dried. Xylem was also dissected out of the whole stems of individual wild-type and transgenic plants, freeze-dried then hammer-milled to pass a 0.5-mm sieve. The milled residues were extracted with toluene:ethanol (9:1, v/v) and air-dried.

Fourier-transform infrared and Raman spectroscopy. The FT-IR spectra were acquired using a Bruker IFS 66 FT-IR spectrometer (Bruker Spectrospin, Coventry, UK) with microspectrometer attachment. The inner and outer sides of the xylem strips were analysed using the FT-IR microspectrometer in the reflectance mode. Routinely, nine areas of xylem were analysed, each spectrum comprising 4000 scans, at a resolution of 4 cm^{-1}. After acquisition the reflectance spectra were averaged, converted to Kubelka-Munk spectra using the Bruker Opus 2 software, and vector-normalized to allow the direct comparison of spectra from different tissues. Diffuse Reflectance FT-IR (DRIFT) spectra of milled whole-stem xylem were obtained on the same FT-IR spectrometer using a DRIFT cell (Graseby-Specac, Orpington, UK). The FT-Raman spectra were acquired using a Perkin-Elmer 1700 series Raman/IR spectrometer (Perkin-Elmer, Beaconsfield, UK) incorporating a Spectron model 301 cw Nd:YAG laser operating at 1064 nm, and an InGsAs detector. Xylem strips were placed across the normal sample holder and held in place by a fastening nut. Milled xylem was analysed using the residue tightly packed into a sample cup. Spectra were recorded using 180° backscattering at 250 mW, 4 cm^{-1} resolution and 300 scans. Spectra were acquired from six areas of both the inner and outer sides of the xylem strips, then averaged. All FT-Raman spectra were converted into JCAMP-DX then Opus 2 formats for manipulation and comparison with the FT-IR spectra. All spectra were baseline-corrected using the scattering correction method employing 100 baseline points.

Estimation of lignin and carbonyl groups. Lignin contents were estimated by the acetyl bromide method of Morrison (1972) and carbonyl contents by the method of Šťrancar and Perdith (1992).

Results and discussion

Although the FT-IR spectra of xylem strips of wild-type tobacco are essentially similar, slight differences can be noted depending on the source of the xylem within the stem (apical, intermediate and basal) and whether the inner (more developmentally mature) or outer (younger) surfaces were examined (Fig. 2). For example, the relative absorbances in the 1050–1200 cm^{-1} region, the principal absorbance region for polysaccharide absorbance (Stewart and Morrison 1992; Stewart et al. 1995), are different between the outer and inner surfaces. The

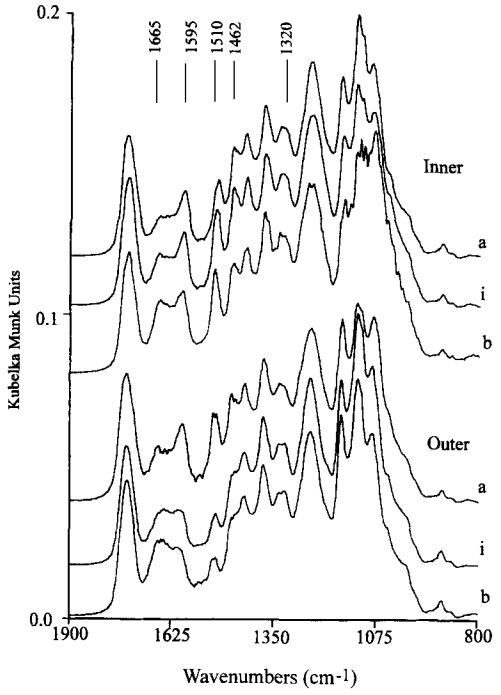

Fig. 2. Infrared spectra of the inner and outer surfaces of xylem strips dissected from wild-type tobacco plants. (*a*, apical; *i*, intermediate; *b*, basal). The absorbances at 1665 cm^{-1} is associated with conjugated carbonyl groups and the peaks at 1595 and 1510 cm^{-1} are lignin-related absorbances. The absorbances at 1462 and 1320 are related to methyl groups and condensed guaiacyl rings' respectively

inner xylem surfaces also have more prominent absorbances at 1510 and 1595 cm^{-1}, the two absorbances characteristic of lignin or lignin-like material (Hergert 1969, 1970; Stewart and Morrison 1992; Stewart et al. 1995) and the ratio of the absorbances 1510:1595 cm^{-1} is greater in the inner, more developmentally mature xylem than the outer, younger tissues.

The absorbance at 1510 cm^{-1} has been reported to be more intense in condensed, highly cross-linked lignin structures (Sarkanen et al. 1967; Hergert 1969, 1970; Akin et al. 1993) and the high 1510:1595 cm^{-1} ratio suggests that the inner xylem surfaces contain more cross-linked lignin than the younger, outer xylem. That the ratio is less than 1 for the outer surfaces in the spectra of the xylem from the intermediate and basal parts of the stem may reflect the small developmental difference between these zones (Table 1). The inner and outer surfaces of these zones are distinct whereas the corresponding tissue from the apical region is less well defined and lignin in these outer and inner surfaces may be closer in developmental origin than in the basal and intermediate sections.

In the majority of cases, the FT-IR spectra of the xylem strips from clone 49 (Fig. 3) and clone 37 (Fig. 4) have greater absorbances at 1595 cm^{-1} than 1510 cm^{-1} (Table 1). This relatively lower 1510:1595 cm^{-1} ratio suggests that the lignin in these transformed plants may not be as extensively cross-linked as in the wild-type tissues. The exceptions are the spectra of the inner surfaces of the basal parts of the stems from clones 49 and 37 which have a greater absorbance at 1510 cm^{-1} than 1595 cm^{-1}. Since these are the most mature tissues, this could be the result of peroxidase-catalysed condensation reactions occurring during maturation (Higuchi 1985).

Absorbances centred at 1320 cm^{-1} have been assigned to condensed guaiacyl rings (Faix 1991). The reduction in the intensity of this absorbance, particularly in the spectra of clone 37 (Fig. 4), indicates that the degree of lignin cross-linking in these transformed plants is reduced.

The FT-IR spectra of the transformed tissues also exhibit notably greater absorbances centred at 1665 cm^{-1} than those of the wild-type (Figs. 2–4). Absorbances in

Table 1. Absorbance and peak intensities for the infrared and Raman spectra of the xylem strips and the corresponding milled xylem

	Infrared intensity						Relative Raman intensity			
Surface	Inner			Outer			Inner		Outer	
Wavenumber (cm^{-1})	1510	1595	1510/1595	1510	1595	1510/1595	1595	1630	1595	1630
Xylem strips										
Wild type										
Apical	0.026	0.022	1.182	0.028	0.025	1.120	0.0081	0.0047	0.0076	0.0041
Intermediate	0.032	0.024	1.333	0.017	0.018	0.944	0.0129	0.0047	0.0070	0.0038
Basal	0.033	0.026	1.269	0.018	0.020	0.900	0.0064	0.0035	0.0068	0.0035
Clone 49										
Apical	0.022	0.028	0.786	0.025	0.028	0.893	0.0117	0.0070	0.0105	0.0072
Intermediate	0.018	0.021	0.850	0.033	0.030	1.100	0.0105	0.0064	0.0164	0.0117
Basal	0.034	0.032	1.062	0.015	0.017	0.883	0.0129	0.0063	0.0076	0.0094
Clone 37										
Apical	0.024	0.030	0.800	0.026	0.028	0.928	0.0269	0.0216	0.0274	0.0210
Intermediate	0.028	0.030	0.934	0.023	0.024	0.958	0.0346	0.0263	0.0260	0.0189
Basal	0.033	0.026	1.269	0.025	0.027	0.926	0.0333	0.0257	0.0257	0.0187
Milled xylem										
Wild type	0.052	0.039	1.333				0.0690	0.0300		
Clone 49	0.044	0.034	1.294				0.0700	0.0370		
Clone 37	0.043	0.046	0.934				0.1210	0.0960		

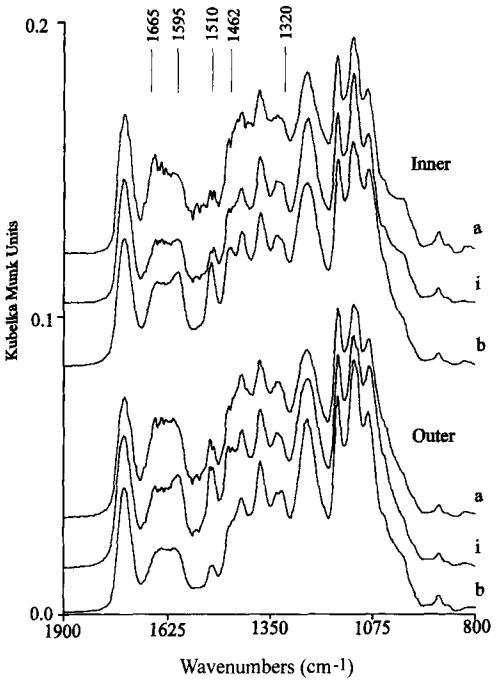

Fig. 3. Infrared spectra of the inner and outer surfaces of xylem strips dissected from antisense CAD tobacco plants (clone 49). (*a*, apical; *i*, intermediate; *b*, basal). The effect of CAD down-regulation is evident by the change in the 1595:1510 cm^{-1} ratio

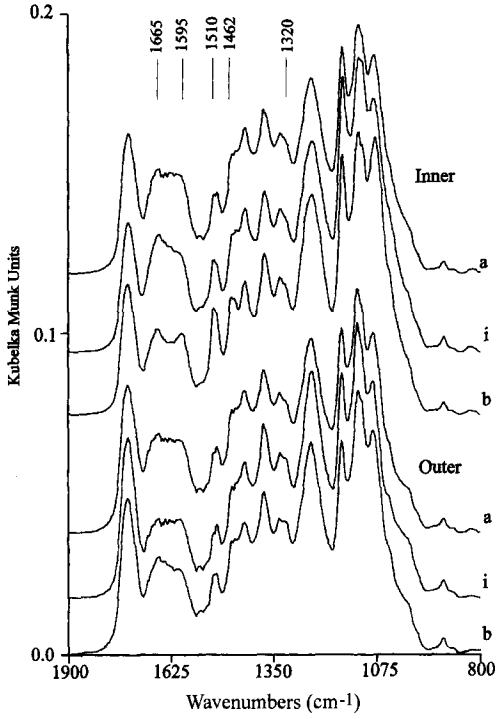

Fig. 4. Infrared spectra of the inner and outer surfaces of xylem strips dissected from antisense CAD tobacco plants (clone 37). (*a*, apical; *i*, intermediate; *b*, basal). The effect of CAD down-regulation is evident by the change in the 1595:1510 cm^{-1} ratio. There is also an increase in the conjugated carbonyl absorbance at 1665 cm^{-1}

this region can be assigned to the carbonyl groups of amides (proteins) and/or conjugated aldehyde and ketones (Williams and Fleming 1976; Kemp 1991) but the contribution from amides can be eliminated as the characteristic accompanying amide N-H stretch at 1540 cm^{-1} is not present. This suggests that the transformed plants have enhanced levels of conjugated aldehyde/ketone groups compared with the wild-type.

Although they contain the noticeable differences highlighted above, it is perhaps surprising that the FT-IR spectra of the wild-type and transformed tissues are so similar. This suggests that no fundamental changes in cell-wall structure, e.g. those involving lignin-polysaccharide cross-linkages, have occurred and that the manipulation of CAD expression has not indiscriminately influenced cell-wall architecture or composition.

Fourier-transform Raman spectra result from radiation scattered from a sample after excitation with visible or, increasingly more commonly, near infrared sources. In general, bonds which are polarized or contain a dipole, such as C = O, O-H etc. do not show up strongly in Raman spectra, whereas non-polarized bonds, such as C-H, C = C etc, do. As this is basically the reverse of that found in infrared spectroscopy, (FT-)Raman spectroscopy can provide complementary chemical and structural information. The FT-Raman spectra of the isolated xylem strips from the wild-type and transformed clones all contain maxima at 1610–1595 cm^{-1} and 1630 cm^{-1} (Fig. 5) with clone 37 exhibiting the most intense maxima (Table 1). Maxima at 1610–1595 cm^{-1} have been associated with aromatic rings (Agarwal and Atalla 1986, 1994, Agarwal et al. 1995) and that at 1630 cm^{-1} with conjugated double bonds (Bond et al. 1990; Agarwal and Atalla 1994; Agarwal et al. 1995). It has been reported that the intensity of these maxima are influenced by conjugation (Bond et al. 1990; Agarwal and Atalla 1994; Agarwal et al. 1995) and, in particular, Bond et al. (1990) noted that conjugation of a guaiacyl ring to a carbon-carbon double bond and a carbonyl group resulted in 10- and 12-fold increases, respectively, in the intensity of the maximum at 1595 cm^{-1}.* The accentuated maxima in the Raman spectra of clone 37 suggest that conjugated aromatic aldehydes, possibly coniferaldehyde, are present. In addition, although the absolute frequencies of the maxima in the spectrum of clone 49 are closer to those of the wild-type than to those of clone 37, the absolute frequencies of the maxima in the spectrum of clone 37 are similar to those reported for coniferaldehyde (Takei et al. 1995).

This increase in maxima at 1595 and 1620 cm^{-1} (Table 1) appears to be more pronounced in the oldest tissues, suggesting that aldehyde conjugation in lignin increases during plant development. The most important conclusion concerns the comparison between the two transformed clones: clone 37, which exhibits a lower

*It has been reported (Bond et al. 1990) that the use of radiation with wavelengths in the visible region for excitation also produces enhancement of scattering in the Raman spectrum at 1595 cm^{-1}. However, in the present study the exciting wavelength used was in the near-infrared region, negating the possibility of such absorbance enhancement.

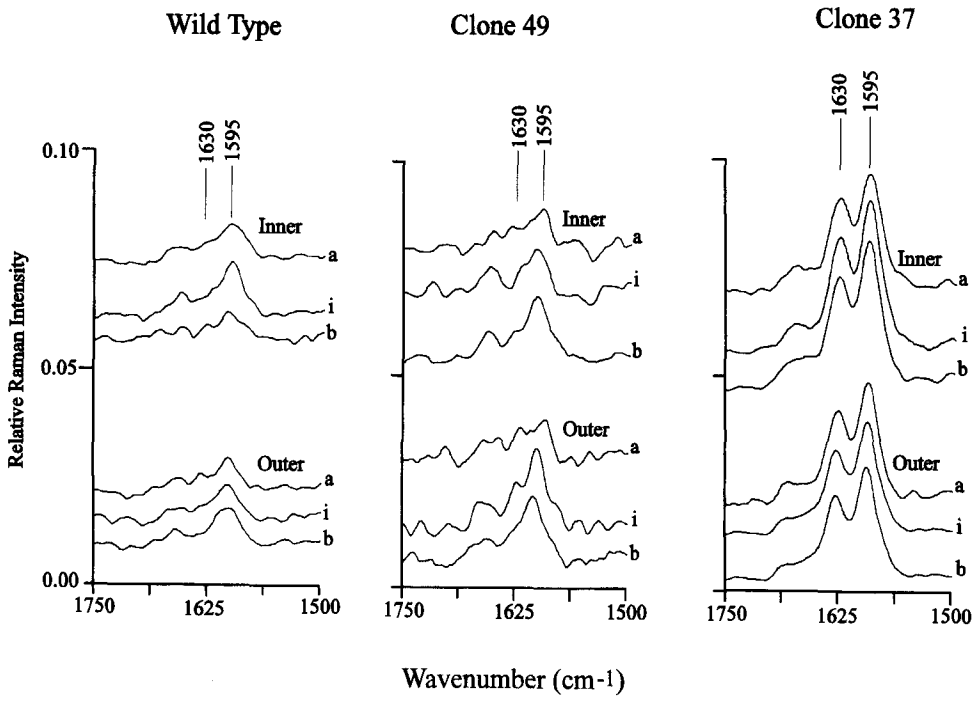

Fig. 5. FT-Raman spectra of the inner and outer surfaces of xylem from wild-type and antisense CAD clones 49 and 37 tobacco plants (*a*, apical; *i*, intermediate; *b*, basal). The incorporation of coniferaldehyde-like moieties into the lignins of the CAD down-regulated plants is reflected in the concurrent increase in intensity at 1630 and 1595 cm^{-1} due to conjugated carbon-carbon double bonds and aromatic rings, respectively

level of residual CAD activity than clone 49, appears to have a greater amount of conjugated aldehydes incorporated into lignin than clone 49.

The content of carbonyl groups in the milled whole-stem xylem was estimated using the oximation method of Štrancar and Perdith (1992). This showed that clone 37 contains a considerably higher level of carbonyl groups than clone 49 (clone 37:clone 49:wild-type, 2.4:1.6:1). These results confirm the relationship between carbonyl content and the level of residual CAD activity as previously described (Halpin et al. 1994; Hibino et al. 1995). This dose-response theory is confirmed by the DRIFT spectra of the milled xylem (Fig. 6) as the spectrum of clone 37 had an enhanced absorbance at 1665 cm^{-1}. However, the DRIFT spectrum of clone 49 shows no appreciable increase at this frequency.

The DRIFT spectrum of clone 37 has a 1510:1595 cm^{-1} ratio of < 1.0 whereas this ratio is > 1.0 in the spectra of the wild-type and clone 49 tissues (Fig. 6, Table 1). This may reflect reduced cross-linking of the lignin in clone 37. In addition, the absorbance at 1462 cm^{-1} present in the spectra of the wild-type and clone 49 is reduced to a shoulder in the spectrum of clone 37. This difference is also seen in the spectra of the xylem strips (compare Figs. 2–4) although the absorbance is present in the most developmentally mature xylem (inner surfaces of the basal part of the stem). Absorbance in this region has been assigned to C-H deformations of alkyl ($-CH_3$, $-CH_2-$) groups (Williams and Fleming 1976; Kemp 1991). Along with the FT-Raman evidence for the presence of carbon-carbon double bonds in the xylem of clones 49 and, in particular, 37, the reduction in the intensity of the absorbance at 1462 cm^{-1} may reflect that the lignin in this clone may have fewer saturated alkyl sidechains than that of the wild-type or clone 49.

The FT-Raman spectra of the milled whole-stem xylem samples (Fig. 7) confirm the observations drawn from the study of the xylem strips. Milled whole-stem samples from clone 49 and the wild-type displayed similar spectra, as was the case for the DRIFT spectra. In contrast the spectrum of clone 37 (Fig. 7) had greatly enhanced double-bond and aromatic-ring maxima at 1630 cm^{-1} and 1595 cm^{-1}, respectively, compared with spectra of the wild-type and clone 49. In fact, the FT-IR

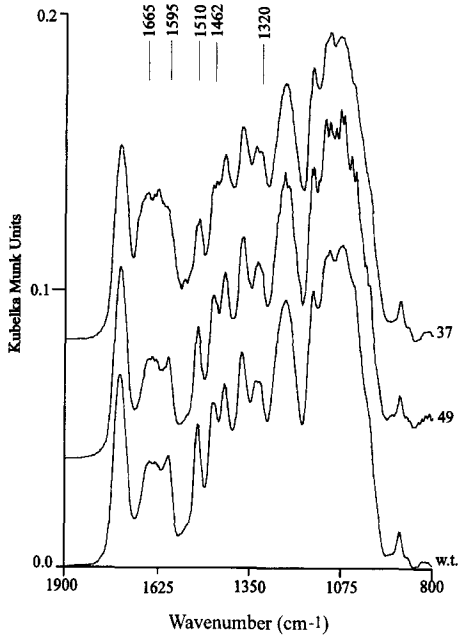

Fig. 6. DRIFT spectra of milled whole-stem xylem from wild-type (*w.t.*) and clone 49 (*49*) and clone 37 (*37*) antisense CAD tobacco plants. Only clone 37, with the lowest CAD activity exhibits a 1595:1510 cm^{-1} ratio > 1 indictaive of a less condensed lignin

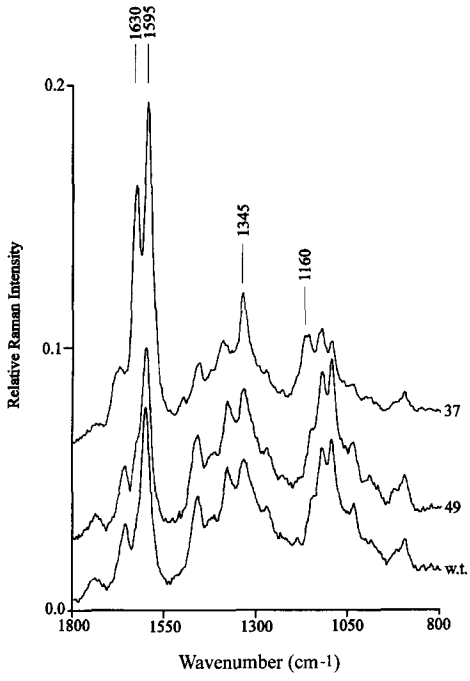

Fig. 7. FT-Raman spectra of milled whole stem xylem from wild-type (*w.t.*) and clone 49 (*49*) and clone 37 (*37*) antisense CAD tobacco plants showing an increased incorporation of conjugated, unsaturated moieties into the lignins of the CAD down-regulated plants, particularly clone 37. This is reflected in the concurrent increase in intensity at 1630 and 1595 cm^{-1} due to conjugated carbon-carbon double bonds and aromatic rings, respectively

and Raman spectra of the milled whole-stem xylem samples from clone 49 and the wild-type are very similar. Additional support for cinnamaldehyde incorporation in clone 37 comes from the enhanced maxima centred at 1160 cm^{-1}, the conjugated aromatic C-H out-of-plane deformation (Agarwal et al. 1995).

Concluding remarks

While the single-copy clone 49 and multiple-copy clone 37 had CAD activities reduced to 25 and 9%, respectively, of that of the wild-type this level of down-regulation did not reduce lignin content as measured by the acetyl bromide method (wild-type:clone 49:clone 37, 19.0 ± 0.5: 18.0 ± 0.5: 21.2 ± 0.7%, respectively). This confirms the findings of Halpin et al. (1994) and Higuchi et al. (1994) and suggests that the reduction of cinnamaldehydes to cinnamyl alcohols is not a limiting step in the biosynthesis of lignin. In our case, only clone 37, had the reddish-brown xylem found in other transgenic plants with greatly reduced CAD activity (Halpin et al. 1994; Higuchi et al. 1994) and in the brown mid-rib mutants of *Sorghum* which also have greatly reduced CAD activities (Pillonel et al. 1991). This distinct red phenotype indicates that chemical changes have occurred within the lignin polymer.

Both clones were found to have elevated levels of carbonyl groups incorporated into lignin as determined by the oximation method. The presence of elevated levels of aldehydic groups in the xylem of clones 37 and 49 was also shown by FT-IR spectroscopy (Figs. 2–4, 6) and the presence of coniferaldehyde moieties was implicated by greatly elevated maxima in the FT-Raman spectra of the xylem strips (Fig. 5) and whole-stem milled xylem (Fig. 7). However, clone 49 (with higher residual CAD activity than clone 37) did not show such obvious maxima in the DRIFT spectrum, perhaps reflecting the lower incorporation of cinnamaldehydes into lignin in these tissues. Halpin et al. (1994), using pyrolysis-mass spectrometry, also noted that the cinnamaldehyde content was appreciably higher in the lines of anti-sense-CAD plants which displayed low levels of residual CAD activity (7% of wild-type) as compared to the other lines where CAD activity was less constrained (≈ 20%) and where cinnamaldehyde content was not greatly different from that of the wild-type.

Similar conclusions have been reached by Higuchi et al. (1994) using thioacidolysis. They detected a higher level of coniferaldehyde derivatives in the thioacidolysis products of transgenic plants with decreased CAD activity. In contrast, no appreciable yield of such products was found among the monomers recovered from the non-condensed part of the lignin obtained from CAD down-regulated tobacco and poplar plants (B. Chabbert et al., INRA, France, personal communication). This suggests that cinnamaldehydes are incorporated into C-C linked structures which are not degraded by thioacidolysis or that the aldehydes can only be detected at a certain developmental stage. Our results support, through a new experimental approach, the enrichment of these transgenic lignins in cinnamaldehyde units.

The use of FT-IR spectroscopy also indicated that the lignin of both single-copy clone 49 and multiple-copy clone 37 may be less condensed, or cross-linked, than in the wild-type. This agrees with the findings of Halpin et al. (1994) who found that lignin from the antisense-CAD plants was more easily and more completely extracted by conventional pulping methods and was less stable to thermal breakdown during Pyrolysis/mass spectrometry. Lignin from transgenic poplar with reduced CAD expression has recently been shown to have a lignin that is easily extracted, and the pulp and paper produced from these trees has enhanced quality factors (Jouanin 1995).

It can be argued that the incorporation of large amounts of cinnamaldehydes into lignin would result in less aromatic-ring cross-linking through more complete delocalisation of unpaired electrons in the phenoxy radicals formed by peroxidases/oxidases during lignin biosynthesis. The presence of the carbonyl moiety in coniferaldehyde means that the unpaired electron would reside, to a greater degree, in the sidechain in comparison to an equivalent coniferyl alcohol-derived radical. Indeed, FT-IR and FT-Raman evidence suggests that the lignin of clone 37 contains higher levels of sidechain carbon-carbon double bonds than are found in native lignins (Himmelsbach and Barton 1980; Lewis et al. 1987; Ede et al. 1990; Lundquist 1991). The lower levels of incorporation of cinnamaldehyde structures into the

lignin of clone 49 may influence cross-linking by a similar mechanism but to a lesser degree.

In conclusion, the use of FT-IR and FT-Raman spectroscopies has confirmed that cinnamaldehyde moieties are incorporated into the lignins of transgenic plants with greatly depleted expression of CAD. Although essentially comparative, the results of these methods indicate that there are substantial structural differences between the lignin of CAD down-regulated transgenic plants with wild-type-like or red-coloured xylem. In particular, these methods may be used to rapidly estimate the extent of cross-linking of lignins within genetically manipulated plants and to predict the ease of extractability of lignin for industrial purposes.

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References

Agarwal UP, Atalla RH (1986) In-situ Raman microprobe studies of plant cell walls: macromolecular organization and compositional variability in the secondary wall of *Picea mariana* (Mill.) B.S.P. Planta 169: 325–332

Agarwal UP, Atalla RH (1994) Raman spectral features associated with chromophores in high-yield pulps. J. Wood Chem Technol 14: 227–241

Agarwal UP, Atalla RH, Forsskåhl I. (1995) Sequential treatment of mechanical and chemi-mechanical pulps with light and heat: a Raman spectroscopic study. Holzforschung 49: 300–312

Akin DE, Himmelsbach DS, Carl RT, Hanna WW, Barton II FE (1993) Mid-infrared microspectroscopy to assess lignin in plant tissues related to digestibility. Agron J 85: 171–175

Atanassova R, Favet N, Maitz F, Chabbert B, Tollier MT, 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

Bond JS, Agarwal UP, Atalla RH (1990) Contributions of pre-resonance Raman and conjugative effects to the Raman spectrum of native lignin. In: Durig JF, Sullivan JF (eds) The Twelfth International Conference on Raman Spectroscopy. South Carolina, USA, pp 652–653

Boudet AM, Lapierre C, Grima-Pettenari J (1995) Biochemistry and molecular biology of lignification. New Phytol 129: 203–236

Campbell MM, Sederoff RS (1996) Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. Plant Physiol 110: 3–13

Cui F, Dolphin D (1991) Iron porphyrin catalyzed oxidation of lignin model compounds: the oxidation of veratryl alcohol and veratryl acetate. Can J Chem 70: 2314–2318

Dean JFD, Eriksson KEL (1992) Biotechnological modification of lignin structure and composition in forest trees. Holzforschung 46: 135–147

van Doorsselaere JV, Baucher M, Chogrot E, Chabbert B, Tollier MT, Petit Conil M, Leplé JC, Pilate G, Cornu D, Monties B, Van Montagu M, Inzé D, Boerjan W, Jouanin L (1995) A novel lignin in poplar with a reduced caffeic acid/5-hydroxyferulic acid O-methyltransferase activity. Plant J 8: 855–864

Dwivedi UN, Campbell WH, Yu J, Dalta RSS, Bugos RC, Chiang VL, Podila GK (1993) Modification of lignin biosynthesis in transgenic tobacco through expression of an antisense O-methyltransferase gene from *Populus*. Plant Mol Biol 26: 61–71

Ede RM, Brunow G, Simola LK, Lemmetyinen J (1990) Two-dimensional ^{1}H-^{1}H chemical shift correlation and *J*-resolved NMR studies on isolated and synthetic lignins. Holzforschung 44: 95–101

Elkind Y, Edwards R, Mavandad M, Hendrick SA, 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 87: 9057–9061

Faix O (1991) Classification of lignins from different botanical origins by FT-IR spectroscopy. Holzforschung 45 [Suppl]: 21–27

Gellerstedt G, Pettersson EL (1975) Light-induced oxidation of lignin. Part 2. The oxidative degradation of aromatic rings. Svensk Papperstidn 80: 15–21

Goffner D, Campbell MM, Campargue C, Clastre M, Borderies G, Boudet A, Boudet AM (1994) Purification and characterization of cinnamoyl-CoA: NADP oxidoreductase in *Eucalyptus gunnii*. Plant Physiol 106: 625–632

Halpin C, Knight ME, Grima-Pettenari J, Goeffner D, Boudet AM, Schuch W (1992) Purification and characterization of cinnamyl alcohol dehydrogenase from tobacco stems. Plant Physiol 98: 12–16

Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier MT, Schuch W (1994) Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. Plant J 6: 339–350

Hergert HL (1969) Infrared spectra of lignin and related compounds. II. Conifer lignin and model compounds. J Org Chem 25: 405–413

Hergert HL (1970) Infrared spectra. In: Sarkanen KV, Ludwig CH (eds) Lignins; occurrence, formation, structure and reactions. Wiley Interscience, New York, pp 267–297

Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T (1995) Increase of cinnamaldehyde groups in lignin of transgenic plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. Biosci Biotech Biochem 59: 929–931

Higuchi T (1985) Biosynthesis of lignin. In: Kratzl K, Billek G (eds) Biosynthesis and biodegradation of wood components. Academic Press, London, pp 141–160

Higuchi T, Ito T, Umezawa T, Hibino T, Shibata D (1994) Red-brown colour of lignified tissues of transgenic plants with antisense CAD gene: Wine-red lignin from coniferaldehyde. J Biotechnol 37: 151–158

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

Jouanin L (1995) Modification of lignin quality in poplar trees using antisense strategy. Polyphenols Actualites 13: 5–7

Jung HJG, Deetz DA (1993) Cell wall lignification and degradability. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) Forage cell wall structure and digestibility, ASA-CSSA-SSSA, Madison, pp 315–346

Kemp W (1991) Organic spectroscopy. Macmillan, London, pp 19–100

Lewis NG, Newman J, Just G, Ripmeister J (1987) Determination of bonding patterns of ^{13}C specifically enriched dehydrogenatively polymerised lignin in solution and solid state. Macromolecules 20: 1752–1756

Lundquist K (1991) ^{1}H NMR spectral studies of lignins. Nordic Pulp Paper Res J 3: 140–146

McDougall GJ, Morrison IM, Stewart D, Weyers JDB, Hillman JR (1993) Plant fibres: botany, chemistry and processing for industrial use. J Sci Food Agric 62: 1–20

Morrison IM (1972) A semi-micro method for the determination of lignin and its use in predicting the digestibility of forage crops. J Sci Food Agric 23: 455–463

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

Pillonel C, Mulder MM, Boon JJ, Forster B, Binder A (1991) Involvement of cinnamyl alcohol dehydrogenase in the control of lignin formation in *Sorghum bicolor* L. Moench. Planta 185: 538–544

Sarkanen KV, Chang HM, Ericsson B (1967) Species variations in lignins. Part 1. Infrared spectra of guaiacyl and syringyl models. Tappi 50: 572–575

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: Ellis B (ed) Genetic engineering of plant secondary metabolism. Plenum Press, New York, pp 313–355

Stewart D, Morrison IM (1992) FT-IR spectroscopy as a tool for the study of biological and chemical treatments of barley straw. J Sci Food Agric 60: 431–436

Stewart D, Wilson HM, Hendra PJ, Morrison IM (1995) Fourier-transform infrared and Raman spectroscopic study of biochemical and chemical treatments of oak (*Quercus rubra*) wood and barley (*Hordeum vulgare*) straw. J Food Agric Chem 43: 2219–2225

Štrancar A, Perdith A (1992) Oximation of lignin carbonyl groups and their potential use for lignin determination. Holzforschung 46: 391–393

Takei T, Kato N, Iijima T, Higaki M (1995) Raman spectroscopic analysis of wood and bamboo lignin. Mokuzai Gakkaishi 41: 229–236

Thomas BB (1970) Pulp properties. In: Britt KW (ed) Handbook of pulp and paper technology. Van Nostrand Reinhold Co., New York, pp 225–238

Whetton R, Sederoff R (1991) Genetic engineering of wood. Forest Ecol Manage 43: 301–316

Williams HD, Fleming I (1976) Spectroscopic methods in organic chemistry. McGraw-Hill, Maidenhead, pp 35–73