Alterations in the Biosynthesis of Lignin in Transgenic Plants with Chimeric Genes for 4-Coumarate:Coenzyme A Ligase

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The introduction of chimeric sense and antisense gene constructs for 4-coumarate:coenzyme A ligase into tobacco plants caused the reduction of the 4CL activity in the transgenic plants. In the transgenic plants, the cell walls of the xylem tissue in stems were brown and the molecular structure of lignin in the colored cell walls was dramatically different from that in the control plants. Analysis with different types of stain revealed that levels of cinnamyl aldehyde residues and syringyl units in lignin were depressed in the brownish cell walls. Furthermore, the lignin content in colored tissue was lower than that in the normal tissue. Our results indicate that 4CL has important roles in the determination of the composition and the amount of lignin in tobacco plants.

Key words: 4-Coumarate:CoA ligase — Genetic manipulation — Lignin biosynthesis — Transgenic tobacco.

Lignin is one of the major components of plant cell walls and it is synthesized by the dehydrogenative polymerization of three monolignols, namely, 4-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 1). The p-hydroxyphenyl, guaiacyl, and syringyl residues of lignin are derived from 4-coumaryl, coniferyl, and sinapyl alcohol, respectively. Lignin in angiosperms is composed of guaiacyl and syringyl units and that in gymnosperms consists mainly of guaiacyl units. Many p-hydroxyphenyl units are found in the compression wood of gymnosperms and grasses (Lewis and Yamamoto 1990). It is thought that the differences in the monomeric composition of lignins between angiosperms and gymnosperms are due to the presence or absence of hydroxylases and O-methyltransferases that are able to use ferulate and 5-hydroxyferulate, respectively, as substrates (Whetten and Sederoff 1995).

Monolignols are considered to be generated from the corresponding cinnamate derivatives, namely, 4-coumarate, ferulate, and sinapate. It is thought that three enzymatic reactions are involved in the synthesis of the monolignols from these cinnamate derivatives. In the first step in these reactions, the carboxyl group is activated by coenzyme A. This reaction is catalyzed by 4-coumarate: coenzyme A ligase (4CL; Luderitz et al. 1982, Higuchi 1990). The level of the enzymatic activity and that of the transcripts for 4CL reflect the rate or extent of the deposition of lignin in some plants (Church and Galston 1988, Hennion et al. 1992, Wilkinson and Butt 1992). The genes for 4CL have been isolated from many plants and the expression of these genes in response to various stresses has been well characterized (Douglas et al. 1987, Lozoya et al. 1988, Becker-Andre et al. 1991, Douglas et al. 1991, Uhlmann and Ebel 1993, Lee et al. 1995, Yazaki et al. 1995). However, the role of 4CL in the biosynthesis of lignin has not been well characterized.

The isoenzymes of 4CL can react with a variety of cinnamate derivatives in many plants (Sederoff et al. 1994). However, in some angiosperms that have syringyl-guaiacyl lignin in their cell walls, 4CL cannot recognize sinapate as a substrate (Kutsuki et al. 1982, Higuchi 1990). This result suggests that sinapoyl-CoA ester is not formed directly from sinapate in these plants. In recent studies (Kneusel et al. 1989, Kuhnli et al. 1989, Pakusch et al. 1989), an altered biosynthetic pathway for monolignols was proposed and novel hydroxylases and O-methyltransferases, which can use derivatives of cinnamoyl-CoA ester as substrates, were isolated from several plants and characterized. The transcripts of genes for CoA-ester specific O-methyltransferases have been detected predominantly in differentiating xylem tissues (Ye and Varner 1995). In the alternative pathway, sinapoyl-CoA ester might be synthesized from 5-hydroxyferuloyl-CoA and not from sinapate (Kutsuki et al. 1982). Therefore, we predicted that CoA-esterification of cinnamate derivatives by 4CL could be important for the biosynthesis of different types of monolignol and for tissue type-specific lignification.

From an industrial perspective, manipulation of lignins in woody and forage plants is important for pulp and paper making and for the production of dairy products. Therefore, much effort has been made to alter the
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![Biosynthetic pathways to lignin](https://example.com/pathways)

Fig. 1  Biosynthetic pathways to lignin. The arrows indicate reactions that are catalyzed by specific enzymes.

quantity and quality of lignin in plants by genetic manipulation. Manipulation of the lignin content and the monomeric composition in transgenic plants has been achieved by introducing antisense genes for O-methyltransferases that can use cinnamate derivatives as substrates (Dwivedi et al. 1994, Ni et al. 1994, Atanassova et al. 1995). Altered lignin, with improved chemical extractability, was produced in plants that had been transformed with an antisense gene for cinnamyl alcohol dehydrogenase (Halpin et al. 1994, Hibino et al. 1995). The published information about 4CL suggests that the regulation of 4CL activity by the introduction of 4CL transgenes might also allow the manipulation of the content and the monomeric composition of lignin.

To elucidate the role of 4CL in the biosynthesis of the monolignols or lignin polymers in plants, we transformed tobacco plants with chimeric genes for 4CL. In the transgenic plants with low 4CL activity that was induced by the introduction of the chimeric genes, the cell walls in the xylem tissue were brown, rather than pale white as in the control plant. The lignin content and the composition of cell walls of the colored tissue were quite different from those of the control plants. It appears that the expression of genes for 4CL plays an important role in the determination of the content and the monomeric composition of lignin in plant cell walls.

Materials and Methods

Production of transgenic plants—An Agrobacterium-mediated transformation system was used for the production of transgenic plants (Bevan 1984). A near full-length cDNA for 4CL (1,855 bp; accession number D43773 in the DDBJ database), including the entire coding region, was used for the preparation of two chimeric genes for 4CL: a 'sense' and an 'antisense' construct. Plasmids pSNT4CL and pANT4CL contained a chimeric gene that consisted of the cauliflower mosaic virus (CaMV) 35S promoter sequence, 4CL cDNA in the regular (pSNT4CL) or the opposite (pANT4CL) orientation with respect to the CaMV promoter sequence, and the terminator sequence of a gene for nopaline synthase (Fig. 2). Plasmid pBI121DG, namely, pBI121 (Clontech Laboratories, Palo Alto, CA, U.S.A.) without the β-glucuronidase-coding (GUS-coding) region, was used for the production of the control transgenic plants. pSNT4CL, pANT4CL and pBI121DG were separately introduced into Agrobacterium tumefaciens LBA4404 by triparental mating with pRK2013 as a helper plasmid. Leaf disks of tobacco (Nicotiana tabacum L. var. SR1) were infected with the A. tumefaciens cells that harbored each plasmid by the standard procedure. Transformed calli were selected on kanamycin-containing Murashige-Skoog medium (200 mg l⁻¹ kanamycin; Murashige and Skoog 1962). Transgenic plants were regenerated from the transformed calli.

Histochemical analysis—Wiesner and Maule reactions, which are the standard colorimetric reactions for the detection of lignin, were used to stain 100-μm-thick sections of stems (Srivastava 1966). Transverse sections of stems were incubated in a mixture of phloroglucinol (5% in ethanol) and 12 M HCl (1:1, v/v) for 1 min for the Wiesner reaction. The sections were treated successively with 1% KMnO₄, 3% HCl, and a concentrated solution of am-
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Fig. 2  T-DNA regions of vectors that were used for the generation of transgenic plants. The binary vectors pSN4CL (A) and pANT4CL (B) contained CDNA for tobacco 4CL in the regular and the reverse orientation, respectively, relative to the cauliflower mosaic virus 35S promoter (CaMV35S) and the terminator of the gene for nopaline synthase (NOST). pBI121DG (C) was derived from pBI121 by removal of the coding region for β-glucuronidase. These vectors carried the gene for neomycin phosphotransferase (NPTII) as a selectable marker. NOSP represents the promoter of the gene for nopaline synthase.

Southern blot analysis—Total DNA was extracted from leaf tissues (1 g) of each plant by the cetyltrimethyl-ammonium bromide procedure. The DNA samples (10 μg) were digested with XbaI and then subjected to electrophoresis in a 0.8% agarose gel. After staining of the gel with ethidium bromide, a photograph of the gel was taken and then the DNA was blotted on a nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany). Blotted DNA on the membrane was fixed by exposure to UV light for 3 min. The probe, namely, a fragment of tobacco 4CL cDNA fragment, was labelled with digoxigenin-DUTP in a DIG DNA labelling kit (Boehringer Mannheim GmbH). Hybridization was performed according to the instructions from Boehringer Mannheim GmbH. The membrane was washed once with 1 × SSC, 0.1% SDS and once with 0.1 × SSC, 0.1% SDS at 65°C, and then it was exposed to X-ray film for 1 h at room temperature.

Assay of enzymatic activity of 4CL—All procedures for preparation of the crude enzyme for measurements of the enzymatic activity of 4CL were carried out at 4°C. About one gram of stem tissue from each transgenic tobacco plant was ground in a mortar with liquid nitrogen. Then the powder was added to 4 ml of extraction buffer (200 mM Tris-HCl, pH 7.8, 14 mM β-mercaptoethanol). After the mixture had been centrifuged at 12,000 × g for 10 min, the precipitate from the supernatant, obtained between 30% and 75% saturation with ammonium sulfate, was collected. The precipitate was resuspended in 1 ml of extraction buffer and the solution was desalted overnight by dialysis against the extraction buffer. Then 30–40 μg of protein in each crude extract were used for each assay of enzymatic activity. Total protein was quantitated with a protein assay kit (Nippon Bio-Rad Laboratories KK, Tokyo, Japan) with bovine serum albumin as the standard.

The enzymatic activity of 4CL was determined at 30°C with 4-coumarate as substrate. The reaction mixture and the procedure for the measurement of the enzymatic activity were those described by Grand et al. (1983).

Northern blot analysis—Total RNA (5 μg) from the stem tissue of each plant was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and then bands of RNA were transferred to a nylon membrane. The membrane was allowed to hybridize at 65°C with the same probe as that used for Southern analysis. The membrane was washed once with 2 × SSC, 0.1% SDS and once with 1 × SSC, 0.1% SDS at 65°C.

Quantitation of lignin—Xylem tissue in stems was separated from all other living tissues of individual plants. The xylem tissue was ground into particles that passed through a 355-μm screen and dried under a vacuum for three days. The dried sample of xylem tissue was extracted with a mixture of ethanol and toluene (1 : 2, v/v) for 6 h, with ethanol for 3 h, and with hot water for 2 h, successively, in a Soxhlet apparatus and then it was dried again under a vacuum for one week.

Spectrophotometric determinations of lignin content were made by the acetyl bromide method (Iiyama and Wallis 1990). The dried and extracted powder (5 mg) was placed in a glass reaction bottle with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 ml). Perchloric acid (70%, 0.1 ml) was added, and the bottle was capped and placed in an oven at 70°C for 30 min. After digestion, the solution was transferred to a 50-ml volumetric flask that contained 10 ml of 2 M NaOH and 12 ml of acetic acid. The bottle was rinsed and the solution was made up to 50 ml with acetic acid. The UV absorption spectrum of the solution was recorded against a blank solution, prepared without the sample, that was monitored in conjunction with the sample. The lignin content of xylem tissue was determined from the absorbance at 280 nm and a specific absorption coefficient of 20.0 (liter g⁻¹ cm⁻¹; Iiyama and Wallis 1990).

Alkaline nitrobenzene oxidation of xylem tissue—Ten mg of the extracted powder obtained from the xylem tissue were mixed with 4 ml of 2 M NaOH and 240 μl of nitrobenzene. The mixture was heated in an autoclave at 170°C for 3 h. After cooling, the nitrobenzene in the mixture was removed by three extractions with ethyl ether. An internal standard (1 mg of acetovanillone; Aldrich Chemical Company Inc., Milwaukee, WI, U.S.A.), was added to the aqueous solution, which was then acidified to pH 2 with 1 M HCl. The acidic solution was saturated with NaCl and then extracted three times with ethyl ether. The ether solution was then dried over anhydrous sodium sulfate overnight. After the evaporation of ethyl ether, the products were dissolved in pyridine (1 ml) and analyzed by gas chromatography (Gas Chromatograph G1800A; Hewlett Packard, Palo Alto, CA, U.S.A.). The separation was carried out on a capillary column (HP-INNOWax; Hewlett Packard) with an electron ionization detector. The temperatures of the column, injector, and detector were 220, 250, and 260°C, respectively.
Results

Transgenic plants with chimeric 4CL genes—Prior to this study, we had already isolated a near full-length cDNA for 4CL from tobacco (accession number D43773 in the DDBJ database). This fragment was sufficient for production of active 4CL protein in E. coli (Kajita et al. in preparation). We used this cDNA fragment for construction of chimeric genes for 4CL. The fragment of cDNA for 4CL of tobacco was ligated, in the sense or the antisense orientation, with the 35S promoter of CaMV in pBl121DG (Fig. 2) and the constructs were used for production of transgenic plants. We picked randomly four three-week-old transgenic plants that had been transformed with pSNT4CL (designated S1, S2, S3, and S4; S=sense), five that had been transformed with pANT4CL (A1, A2, A3, A4, and A5; A=antisense), and three that had been transformed with pBl121DG (C1, C2, and C3; C=control) and we cultivated these plants for further experiments. No morphological differences were seen among these transgenic plants when the plants were selected. At the flowering stage, dwarf stems were observed in three (S1, S2, and S3) of the transgenic plants. However, no obvious relationship between changes in morphology and expression of the gene for 4CL in these plants was recognizable from our observations.

The presence of a 4CL transgene in each transgenic plant was confirmed by Southern blot analysis. A fragment of approximately 1.9 kbp hybridized to the DIG-labelled 4CL cDNA probe during Southern analysis in the case of plants transformed with pSNT4CL and pANT4CL but not in the case of controls (Fig. 3). This fragment represented the introduction of the XbaI fragment of the tobacco 4CL cDNA sequence into these plants. The other bands observed in Figure 3 presumably represent native tobacco genes for 4CL.

The lower parts of the stems (internodes below the fifth or sixth internode) of the S1, S2, S3, S4, and A5 plants were brownish-green as compared to the green stems of the control plants. This change in color of the stems was due to the formation of brown xylem tissues in these plants (Fig. 4). These plants had both brown and pale white (normal color) cell walls in the xylem tissues. The differences in color between the brownish and the normal-colored xylem tissues was most conspicuous in the S4 plant among the various plants (Fig. 4-A). The change in color of the xylem tissue in the A5 plant (Fig. 4-B) was relatively minor as compared to that in the other four plants that had been transformed with pSNT4CL. The xylem tissues in the other antisense plants (A1, A2, A3 and A4) were normal in color (data not shown).

Histochemical analysis of xylem tissues in the C1, S4 and A5 plants was performed with reagents that are known to stain lignin. Lignin was stained bright red with Wiesner's reagent, which reacts with cinnamyl aldehyde units in lignin (Srivastava 1966), while the syringyl units in lignin could be detected specifically after the Mäule reactions reddish-brown deposits (Monties 1989).

Figure 5 shows that, in the S4 plant (Fig. 5-A), some of the brownish cell walls in the xylem (left side of the xylem region in the photograph) were not stained with Wiesner's reagent while cell walls with a normal color (whitish; right side of the xylem region in the photograph) were stained light pink, as compared to the normal deeper pink color observed after staining of the control plants. Transverse sections of the A5 plant stained pink and the color was almost the same that observed in the S4 plant (Fig. 5-B).

The xylem tissue in the control plants was reddish-brown after the Mäule reaction (Fig. 5-F). The brownish part of the xylem in the S4 plant (left side) did not stain normally while the whitish xylem (right side) stained brick red after the Mäule reaction (Fig. 5-D). In the A5 plant, the outer part of the xylem tissue was stained yellow while the inner part was brick red after the Mäule reaction (Fig. 5-E). The brownish xylem tissues in the S1, S2, and S3 plants were also stained similarly to those of the S4 plant after the two reactions (data not shown). In terms of color, the stained xylem tissues in the A1, A2, A3, and A4 plants of the two reactions were indistinguishable.

4CL activity in transgenic plants and Northern blot analysis—The enzymatic activity of 4CL in individual

![Fig. 3 Southern blot analysis with the tobacco cDNA for 4CL as the probe. Genomic DNA (10 µg), digested with XbaI, from each transgenic plant was used for the analysis. Molecular size standards are indicated on the left side of the panel. Lanes 1-3 represent the C1, C2 and C3 plants, respectively. Lanes 4-7 represent the S1, S2, S3 and S4 plants, respectively. Lanes 8-12 represent the A1, A2, A3, A4 and A5 plants, respectively. ]
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plants was determined by the photometric detection of the formation of 4-coumaroyl-CoA from 4-coumarate in vitro. The enzymatic activity of 4CL was detected at appropriate levels in crude extracts from mature stem tissues of wild-type tobacco plants, as observed in Arabidopsis thaliana (Lee et al. 1995). Therefore, we used parts of the lowest four internodes of the transgenic plants for the extraction of the crude enzyme. The 4CL activities in crude extracts from the stems of transgenic plants that had brownish xylem tissue were dramatically reduced (Fig. 6). In particular, in the extracts from the S1, S3, and S4 plants, very low activity was detected and it was less than 1% of the activity in the controls. 4CL activity of the A1, A2, A3 and A4 plants, which had normal xylem tissues, was not significantly different from those of the controls. Northern blot analysis indicated that the level of transcripts for 4CL was

Fig. 4 Xylem of stems of transgenic plants. Brown coloration is visible in the S4 (A; transformed with the sense construct) and A5 (B; transformed with the antisense construct), as compared with the C1 plant (C; transformed with the control construct). Bar = 5 mm.

Fig. 5 Histochemical analysis of transverse section of stems of transgenic plants. Sections from the S4 (A), A5 (B) and C1 (C) plants were stained by Wiesner's reaction. Sections from the S4 (D), A5 (E) and C1 (F) plants were stained by Mäule's reaction. Bar = 250 μm.
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Fig. 6 The enzymatic activity of 4CL in the control plants and in the transgenic plants transformed with pSNT4CL and with pANT4CL. Three control plants (C1, C2 and C3), four transgenic plants with a sense 4CL gene construct (S1, S2, S3 and S4) and five with an antisense construct (A1, A2, A3, A4 and A5) were used for this analysis.

Reduced lignin was observed in the transgenic plants that had low 4CL activity (Fig. 7). Thus, the reductions in 4CL activity in the S1, S3 and S4 plants were due to the low levels of transcripts for 4CL. Our data suggest that the change in the color of the xylem tissue in the transgenic plants might have been caused by a reduction in 4CL activity that was induced by the down-regulation of the endogenous gene(s) for 4CL.

Characterization of lignin in transgenic plants—The analysis of the level and the monomeric composition of lignin in the transgenic plants was performed with the cell wall residues (CWR) of the xylem tissues in stems. When we applied this method, we found that the level of lignin in the control plants was approximately constant (Table 1). By contrast, in the transgenic plants that had been transformed with the 4CL transgenes, levels of lignin in the CWR varied among plants. The levels of lignin in the plants that had brownish xylem tissues were much lower than those in the control plants. The lignin in the CWR from the S1, S2, S3, S4, and A5 plants were 65.5%, 79.7%, 78.6%, 77.6% and 81.3% of the average value for the control plants, respectively (Table 1). The levels of lignin in the other transgenic plants that had been transformed with pANT4CL (A1, A2, A3 and A4) did not differ from that of the control plants. Thus, the level of lignin in the CWR

Table 1 Chemical analysis of lignin from transgenic plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>Lignin (^a)</th>
<th>Vanillin (^b)</th>
<th>Syringaldehyde (^b)</th>
<th>Total aldehydes (^a)</th>
<th>Relative amount (^c)</th>
<th>S/V ratio (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>21.6 ± 0.1</td>
<td>183 ± 3.4</td>
<td>143 ± 2.8</td>
<td>326</td>
<td>100</td>
<td>0.78</td>
</tr>
<tr>
<td>C2</td>
<td>22.1 ± 0.4</td>
<td>169 ± 3.3</td>
<td>128 ± 2.0</td>
<td>297</td>
<td>88.7</td>
<td>0.76</td>
</tr>
<tr>
<td>C3</td>
<td>21.3 ± 0.0</td>
<td>168 ± 0.9</td>
<td>145 ± 0.6</td>
<td>313</td>
<td>97.4</td>
<td>0.86</td>
</tr>
<tr>
<td>S1</td>
<td>14.2 ± 0.5</td>
<td>22 ± 0.7</td>
<td>10 ± 0.5</td>
<td>32</td>
<td>15.2</td>
<td>0.45</td>
</tr>
<tr>
<td>S2</td>
<td>17.3 ± 1.0</td>
<td>47 ± 2.9</td>
<td>88 ± 3.2</td>
<td>135</td>
<td>58.3</td>
<td>1.9</td>
</tr>
<tr>
<td>S3</td>
<td>17.0 ± 0.6</td>
<td>50 ± 1.0</td>
<td>100 ± 2.0</td>
<td>150</td>
<td>51.7</td>
<td>2.0</td>
</tr>
<tr>
<td>S4</td>
<td>16.8 ± 0.3</td>
<td>100 ± 1.0</td>
<td>79 ± 1.1</td>
<td>179</td>
<td>70.2</td>
<td>0.79</td>
</tr>
<tr>
<td>A1</td>
<td>20.5 ± 0.4</td>
<td>139 ± 2.3</td>
<td>121 ± 1.4</td>
<td>260</td>
<td>84.1</td>
<td>0.87</td>
</tr>
<tr>
<td>A2</td>
<td>22.9 ± 0.2</td>
<td>176 ± 1.1</td>
<td>134 ± 1.3</td>
<td>310</td>
<td>90.0</td>
<td>0.76</td>
</tr>
<tr>
<td>A3</td>
<td>20.5 ± 0.0</td>
<td>154 ± 1.4</td>
<td>111 ± 0.5</td>
<td>265</td>
<td>85.4</td>
<td>0.72</td>
</tr>
<tr>
<td>A4</td>
<td>21.9 ± 0.7</td>
<td>157 ± 0.7</td>
<td>125 ± 0.8</td>
<td>282</td>
<td>85.4</td>
<td>0.80</td>
</tr>
<tr>
<td>A5</td>
<td>17.6 ± 0.1</td>
<td>89 ± 0.1</td>
<td>92 ± 1.2</td>
<td>181</td>
<td>68.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Lignin content was determined by the acetyl bromide procedure and is expressed as a percentage (w/w) of the cell wall residue (CWR).

\(^b\) Vanillin and syringaldehyde were obtained when the CWR was treated by the alkaline nitrobenzene oxidation procedure. Total aldehydes are the sum of amounts of vanillin and syringaldehyde. The values are expressed as \(\mu\)mol per gram of CWR.

\(^c\) Relative amount indicates the relative amount of total aldehydes per unit of lignin for each plant. The values are given relative to that for the C1 plant.

\(^d\) The S/V ratio is the ratio of the amount of syringaldehyde to that of vanillin.
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Table 2 Analysis of lignin in colored and normal xylem tissues of the S4 plant

<table>
<thead>
<tr>
<th>Color of cell walls</th>
<th>Lignin content</th>
<th>Vanillin</th>
<th>Syringaldehyde</th>
<th>Total aldehyde</th>
<th>S/V ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>16.8±0.2</td>
<td>41±0.7</td>
<td>64±0.2</td>
<td>105±17.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Normal (whitish)</td>
<td>24.1±0.1</td>
<td>157±5.1</td>
<td>142±8.0</td>
<td>299±49.7</td>
<td>0.90</td>
</tr>
</tbody>
</table>

a The cell wall residues (CWR) differently colored xylem tissues of the S4 plant were used as starting material.
b Lignin content was determined by the acetyl bromide procedure and is expressed as a percentage (w/w) of the CWR.
c The values are expressed as μmol per gram of CWR.

seemed roughly to reflect the enzymatic activity of 4CL in the individual plants.

To determine the monomeric composition of lignin in the CWR, we examined the CWR from each plants by alkaline nitrobenzene oxidation analysis. Vanillin and syringaldehyde are generated from the non-condensed fraction of lignin by this procedure (Chiang and Funaoka 1988). These benzaldehyde derivatives are considered to be derived from the guaiacyl and syringyl units in lignin, respectively. The relative abundance of guaiacyl and syringyl units in lignin can be summarized as the S/V ratio (the ratio of the amounts of syringaldehyde and vanillin). The ratios for S2 and S3 were very much higher than those for the other plants, while the ratio for S1 was lower than those for the control plants. These results indicate that the reduction in the enzymatic activity of 4CL in transgenic plants altered not only the level but also the monomeric composition of the lignin.

The relative levels of total aldehydes, detected by oxidation analysis, per unit of lignin from plants with the brownish xylem tissue were much lower than those in the control plants. In particular, the level of total aldehydes in lignin from the S1 plant was very much lower than the control values (e.g., 15% of the level in the C1 plant). These data indicate that lignin in the plants with brownish tissue contained more condensed units that could not be degraded by the oxidation procedure.

Table 2 shows the results of the chemical analysis of the lignin in the differently colored xylem tissues from the S4 plant. The characteristics of lignin in these two tissues are quite different from each other. The levels of lignin in the brownish tissue was 65% and the level of total aldehydes per unit of lignin was 53% of the corresponding levels in the whitish tissue. These results indicate that heterogeneous lignification occurred in the differently colored tissues of the S4 plant and, moreover, that the change in the structure of lignin in the S1, S2, S3, S4 and A5 plants was the reason for brownish tissues in these plants.

Discussion

This report is the first, to our knowledge, of the alteration of lignin by the down-regulation of an endogenous gene for 4CL. The aim of our work was to study a role of 4CL in the biosynthesis of the monolignols and lignin polymers. The tobacco plant is a good material for analysis of the molecular mechanisms of lignin biosynthesis since guaiacyl and syringyl units, the main components of lignin in angiosperms, are present in cell walls and the production of transgenic tobacco plants is easily accomplished. Several groups of researchers have used tobacco as a model plant for studies of the molecular mechanisms of lignin biosynthesis (Dwivedi et al. 1994, Halpin et al. 1994, Hibino et al. 1995, Atanassova et al. 1995).

We produced transgenic tobacco plants with low 4CL activity by introducing chimeric genes for 4CL and we analyzed the structure of lignin in the transgenic plants. Both the sense and antisense gene constructs for 4CL were useful for production of transgenic plants with low 4CL activity. The alteration in 4CL activity in the transgenic plants with these constructs might have been due to cosuppression (sense construct) and to down-regulation by antisense RNA (antisense construct) of the expression of the gene for 4CL (Napoli et al. 1990, Kuipers et al. 1995).

Brownish and normal-colored xylem tissues were observed in several transgenic plants with chimeric 4CL transgenes. A change in the color of the xylem cell walls was also seen in the bm, mutant of corn, which has low cinnamyl alcohol dehydrogenase (CAD) activity (Pillonel et al. 1991) and in transgenic plants transformed with an antisense gene for CAD (Halpin et al. 1994). However, the lignin in plants with an antisense gene for CAD was appropriately stained with Wiesner’s reagent, and the results of Mäule staining of the xylem in the transgenic plants were indistinguishable from those in wild-type plants. These features of the CAD antisense plants were quite different from those of the present transgenic plants with the 4CL transgene. Higuchi et al. (1994) suggested that coniferal aldehyde might be converted to a wine-red lignin by peroxidase and hydrogen peroxide in vitro and, thus, the
brownish color of xylem cell walls in the \( bm \) mutant and in the transgenic plants with an antisense CAD gene might have been due to the accumulation of cinnamyl aldehyde residues in the lignin of these plants. From the results of the staining analysis of our S4 plant (Fig. 5-A), it appeared that quite a low level of the cinnamyl aldehyde units that can react with Wiesner’s reagent was present in the brownish tissue. Thus, the coloration of the xylem tissue in the plants with the 4CL transgenes and in those with the antisense gene for CAD seems to be attributable to different mechanisms.

To elucidate the structure of lignin in the brownish tissue, we performed the chemical analysis of the lignin in the brownish tissue from the S4 plant. Table 2 shows results of the analysis of different color tissues from the S4 plant. The level of lignin in the brownish tissue was 65% of that in the normal-colored tissue. The level of total aldehydes from the brownish tissue was one-third of that from the normal tissue, as indicated by the oxidation analysis, and the S/V ratio for the brownish tissue was greater than that for the normal-colored tissue. The reduction in the level of total aldehydes detected by oxidation analysis seem to suggest that a relatively high level of condensed units, which are rich in carbon-carbon linkages, is present in the lignin of the brownish tissue. Monties (1989) indicated that a low proportion of syringyl units in lignin was likely to lead to a condensed lignin polymer. Thus, the low level of total aldehydes detected by oxidation analysis of the brownish tissue might suggest a reduced proportion of syringyl units in the tissue. This hypothesis is supported by the results of Mäule staining of the brownish tissue in the S4 plant. The brownish part was not stained after the Mäule reaction, which can stain syringyl units red (Monties 1989). By contrast, the results of the oxidation analysis of the brownish tissue suggest that the relative amount of syringyl units in the brownish tissue was much higher than that in the whitish tissue. However, this result represents the high proportion of syringyl units in the non-condensed lignin fraction, and not in the condensed lignin fraction of the brownish tissue. Therefore, we surmise that the relative level of syringyl units relative to that of guaiacyl units in the entire lignin might be lower in the brownish tissue. Further analysis is necessary to clarify the heterogeneity of the monomeric composition of condensed and non-condensed lignin fractions in the brownish tissue.

The chemical analysis of the lignin in each plant indicated that the structure of lignin in the transgenic plants with low 4CL activity (S1, S2, S3, S4 and A5) was very different from that in the controls and that in the transgenic plants without the brownish tissue (Table 1). The change in the lignin structure was due to the presence of the brownish tissue, including abnormal lignin, in the plants (Table 2). The levels of lignin in each plant were roughly correlated with the level of 4CL activity. However, the monomeric composition of the lignin in each plant was not so closely related to the level of the activity of 4CL. The fluctuations in the composition among the transgenic plants with low 4CL activity might be attributable to the different proportions of brownish tissue in each plant and/or to the different degrees of lignin biosynthesis in the brownish tissue of each plant. In our experiment, we used non-divided xylem tissue, which included both the brown and the normal-colored tissue, from each transgenic plant as starting material. Thus, the data from our analysis were complicated by the existence of heterogeneous lignin in the brownish and normal-colored tissues in each sample.

Our data suggest that the reduction in 4CL activity in the transgenic plants induced not only a reduction in lignin content but also alterations in the monomeric composition of lignin in the brownish tissue. The monomeric composition varies among different kinds of cell and tissue in plants (Fukushima et al. 1994). The factors that influence the distribution of the different monomers of lignin are not unknown. Generally, it is thought that the activities of ferulic acid 5-hydroxylase and the related \( O \)-methyltransferase (OMT) are important for the control of the monomeric composition of lignins in angiosperms (Whetten and Sederoff 1995). Recently, a hydroxylase and an \( O \)-methyltransferase that can use cinnamoyl-CoA derivatives as specific substrates were isolated and characterized. In particular, the activity of caffeoyl-CoA 3-\( O \)-methyltransferase (CCoAOMT) was detected in several plants, including tobacco (Ye et al. 1994). The expression of CCoAOMT and that of OMT are regulated differently during lignification in different types of cell (Ye and Varner 1995). It is possible that these two \( O \)-methyltransferases participate in the biosynthesis of different types of lignin monomer in various cells and tissues (Ye et al. 1994). If the CCoAOMT is involved in the channelling of the synthesis of different types of monolignol, the CoA-esterification of cinnamic acid derivatives might also be important for the determination of the monomeric composition. Furthermore, isoenzymes of 4CL with different substrate-specificities exist in several plants and these isoenzymes are active in different types of cell and tissue (Knobloch and Hahlbrock 1975, Grand et al. 1983, Wilkinson et al. 1992). Thus, it is also possible that specific isoenzymes of 4CL play important roles in the synthesis of different types of lignin monomer in the plants. These observations and our present data suggest that 4CL(s) plays important roles in the determination of the monomeric composition of lignin in the tobacco cell wall.

Modification of the structure of lignin in transgenic plants with altered expression of the gene for 4CL is a useful method for investigating the function of 4CL in lignin biosynthesis. Further detailed chemical and spectrometric analysis of the lignin in the brownish tissues should provide more information about the roles of 4CL in
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lignin biosynthesis.

References


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