Modifications in Lignin and Accumulation of Phenolic Glucosides in Poplar Xylem upon Down-regulation of Caffeoyl-Coenzyme A O-Methyltransferase, an Enzyme Involved in Lignin Biosynthesis*

Caffeoyl-coenzyme A O-methyltransferase (CCoAOMT) methylates, in vitro, caffeoyl-CoA and 5-hydroxyferuloyl-CoA, two possible precursors in monolignol biosynthesis in vivo. To clarify the in vivo role of CCoAOMT in lignin biosynthesis, transgenic poplars with 10% residual CCoAOMT protein levels in the stem xylem were generated. Upon analysis of the xylem, the affected transgenic lines had a 12% reduced Klasson lignin content, an 11% increased syringyl/phyenyl groups.

Received for publication, August 1, 2000
Published, JBC Papers in Press, August 8, 2000, DOI 10.1074/jbc.M006415200

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This paper is available on line at http://www.jbc.org

1 The abbreviations used are: G, guaiacyl; 4CL, 4-coumaric acid:CoA ligase; CaMV, cauliflower mosaic virus; CCoAOMT, caffeoyl-CoA O-methyltransferase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) (1). However, the analysis of transgenic tobacco and poplar with suppressed COMT activity has shown that

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FIG. 1. Biosynthetic pathway for cinnamyl alcohols and production of side product phenolics. Enzymatic reactions involved in the cinnamyl alcohol biosynthesis in poplar are indicated in black. The formation of sinapoyl-CoA and its conversion to sinapaldehyde are indicated in blue; the relevance of these conversions in monolignol biosynthesis is uncertain, as mentioned above. Conversions leading to the synthesis of side product phenolics are indicated in red and are markedly up-regulated when CCoAOMT expression is reduced. The reduced flux from caffeoyl-CoA to feruloyl-CoA, caused by the down-regulated CCoAOMT expression causes an elevated production of caffeic acid that is detoxified to GCA. GVA synthesis is probably up-regulated because of an increased availability of caffeic acid (48). Caffeic acid can be converted to sinapic acid by the sequential activity of COMT/AldOMT, ferulic acid 5-hydroxylase (F5H)/coniferyl aldehyde 5-hydroxylase (CAld5H), and COMT/AldOMT. Because of the lack of 4CL activity toward sinapic acid, it cannot take part in syringyl biosynthesis, and it is detoxified and stored as GSA. p-Hydroxybenzoic acid formation can be mediated by a β-oxidation mechanism (conversion 1) (47) or, more probably, by an NAD-dependent p-hydroxybenzaldehyde dehydrogenase (conversion 2) (52) acting on p-coumaric acid. The formation of sinapyl alcohol from coniferyl alcohol remains ambiguous (5, 13, 14, 17, 27). PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; C3H, coumaric acid 3-hydroxylase; CCoA3H, coumaroyl-CoA 3-hydroxylase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.
COMT is mainly or exclusively implicated in the biosynthesis of S monomers (6–9). Later, it was shown that the methylation of the lignin precursors could also occur at the level of the cinnamoyl-CoA esters by caffeoyl-CoA-3-O-methyltransferase (10), and it was proposed that CCoAOMT was predominantly involved in the biosynthesis of G units. Transgenic tobacco plants down-regulated for CCoAOMT, however, had a reduced lignin amount, indicating that CCoAOMT regulates both G and S unit production (11). Subsequently, in vitro activity assays have shown that COMT is also active at the cinnamoyl-CoA, cinnamaldehyde, and cinnamyl alcohol levels (12–14) and that 5-hydroxyconiferaldehyde competitively inhibits the methylation of caffeic acid to ferulic acid and of 5-hydroxyferulic acid to sinapic acid (15). Additionally, coniferyl alcohol has been demonstrated to inhibit the conversion of ferulic acid to 5-hydroxyferulic acid in a noncompetitive manner (13). Together, these data, obtained from in vitro enzymatic assays, have suggested that G and S units are biosynthesized via caffeoyl-CoA and feruloyl-CoA to coniferaldehyde and then diverted to S units via 5-hydroxyconiferaldehyde and sinapaldehyde. Furthermore, these data also suggested that the pathway from caffeic acid to sinapic acid is not used for lignin precursor biosynthesis. Given our sparse knowledge on the cell-specific localization of these enzymes in the different cell types of wood (16), the actual pool sizes of pathway intermediates in the different cell types, their effects on the kinetics of the monolignol biosynthesis enzymes (13, 15, 17), and the expression levels of the corresponding genes (18), the metabolic outcome of altering one particular step of the pathway in vivo still remains largely unpredictable.

Here, we have analyzed the metabolic consequences of reducing CCoAOMT production in transgenic poplar. We show that a down-regulation of CCoAOMT by 90% reduces the Klasson lignin amount by 12%, increases the S/G composition ratio by 11%, and increases the content of p-hydroxybenzoate in the lignin polymer. In addition, we demonstrate by HPLC (along with MS and 1H NMR) that Oβ-β-glucopyranosyl-caffeic acid (GCA), Oβ-β-glucopyranosylvanillic acid (GVA), and Oβ-β-glucopyranosyl-sinapic acid (GSA) accumulate in the methanol-soluble phenolics fraction of the transgenic wood. These data indicate that when the flux toward monolignol biosynthesis is reduced in vivo by down-regulating CCoAOMT, the pathway from caffeic acid to sinapic acid is followed. Furthermore, because the lignin amount is reduced and GSA accumulates up to 10% of the total soluble phenolics, our data indicate that sinapic acid is not used as a primary lignin precursor.

**EXPERIMENTAL PROCEDURES**

**Construction of Sense and Antisense Vectors**

All DNA recombinant techniques were performed essentially as described (19). The binary vector pBIH17 (20) was derived from pBIH10 (21), in which the neomycin phosphotransferase gene is replaced by the hygromycin phosphotransferase gene. This binary vector was used to transform poplar with four different constructs harboring DNA sequences placed downstream from the cauliflower mosaic virus (CaMV) 35S RNA promoter (P70) (22).

**Construction of a 5′ Antisense Construct**

A 5′ CCoAOMT PCR fragment was generated using primers 5′-AGAAGGGATCCACAATATTGGCC-3′ and 5′-GGCATCAGTTGACAGCTTACAAAGC-3′ (priming at positions –17 and +240 relative to the ATG start codon of CCoAOMT-1B, respectively), hereby introducing a BamHI site at the 5′-end of the coding region. After BamHI and HindIII digestion, the fragment was cloned in the BamHI/HindIII sites of vector pLB19. pLB19 is a PUC19-derived vector containing the P70 and the CaMV terminator sequence, kindly provided by L. Jouanin (INRA Versailles, France). Subsequently, the KpnI/XhoI P70-CCoAOMT fragment was cloned into the KpnI/XhoI sites of pBIBHYG, resulting in vector p5′-asCCoAOMT.

**Construction of an Internal Antisense Construct**

A 571-bp HindIII/BamHI CCoAOMT fragment was generated by digesting pccoaomt-1b. This fragment was cloned into the HindIII/BamHI-digested pOMTmutpBSrev, kindly provided by U. Matern (Institute of Pharmaceutical Biology, Marburg, Germany), resulting in plasmid pCCmut. pCCmut was digested with KpnI and BamHI, and the CCoAOMT fragment was ligated in the KpnI/BamHI-digested pUC19. This construct was digested with EcoRI and BamHI and ligated in EcoRI/BamHI-digested plpB19, resulting in plpBR19–571. The 1.9-kb pair KpnI/XhoI P70-CCoAOMT fragment, derived by partial digestion of plpBR19-571, was ligated in the KpnI/XhoI-digested pBIBHYG vector, resulting in vector p5′N-asCCoAOMT.

**Construction of the Full-length Antisense Construct**

The 5′ CC0AOMT PCR fragment, obtained as described above, was cloned in pGEM-T (Promega, Madison, WI). This construct was digested with HindIII/PstI, and the CCoAOMT fragment was transferred into the HindIII/PstI site of pccoaomt-1b. The resulting plasmid was digested with BamHI and the fragment was cloned in the BamHI site of plpBR19 in the antisense orientation. This construct was digested with KpnI/XhoI and the P70-CCoAOMT fragment was ligated into KpnI/XhoI-digested pBIBHYG, resulting in vector p5N-asCCoAOMT.

**Construction of the Full-length Sense Construct**

Plasmid pccoaomt-1b was digested with PstI and KpnI, and the CCoAOMT fragment was ligated in the PstI/KpnI site of pUC19. This plasmid was digested with EcoRI, and the CCoAOMT fragment was ligated in the EcoRI site of pLB19 in the sense orientation. This plasmid was partially digested with KpnI and XhoI, and the 2.5-kb P70-CCoAOMT fragment was ligated into the KpnI/XhoI site of pBIBHYG, resulting in vector p5cCoAOMT.

**Polar Transformation and Regeneration**

Wild-type *Populus tremula* × *Populus alba* (INRA, number 717 1-B4) was transformed with all four constructs. The transformation via *Agrobacterium tumefaciens* and regeneration procedures were essentially as described (23). One shoot was isolated from each stem explant to ensure that the transgenic lines were derived from independent transformation events.

**Production of Antibodies against Poplar CCoAOMT**

The PCR product described above was digested with BamHI/HindIII and ligated together with a HindIII/XhoI fragment from the CCoAOMT-1B cDNA into the BamHI/XhoI site of the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech), resulting in vector pGEX-CCoAOMT. CCoAOMT was expressed as a glutathione S-transferase-CCoAOMT fusion protein. Fusion protein expression was induced with 0.1 mM isopropyl β-d-thiogalactoside after 4 h of bacterial growth, according to the Bulk and RediPack glutathione S-transferase purification protocol (Amersham Pharmacia Biotech). The fusion protein was purified using the glutathione-Sepharose 4B RediPack columns (Amersham Pharmacia Biotech). The fusion protein was cleaved with thrombin. The glutathione S-transferase and CCoAOMT protein fragments were then separated by SDS-polyacrylamide gel electrophoresis. Approximately 200 mg of CCoAOMT protein, cut out from the polyacrylamide gel, was used for immunization of a rabbit according to standard procedures at the Laboratoire d’Hormonologie Animale (Marloie, Belgium).

**Immunoblot Analysis**

Samples were taken from the base of the stem of 6-month-old greenhouse-grown poplars. Xylem tissue was obtained by scraping a 5-cm debarked stem with a scalpel. This tissue was subsequently homogenized in liquid N2. Pink-red-colored and whitish xylem from line asc01 were separated with a scalpel. Proteins were extracted with 60 ml Tris (pH 6.5) containing 0.03% (w/v) dithiothreitol and 0.5% (w/v) polyvinylpolypyrrolidone and the protease inhibitor Complete™ Mini (Roche Diagnostics, Brussels, Belgium). SDS-polyacrylamide gel electrophoresis was carried out according to standard procedures. Proteins were blotted on Hybond C super membranes (Amersham Pharmacia Biotech). Immunodetection was performed according to standard laboratory methods, using an anti-rabbit IgG alkaline phosphatase.
conjugate (Roche Diagnostics) and the color development reagents 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Duchefa, Haarlem, The Netherlands) and p-nitro blue tetrazolium chloride (Duchefa). The titer of the CCoAOmT antibodies and anti-rabbit IgG secondary antibodies in the immunodetection procedure was 1:1500 and 1:2500, respectively. Alternatively, CCoAOmT protein amounts were quantified by measuring the hybridization intensities with a PhosphorImager 445Si (Amer sham Pharmac ia Biotech), after using 25S-labeled anti-rabbit IgG (Amer sham Pharmac ia Biotech) (dilution 1:2500) as secondary antibodies.

**Histology of Stem Sections**

Stem cross-sections (60 µm thick) were made from the base of 6-month-old greenhouse-grown poplars with a sledge microtome (Jung, Heidelberg, Germany). Mäule and Wiesner staining were performed essentially as described (6). Autofluorescence of stem cross-sections was observed with an Axiostar microscope (Zeiss, Jena, Germany) equipped with a fluorescence device using a filter combination of 450–490 nm excitation and 515–565 nm emission in conjunction with a 100-watt mercury burner.

**Lignin Analyses**

Klason lignin analysis, thiocicoidysis, and analysis of low-molecular-weight phenolics released by mild alkaline hydrolysis were essentially as described (9). For lignin isolation and subsequent NMR analysis, three to four stems from each line of 7-month-old poplars were pooled. Before the poplar stem walls were isolated, the stems were split into 1–2-cm pieces and ground to pass a 1.0-mm screen of a cyclone mill (Udy Corp., Fort Collins, CO). Lignin isolations were essentially as described previously (24, 25). Soluble phenolics, carbohydrates, and other components were removed by successive extractions with water, methanol, acetone, and chloroform. Most of the colored material was removed by the water and methanol extraction cycles. The isolated cell wall material was ball-milled, suspended in 50 mM acetate buffer (pH 5.0), and treated with 30 mg of cellulase (Cellulysin; Calbiochem-Novabiochem, Bad Soden, Germany) per 1 g of ball-milled material. Cell wall digestions ran for 8 days with fresh enzyme and buffer being added after 2.5 and 5 days of incubation at 30 °C. The resulting lignin polysaccharide complex was subjected to fractionation in 96:4 (v/v) dioxane/water, of the total cell wall material, and 73, 72, and 69% of the total lignins for wild-type, sccoaomt-16, and sccoaomt-29, respectively. Isolated lignins (200 mg of each) were acetylated overnight with acetic anhydride/pyridine. The solvents were removed by coevaporation with 95% ethanol and traces of ethanol by coevaporation with acetone. The acetylated lignins were then extracted into CHCl3 and washed with aqueous nol and traces of ethanol by coevaporation with acetone. The acetylated lignins were then extracted into CHCl3 and washed with aqueous EDTA (6 mM, pH 8.0) to remove trace metal contaminants. Proton- and carbon-13 NMR spectra were taken on a Bruker DRX-360 instrument (Bruker, Karlsruhe, Germany) fitted with a 5-mm H/H broadband gradient probe with inverse geometry (proton coils closest to the sample). Acetylated lignins (EDTA-washed, 100 mg) were dissolved in 0.4 mL of acetone-d6, unacetylated lignins (not ET DAwashed, 60 mg) were dissolved in acetone-d6 (0.35 mL) and deuterium oxide (D2O) (0.05 mL). The central acetone solvent peak was used as internal reference (δC 29.80). Peaks for unacetylated and acetylated p-hydroxybenzoic acid esters were assigned by comparison with data from methyl or ethyl p-hydroxybenzoates in the NMR Database of Lignin and Cell Wall Model Compounds, entries 17, 18, and 94.

**Analysis of Soluble Phenolics**

Xylem tissue (approximately 100 mg) was obtained by scraping the debarked stem of 4-month-old greenhouse-grown poplars with a scalpel, homogenized in liquid N2, and extracted with 5 mL of methanol. The supernatant was dried, and samples were extracted with cyclohexane/water containing 0.1% trifluoroacetic acid (1:1, v/v). The phenolic compounds in the aqueous phase were separated and quantified by reversed-phase HPLC using a Luna C18 column (Phenomenex, Torrance, CA) (5 μm, 250 × 4.6 mm) with gradient elution by an HPLC Waters 625LC system (Waters, Milford, MA), employing an increasing gradient of methanol-acetonitrile (25:75, v/v) acidified with 0.1% trifluoroacetic acid (solvent B) in 0.1% aqueous trifluoroacetic acid (solvent A). The following gradient elution conditions were used: time = 0 min/0% B; time = 25 min/60% B; time = 27 min/100% B; flow rate = 1.5 mL/min; temperature 40 °C; injection loop = 20 μL. All solvents were of HPLC grade purity. Absorbance spectra were recorded with a Waters 996 diode array detector by scanning from 200 to 450 nm. The peak height was quantified at the maximum absorbance value between 230 and 450 nm. Data collection and integration was done with the Millennium software (Waters).

**Statistical Analyses**

Pearson product-moment correlation analyses were performed using GraphPad Prism version 2.01. Calculation of the correlation coefficient (r²) was based on peak concentration values obtained from two harvests (July 1998 and May 1999), containing several ramets for lines sccoaomt-16 and sccoaomt-29 (12 plants in total).

**Mass Spectrometry**

The molecular weight and the daughter ion spectra of the accumulating glycosides were determined using a nanoelectrospray ionization source operated in the negative mode on a hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom) using borosilicate capillaries (Protana, Odense, Denmark). The conditions were as follows: solvent = acetonitrile/water/formic acid (1:99.95:0.05); concentration loaded = 5 pmol/μL; electrospray ionization probe voltage = −1.4 kV; cone voltage = 40 V; source temperature = 40 °C; collision energy = −30 eV; collision gas = argon; collision pressure = 1 × 10⁻³ torr. The collision-induced dissociation was performed on the [M−H]⁺ ions. MS data were acquired in the m/z range from 100 to 700 Da, and MS/MS data were acquired in the range from 50 to 450 Da.

**1H NMR Spectroscopy**

Approximately 250 μg of the glycosides were dissolved in 0.7 mL of D2O (Aldrich). The spectra were recorded with a UNITY-500 spectrometer (Varian Analytical Instruments, Palo Alto, CA). The experiments were performed with a 5-mm inverse detection probe equipped with a liquid nitrogen-cooled gradient coil. The standard Varian software Vnmr version 5.3b was used throughout. The spectra were run at 27 °C and referenced to the residual water peak at δ 4.76. The 1H-90° pulses were 5.5 μs. Presaturation was chosen to suppress the water peak by using a saturation delay of 2.5 s at a power of 1 dB. Data were analyzed with the software package from Advanced Chemistry Development Inc. (Toronto, Canada). Nuclear Overhauser effect (NOE) difference spectroscopy was performed essentially as described (28).

**Structural Assignment of Compounds X, D, and E**

In MS/MS, compound X had a [M−H]− molecular ion at m/z 329.3 and fragment ions at m/z 108.1, 123.2, 152.2, and 167.2; compound D had a [M−H]− ion at m/z 341.3 and fragment ions at m/z 135.2 and 179.2; and compound E had a [M−H]− ion at m/z 385.3 and fragment ions at m/z 149.2, 164.2, 208.2, and 223.2. All three compounds showed a loss of 162 Da, suggesting an O-glycosidic bond. Molecular masses of 167.2, 179.2, and 223.2 Da gave evidence that the respective aglycones of compounds X, D, and E were isovanillic or vanillic, caffeic, and sinapic acid analogues, respectively. The assignment of the isomeric form was derived from 1H NMR (Table I).

**Structure Assignment of the Glycosylated Vanillic Acid Analogue (Compound X)—The resonances for H-6A′ and H-6B′ were typical for the resonances of an exocyclic CH2OH group in glucopyranose. These data and the data in Table I demonstrated that the sugar residue was a β-D-glucopyranosyl residue (29).**

The NMR data showed three resonances in the region of the aromatic protons (two doublets: δ 7.194 with J = 8.4 Hz and δ 7.577 with J = 1.7 Hz; one doublet of doublets: δ 7.510 with J = 8.4 and 1.7 Hz), which is typical for the substitution pattern of a phenyl ring with two vicinal protons and one isolated proton meta-positioned to one of these. Although two possibilities could be considered, GVA or O-b-D-glucopyranosyl-isonicotinic acid, extensive NOE difference spectroscopy experiments clearly pointed to the former.

**Structure Assignment of the Glycosylated Caffeic Acid Analogue (Compound D)—The nature of the sugar was derived from the 1H NMR data and led to the identification of a β-D-glucopyranosyl residue.**

The NOE pattern of the sugar group was derived from the splitting of the three resonances. Two possibilities for compound D could be considered: Oβ-D-glucopyranosyl-caffeic acid or GCA. The latter structure was confirmed by NOE difference spectroscopy experiments.

The 1H NMR spectrum of compound D was a mixture of the trans and cis isomers of which the parameters were assigned from the magnitude of the vicinal coupling constant of the double bond (16.1 Hz for trans,

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2 The NMR Database of Lignin and Cell Wall Model Compounds (S. A. Ralph, J. Ralph, W. L. Landucci, and L. L. Landucci) is available on the World Wide Web.
was found. The magnitude of these coupling constants pointed to a
trans
was a mixture of
all peak heights in the chromatogram. A peak height,
absorbance value between 230 and 450 nm and expressed as percentage
formed as described above. Quantification was based on the maximum
acids: caffeic acid, ferulic acid, 5-hydroxyferulic acid, or sinapic acid.

Pascual’s tables (30).

12.5 Hz for cis) as well as from the chemical shifts as calculated from
Pascual’s tables (30).

Structure Assignment of the Glycosylated Sinamic Acid Analogue
(Compound E)—At δ 5.038, a disturbed doublet for the anomeric proton
was found. The magnitude of these coupling constants pointed to a
β-D form of the gluco-configuration (29). Integration of the 1H NMR showed
two O-methyl substituents on the aromatic system, collapsing at δ
3.867. A singlet at δ 7.295(d) that integrated for two protons was found in
H-5 (14.5%) irrN H-1
H-2 (12.2%) irrN H-6 (5.6%) irrN H-3
H-4 (23.1%) irrN H-3
H-1
H-4
H-5
H-6
OCH3(8.9%)

Feeding Experiments

Internodes of approximately 15-cm length with one leaf were cut
from 3-month-old, greenhouse-grown wild-type poplars. These
internodes were put for 16 h overnight into Falcon tubes containing 10 ml of
either distilled water or a 1:1 mix concentration of the following cinnamic
acids: caffeic acid, ferulic acid, 5-hydroxyferulic acid, or sinapic acid.
Xylem extraction and HPLC separation of the metabolites were
performed as described above. Quantification was based on the maximum
absorbance value between 230 and 450 nm and expressed as percentage
peak height, i.e. the height of the peak of interest relative to the sum
of all peak heights in the chromatogram.

RESULTS

Identification of Transgenic Poplars with Reduced CCoAOMT
Production—We have previously shown that CCoAOMT is en-
coded by two genes in poplar (31). Both genes are expressed at
similar levels in poplar stems. Because the cDNAs that corre-
spond to both genes share 92% nucleotide identity in the coding
region, one of the cDNAs (CCoAOMT-1B) was chosen to make
sense and antisense constructs to down-regulate the expression
of both CCoAOMT genes. Three CCoAOMT cDNA fragments
containing a 235-bp 5′-coding region, 571-bp internal coding
sequence, or the complete 744-bp coding sequence of
CCoAOMT-1B were cloned in the antisense orientation down-
stream from the constitutive, double CaMV 35S promoter (P70)
in a binary vector. In addition, the entire CCoAOMT-1B se-
quenue was placed behind the P70 promoter in sense orientation.
Stem explants from poplar (P. tremula × P. alba clone 717
1-B4) were transformed with A. tumefaciens harboring the
binary vectors, and 14–20 independent transformants were
obtained for each construct.

The transgenic poplars were screened by protein gel blot
analysis for a reduced or increased CCoAOMT protein level in
the stem xylem. This analysis was chosen to screen the trans-
genic lines because bispecific COMT has been shown to
methylate cinnamoyl-CoA esters too (12, 32) and, consequently,
to interfere with the classical CCoAOMT activity assay (33). The
antibodies used were generated against a recombinant
poplar CCoAOMT protein and reacted with the two poplar
CCoAOMT isoforms (34).

Of all transgenic plants analyzed, only those transformed
with a sense construct had a strongly reduced protein amount.
None of the transgenic lines harboring the sense construct
showed an increased CCoAOMT level in the stem xylem. Out of
14 transgenic lines transformed with the sense construct, the
first screening yielded four lines with reduced CCoAOMT pro-

1H NMR data for products X, D, and E

| Chemical shifts (δ) |
|---------------------|
| Product X | trans | cis |
| Product D | trans | cis |
| Product E | trans | cis |

| Couplings (Hz) | Compound X | Compound D (cis-isomer) | Compound E (trans-isomer) |
|----------------|-------------|-------------------------|--------------------------|
| 3 J αβ         | irrN H-1 \( \rightarrow \) H-5 (14.5%) | irrN H-1 \( \rightarrow \) H-2 (12.2%) + H-3 (23.1%) | irrN H-1 \( \rightarrow \) no NOE |
| 4 J(2,6)       | 1.7         | 2.2                     | 2.1                      |
| 4 J(5,6)       | 8.4         | 8.2                     | 8.4                      |
| 3 J(1,2) \( 3 \) | 7.5         | 7.7                     | 7.9 \( a \)               |
| 3 J(2,3) \( 3 \) | —           | —                       | —                       |
| 3 J(3′,4′)     | —9 \( a \)   | —10                     | —8 \( a \)               |
| 3 J(4′,5′)     | —9 \( a \)   | —10                     | —8 \( a \)               |
| 3 J(5′,6A′)    | —2          | 2.4                     | 2.1                      |
| 3 J(5′,6B′)    | 5.4         | 5.7                     | 5.3                      |
| 3 J(6′A,6B′)   | —12.3       | —12.4                   | —12.4                    |

a Apparent coupling constant.

b —, the patterns of H-1′, H-2′, H-3′, and H-4′ show second-order effect.

c irrN, irradiated nucleus.

d —, responding nucleus (%).

**Accumulation of Phenolic Glucosides**

*3 H. Meyermans, unpublished data.*
Fig. 2. Immunoblot analysis of CCoAOMT protein levels in wild-type and transgenic poplars. Xylem proteins (10 μg) were separated by SDS-polyacrylamide gel electrophoresis (15%) and analyzed by immunoblotting with polyclonal anti-CCoAOMT antiserum at a dilution of 1:1500. Extracts were taken from wild-type (wt) and sense-suppressed lines (sccoaomt-16 and sccoaomt-29) and whitish (w) and pink-red-colored (p-r) xylem parts of line asccoaomt-1.

tein amount. In subsequent harvests, two lines, sccoaomt-16 and sccoaomt-29, were shown to be stably down-regulated (Fig. 2). The amount of residual CCoAOMT protein present in xylem extracts was measured by quantifying the hybridization intensity of the 29-kDa band, corresponding to CCoAOMT, in immunoblot experiments using 35S-labeled secondary antibodies. When the CCoAOMT protein level of wild type was set at 100% (n = 4), the CCoAOMT protein levels of lines sccoaomt-16 and sccoaomt-29 were 10.2 ± 4.0 (n = 3) and 8.4 ± 1.9 (n = 3), respectively.

CCoAOMT Down-regulation Coincides with Altered Xylem Coloration and Enhanced Vessel Cell Wall Fluorescence—After the bark was peeled off, the stem xylem of the transgenic lines with a reduced CCoAOMT protein level displayed a pink-red coloration (Fig. 3D). Line asccoaomt-1, harboring the full-length antisense construct, had a mottled pink-red coloration (data not shown). A strong reduction of the CCoAOMT amount was observed in the pink-red-colored xylem region, whereas a normal CCoAOMT amount was detected in neighboring whitish xylem, showing the direct relation between color change and reduced CCoAOMT protein level (Fig. 2). A similar variable pattern of gene silencing has been observed in transgenic poplars transformed with a sense COMT construct (8). The molecular mechanisms responsible for these variable gene silencing effects are not fully understood yet (35, 36).

Microtome sections of the pink-red-colored stem from lines sccoaomt-16 and sccoaomt-29 showed an intense fluorescence upon UV microscopy, which was not observed in sections of wild-type poplar (Fig. 3, A–C). This intense fluorescence was also seen in the pink-red-colored xylem from line asccoaomt-1 and was absent from the neighboring whitish xylem. The intense fluorescence was predominantly found in the cell walls of mature vessel cells, whereas the cell walls of the vessel cells close to the cambium did not show this fluorescence.

The overall plant morphology of the transgenic lines with a reduced CCoAOMT activity was indistinguishable from wild type. By genomic DNA gel blot, lines asccoaomt-1, sccoaomt-16, and sccoaomt-29 were found to be independent transformants (data not shown).

Analysis of Lignin and Associated Low Molecular Weight Phenolics—To determine the effect of CCoAOMT down-regulation on the quantity and quality of lignin, the Klason method and thioacidolysis were used along with NMR of isolated lignin fractions. The Klasont method quantifies lignin by weighting the residue left after hydrolysis of the cell wall polysaccharides with sulfuric acid. Thioacidolysis and subsequent gas chromatography analysis identify monomers that are released by selectively breaking the intermonomeric β-O-4 bonds, the major interunit linkages in lignin (37). An additional analysis of the released dimeric structures allows the determination of different types of carbon-carbon and diphenyl ether bonds between monomers (9). 13C NMR of isolated lignin fractions provides compositional and structural fingerprints for comparison; detailed studies can identify the nature of individual components (38).

Klason lignin analysis and thioacidolysis were performed on 6-month-old greenhouse-grown wild-type poplars and on lines sccoaomt-16 and sccoaomt-29, harvested in November 1997 and August 1998 (Table II). The lignin analyses showed that the amount of lignin in the stem varied according to the period at which the plants were harvested, as already observed for poplar (7). Overall, a 12% reduction in Klason lignin content was observed in the down-regulated lines. In addition, an increase of 11% in the S/G ratio of β-O-4-linked monomers was found. The type of chemical bonds between monomers was not altered; no difference was observed in the relative frequency of monomers involved in β-O-4 bonds, as given by the S + G/Klason values, nor was there any change in the relative frequency of different types of condensed bonds. The thioacidolysis procedure applied to permethylated samples allows the determination of the relative amount of monomers that have free phenolic end groups (39). Compared with wild-type lignin, the relative amount of free phenolic end groups on G or S monomers did not differ significantly in lignin of poplars from lines sccoaomt-16 and sccoaomt-29 (data not shown).

13C NMR analysis of lignin fractions obtained from 7-month-old poplars indicated that lignin from lines sccoaomt-16 and sccoaomt-29 had only minor structural variations compared with lignin from wild type (Fig. 4). The core lignin was almost indistinguishable, but the lignin-attached p-hydroxybenzoate component was more prominent in the CCoAOMT-reduced lines. Because these lignins are essentially free of carbohydrates, the p-hydroxybenzoate groups are attached to lignin, assumedly at the γ-side chain positions. Like p-coumarates on grass lignin (24), the p-hydroxybenzoates have free phenolic end groups as shown by the chemical shifts of all of the structures’ carbons that move in a predictable way when spectra of acetylated lignins (Fig. 4, A and B) are compared with those from unacetylated lignins (Fig. 4, C and D); see compounds 17 (methyl 4-acetoxybenzoate), 18 (methyl 4-hydroxybenzoate), and 94 (ethyl 4-hydroxybenzoate) in the NMR Database of Lignin and Cell Wall Model Compounds.

Alkaline hydrolysis of extract-free wood followed by gas chromatography-MS analysis was performed to recover hydroxybenzaldehydes and hydroxybenzoic acids, associated with lignified cell walls (9, 40). The yield of vanillin or syringaldehyde in the transgenic poplars did not differ compared with wild type. However, in agreement with the lignin characterization by 13C NMR, the yield of p-hydroxybenzoic acid, calculated on the basis of lignin content, increased 2-fold in both transgenic lines as compared with wild type (Table II).

Cross-sections of the stem were taken, but neither the Mäule reaction, which stains S units red, nor the Wiesner reaction, which is indicative for cinnamaldehydes (41), revealed any differences between the wild-type poplars and the lines with reduced CCoAOMT expression (data not shown).

Taken together, these data suggest that a reduced CCoAOMT production leads to a reduced synthesis of both G and S units. Whereas the type of interunit bonds and the amount of free phenolic groups on monomers were not affected, a slight increase in the S/G ratio in the noncondensed lignin fraction was detected. Except for an increase in associated p-hydroxybenzoate, no differences were observed in lignin-as-
associated phenolics in the CCoAOMT-reduced lines compared with wild type.

Changes in the Accumulation of Soluble Phenolics in Wood of Transgenic Poplars—The lignin analyses described above suggest that a significant fraction of the phenylpropanoids that can potentially be produced are not incorporated into the lignin polymer. Complementary to the analysis of lignin composition, we compared the phenolic metabolites present in the methanol-extractable fraction of xylem cells of wild-type and lines sccoaomt-16 and sccoaomt-29 by HPLC (Fig. 5). Three peaks (X, D, and E) were found to be more abundant in extracts of the CCoAOMT-down-regulated lines. Remarkably, the HPLC fractions containing compounds D and E were fluorescent, whereas no other HPLC fraction, derived either from wild type or from the transgenics, showed this fluorescence.

The absorbance spectra (Fig. 6) of compound X (\(\lambda_{\text{max}} = 253.5\) and 291.3 nm) and compound D (\(\lambda_{\text{max}} = 314.9\) nm) indicated X to be a dihydroxybenzoic acid analogue and D a cinnamic acid analogue, respectively. No structural assumptions could be made for compound E (\(\lambda_{\text{max}} = 299.5\) nm). Mild acid hydrolysis of the isolated compounds resulted in more hydrophobic compounds: x, d, and e (Fig. 6). The retention time and spectra of x, d, and e were consistent with those of isovanillic or vanillic acid (\(\lambda_{\text{max}} = 260.6\) and 292.4 nm), caffeic acid (\(\lambda_{\text{max}} = 241.7\) and 324.4 nm), and sinapic acid (\(\lambda_{\text{max}} = 324.4\) nm) analogues, respectively. To elucidate the structure of these compounds, they were analyzed by electrospray ionization mass spectrometry (Fig. 7) and \(^1\)H NMR and NOE difference spectroscopy (see “Experimental Procedures”). These analyses revealed unambiguously that compound X was GVA, compound D was GCA, and compound E was GSA.

In wild-type poplars, GCA and GSA were only detected in traces amounts, at concentrations below 0.05 mg/g. In contrast, the concentration of GCA and GSA in the wood of CCoAOMT-down-regulated poplars was 1–2.5 mg/g and 4–20 mg/g, respectively. The concentration of GVA was 0.2–0.4 mg/g in wild-type wood and increased to 1–2.5 mg/g in the transgenic wood. Fig. 8 shows the relative concentrations of GCA, GVA, and GSA observed in ramets of the transgenic lines. Pearson product-moment correlation analysis showed a correlation coefficient (\(r^2\)) of 0.71 (\(p = 0.0006\), significance level \(\alpha = 0.05\)) between the concentrations of GCA and GVA in the transgenic

**TABLE II**

Lignin characteristics and analysis of low molecular weight phenolics associated to lignin from wild-type and transgenic poplars with reduced CCoAOMT expression

|                   | November 1997 | August 1998 |
|-------------------|---------------|-------------|
|                   | Wild type     | sccoaomt-16 | sccoaomt-29 |
| Klasson lignin\(a\) | 19.90 ± 0.85  | 17.56 ± 0.42\(b\) | 17.54 ± 0.27\(b\) |
| Weight (%)        | 17.78 ± 0.57  | 15.52 ± 0.56\(b\) | 16.85 ± 0.74 |
| \(\beta-O-4\) interunit linkage\(c\) | 1.77 ± 0.07  | 1.97 ± 0.01\(b\) | 1.91 ± 0.11 |
| S/G (%)           | 56 ± 1        | 51 ± 4      | 52 ± 2\(c\) |
| \((S+G)/Klasson\) | 2137 ± 30     | 2037 ± 233  | 2077 ± 155 |
| Condensed bond type (%)\(d\) |                      |                              |
| \(\beta-1\)       | 30            | 33          | 34          |
| \(\beta-\beta\)   | 27            | 23          | 21          |
| \(\beta-5\)       | 25            | 23          | 25          |
| \(4-O-5\)         | 9             | 10          | 9           |
| \(5-5\)           | 9             | 11          | 11          |
| p-Hydroxybenzoeic acid\(e\) |            |              |
| Yield (\(\mu\)mol/g Klasson lignin) | 48 ± 11     | 105 ± 14\(b\) | 84 ± 3\(b\) |

\(a\) Klasson lignin content was calculated as weight percentage of the extractive free wood.

\(b\) Different from control.

\(c\) Analysis of the main lignin-derived monomers obtained by thioacidolysis: S/G ratio, percentage of monomers only involved in \(\beta-O-4\) bonds, and \((S+G)/Klasson\) lignin ratio (\(\mu\)mol/g Klasson lignin).

\(d\) Relative frequencies of the main dimers obtained by thioacidolysis, representing the lignin-condensed interunit linkages.

\(e\) Release by alkaline hydrolysis of extractive free poplar wood.

Transgenic Poplars—The lignin analyses described above suggested that a significant fraction of the phenylpropanoids that can potentially be produced are not incorporated into the lignin polymer. Complementary to the analysis of lignin composition, we compared the phenolic metabolites present in the methanol-extractable fraction of xylem cells of wild-type and lines sccoaomt-16 and sccoaomt-29 by HPLC (Fig. 5). Three peaks (X, D, and E) were found to be more abundant in extracts of the CCoAOMT-down-regulated lines. Remarkably, the HPLC fractions containing compounds D and E were fluorescent, whereas no other HPLC fraction, derived either from wild type or from the transgenics, showed this fluorescence.

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In wild-type poplars, GCA and GSA were only detected in trace amounts, at concentrations below 0.05 mg/g of wood. In contrast, the concentration of GCA and GSA in the wood of CCoAOMT-down-regulated poplars was 1–2.5 mg/g and 4–20 mg/g, respectively. The concentration of GVA was 0.2–0.4 mg/g in wild-type wood and increased to 1–2.5 mg/g in the transgenic wood. Fig. 8 shows the relative concentrations of GCA, GVA, and GSA observed in ramets of the transgenic lines. Pearson product-moment correlation analysis showed a correlation coefficient (\(r^2\)) of 0.71 (\(p = 0.0006\), significance level \(\alpha = 0.05\)) between the concentrations of GCA and GVA in the transgenic
In contrast, the $r^2$ value for the relationship between GCA and GSA was 0.36 ($p = 0.0398$), and a nonsignificant $r^2$ value of 0.20 ($p = 0.1460$) was obtained between GVA and GSA. The concentrations for GCA and GVA seemed to depend to a large extent on environmental factors; poplars harvested in July 1998 had a relatively low amount of these compounds, whereas higher concentrations were observed when the same transgenic lines were harvested in May 1999. The accumulation of GSA was especially characteristic of the transgenic trees; it always accounted for more than 10% of the total phenolics in the CCoAOMT-suppressed poplars (Fig. 8).

To investigate whether GCA, GVA, and GSA were derived from free cinnamic acids, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid were fed to young stems of poplar. As shown in Table III, feeding caffeic acid and sinapic acid resulted in the accumulation of GCA and GSA, respectively. Glucosides of ferulic acid or 5-hydroxyferulic acid were not detected. Due to the variable concentrations of GVA in wild-type plants, it could not be conclusively determined from the feeding experiments whether the precursor of GVA was any of the free cinnamic acids.

**DISCUSSION**

**CCoAOMT Is Involved in the Biosynthesis of G and S Monomers**—A down-regulation of CCoAOMT by 90% in poplar xylem suppressed the amount of lignin by 12%. This lignin depletion is caused by a reduced synthesis of both G and S monomers. As determined by thioacidolysis, the noncondensed fraction of the

![Fig. 4. $^{13}$C NMR spectra of isolated lignins.](image)

![Fig. 5. HPLC profiles of soluble phenolics derived from poplar xylem.](image)

![Fig. 6. Absorbance spectra of three soluble xylem phenolics and their aglycones.](image)
lignin had an 11% increased S/G ratio. Recently, Zhong et al. (11) have shown that a down-regulation of CCoAOMT activity by 70% in tobacco resulted in a decrease of 50% in lignin amount. Also in tobacco, a slight increase in the S/G ratio has been observed. The fact that the lignin in the two transgenic species is depleted in both G and S monomers argues against the idea that the methylation step accomplished by CCoAOMT is specific for G monomer synthesis as suggested earlier (7, 10). Indeed, recent in vitro enzymatic assays and feeding experiments have shown that monomethoxylated cinnamyl alcohols and cinnamaldehydes can be converted to S units (13, 17, 31, 42), although the poplar COMT/AldOMT did not methylate 5-hydroxyconiferyl alcohol in vitro (15). These observations imply that methylation of caffeoyl-CoA by CCoAOMT can contribute equally to the synthesis of both G and S units (Fig. 1). Consequently, the reduced synthesis of both G and S units in the transgenic plants can be explained by the reduced flux from caffeoyl-CoA to feruloyl-CoA and does not necessarily involve the methylation of 5-hydroxyferuloyl-CoA, a reaction that is carried out by CCoAOMT in vitro.

**Accumulation of Phenolic Acid Glucosides**—To obtain insight into the flux of metabolites through the monolignol biosynthesis pathway, we have compared the methanol-soluble phenolic fraction from xylem of wild-type plants and transgenic plants down-regulated for CCoAOMT. Reduced CCoAOMT caused a strong accumulation of GVA, GCA, and GSA, of which the latter two were barely detectable in wild-type wood. Their identities were unambiguously established by UV, MS, and $^1$H NMR analysis. Glucosylated forms of hydroxycinnamic acids may occur as esters (where the glucose is attached to the carboxyl group) or as glucosides (where the glucose is attached to the phenolic hydroxyl group). The hydroxycinnamic acid glucose esters have a high group transfer potential and are often found to be intermediates in phenylpropanoid metabolism (43). Hydroxycinnamic acid glucosides have been suggested to arise from a common detoxification mechanism employed in plants against reactive phenylpropanoids (44). GCA has been previously detected after infiltration of poplar leaves with caffeic acid. Also in these studies, it was shown that GCA was specifically glucosylated at the $O_3$ position (45). Experiments with poplar stems indeed showed that GCA accumulates upon feeding with caffeic acid. Similarly, feeding sinapic acid resulted in the accumulation of GSA (Table III). Nevertheless, the corresponding glucosides of ferulic acid and 5-hydroxyferulic acid were not detected upon feeding with ferulic acid and 5-hydroxyferulic acid, respectively. Taken together, our data suggest that down-regulation of CCoAOMT results in a decreased flux of caffeic acid to lignin, leaving a larger pool of caffeic acid to be shunted to GCA and, via the intermediate formation of sinapic acid, to GSA. The variable levels of GVA in the wild-type plants did not allow us to determine whether GVA was derived from any of the free cinnamic acids.

For the biosynthesis of vanillic acid, two biosynthetic pathways have been proposed. Because vanillic acid and ferulic acid have identical substitutions on the aromatic ring, vanillic acid could be derived from feruloyl-CoA by a $\beta$-oxidation mechanism, in analogy with the synthesis of $p$-hydroxybenzoic acid from $p$-coumaroyl-CoA (Fig. 1) (46). This pathway is unlikely because feruloyl-CoA is the product of CCoAOMT and is ex-
expected to accumulate only when it is made through the subsequent action of COMT and 4-coumaric acid:CoA ligase (4CL). If this were the case, the putative transient excess of feruloyl-CoA would probably be shunted into the monolignol rather than into the vanillic acid biosynthesis pathway. In addition, in vitro experiments have shown that in the presence of caffeic acid, ferulic acid is not a good substrate for the poplar 4CL. 4 Our data are in favor of another pathway that is active in Vanilla planifolia and has been proposed by Funk and Brodelius (47); caffeic acid is shunted into the vanillic acid biosynthesis pathway by O-methylation to produce isofeluric acid. Subsequently, O-methylation, oxidative decarboxylation, and demethylation at the p position ultimately result in vanillic acid (47). This pivotal role of caffeic acid as both a lignin and vanillic acid intermediate has been suggested by experiments, in which the addition of a 4CL inhibitor, 3,4-(methylenedioxy)cin- namic acid, to V. planifolia cell suspension cultures resulted in an increase of vanillic acid formation with a simultaneous decrease in the biosynthesis of ligneous material (48).

The accumulation of GCA, GVA, and GSA in the CCoAOMT-down-regulated plants is in agreement with recent data obtained from in vitro enzyme studies. It has been shown that coniferaldehyde inhibits the hydroxylation of ferulic acid to 5-hydroxyferulic acid in a noncompetitive manner and that 5-hydroxyconiferaldehyde competitively inhibits the methylation of caffeic acid and 5-hydroxyferulic acid (13, 15). Thus, when the concentrations of coniferaldehyde and 5-hydroxyco- niferaldehyde are sufficiently high, the pathway from caffeic acid to sinapic acid is not active. Our data suggest that because of the reduced expression of CCoAOMT, the concentrations of coniferaldehyde and 5-hydroxyconiferaldehyde are reduced and, as a consequence, the path from caffeic acid to sinapic acid is used, leading to the accumulation of GCA, GVA, and GSA, which are storage and/or detoxification products of the acids formed.

Sinapic Acid May Not Participate in S Monomer Biosynthesis in Vivo—Whereas the concentrations of GCA and GVA sometimes approached the detection limit in the transgenic plants, GSA concentrations always largely exceeded those found in wild-type poplars. In Brassicaceae, sinapic acid is used for the synthesis of sinapic acid esters (43). However, the produced sinapic acid in the CCoAOMT-down-regulated poplars is converted into its O^4\-acylated units in the lignin (38, 54). Sinapic acid is most strongly derived from sinapic acid and accumulates up to 10% of the soluble phenolics; this result, together with the observation of a reduced lignin amount in the CCoAOMT-down-regulated plants, however, indicate that endogenously produced sinapic acid does not play a major role in the conversion of sinapic acid toward S unit synthesis in vivo.

Down-regulation of CCoAOMT Results in an Increased Incorporation of p-Hydroxybenzoate—Our ^13^C NMR and alkaline hydrolysis experiments have shown the increased incorporation of p-hydroxybenzoate in lignin of CCoAOMT-down-regulated poplars. p-Hydroxybenzoic acid formation is mediated by a \(\beta\)-oxidation mechanism acting on p-coumaroyl-CoA (Fig. 1, conversion 1) (46) or, more probably, by an NAD-dependent p-hydroxybenzaldehyde dehydrogenase (conversion 2) (52) acting on p-coumaric acid. The p-hydroxybenzoate units are attached to lignin units and are assumed to arise from the incorporation of p-hydroxybenzylated monolignols during the lignification process, giving rise to \(\gamma\)-acylated units in the lignin (53). Analogous \(\gamma\)-coumarate units are known to be exclusively attached to lignin side chain \(\gamma\)-positions (24), predominantly on syringyl units (38, 54).

Down-regulation of CCoAOMT Results in Increased Vessel Wall Fluorescence—It remains to be investigated whether the quantitative changes, i.e., the accumulation of the phenolic glucosides, the increased incorporation of p-hydroxybenzoate, and the reduced lignin amount, occur in all cell types or only in the vessels. Promoter-GUS analyses and immunolocalizations have shown that in poplar xylem, CCoAOMT is most strongly expressed in developing vessels and in contact ray cells, whereas it is barely detectable in isolation rays and fibers (16). Consequently, the reduction in CCoAOMT is expected to affect lignin biosynthesis in a cell-specific manner. This cell-specific effect is exemplified by UV microscopy that showed an increased fluorescence specifically in the vessel cell walls. At present, it is still unclear whether the altered vessel cell wall fluorescence is due to the glucosides or to the increased incorporation of p-hydroxybenzoate, and the reduced lignin amount, occur in all cell types or only in the vessels. Promoter-GUS analyses and immunolocalizations have shown that in poplar xylem, CCoAOMT is most strongly expressed in developing vessels and in contact ray cells, whereas it is barely detectable in isolation rays and fibers (16). Consequently, the reduction in CCoAOMT is expected to affect lignin biosynthesis in a cell-specific manner. This cell-specific effect is exemplified by UV microscopy that showed an increased fluorescence specifically in the vessel cell walls.

\begin{table}[h]
\caption{Accumulation of Phenolic Glucosides} \label{Table III}
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Table III} & \textbf{Substrate feeding experiments} & \textbf{No. of individuals} & \textbf{CA} & \textbf{SA} & \textbf{GCA} & \textbf{GSA} \\
\hline
\textbf{WT} & 2 & ND & ND & ND & ND & 0.8 ± 0.3 \\
\textbf{WT + CA} & 4 & 5.88 ± 2.9 & ND & 4.45 ± 1.7 & ND & ND \\
\textbf{WT + SA} & 5 & ND & 7.82 ± 1.8 & ND & ND & 9.76 ± 1.2 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{4} V. Chiang and C. Tsai, personal communication.

\textsuperscript{5} K. Fukushima and K. Yamauchi, personal communication.
count for 10% of the total cell wall volume of wood (56). Although not conclusive, the polymerization of \( p \)-hydroxybenzoic acid to sections from wild-type stems using horseradish peroxidase and \( \text{H}_2\text{O}_2 \) intensified the fluorescence of xylem cell walls (data not shown). Further lignin structural analyses are necessary to clarify unambiguously the nature of the fluorescence.

Similarly, the presumed cell-specific effect of down-regulating CCoAOMT could also explain why only a 12% reduction in total lignin content was observed in the transgenic lines with 90% reduced CCoAOMT protein amount. Because vessel cell walls are richer in G units than fiber cell walls, the preferential effect in vessel cell walls may also be the reason why the S/G lignin composition is slightly higher. If the lower lignin content could be ascribed mainly to the vessels, it remains curious that the overall structure of the cell walls is not affected. In tobacco, a reduction of 50% in Klasson lignin amount by down-regulating cinnamoyl-CoA reductase results in a collapse of cell wall structure (57). In contrast, down-regulation of 4CL in poplar yielded a 45% reduced lignin amount, but without detrimental effect on cell wall structure. In the latter plants, the reduced lignin content is compensated for by an increased cellulose deposition (58). Further experiments are needed to investigate whether an increase in cellulose content also compensates the reduced lignin content in the CCoAOMT-down-regulated poplars.

In conclusion, our data show that when CCoAOMT is down-regulated \( \text{in vivo} \), the flux through the path toward monolignol biosynthesis is reduced, resulting in a lower lignin amount. Concomitantly, the route from caffeic acid to sinapic acid is followed, leading to the accumulation of cinnamic acid glucosides. The observation that the lignin amount is reduced while GSA accumulates to high levels strongly indicates that sinapic acid is not a main precursor for S unit biosynthesis. Our \( \text{in vivo} \) data complement the recently obtained data from \( \text{in vitro} \) enzymatic assays that suggested that the route from caffeic acid to sinapic acid is not involved in lignin biosynthesis (13, 15).

Acknowledgments—We thank Frédéric Legée for the determination of Klasson lignin, Ulrich Matern for providing the OMTmutpBsrev vector, Lise Jouanin for providing the pLB19 vector, Geert Goeminne for technical assistance, Jan Van Doorselaere and Marie Baucher for critical reading of the manuscript, Martine De Cock for help in preparing the manuscript, and technical assistance, Jan Van Doorsselaere and Marie Baucher for critical reading of the manuscript, and Martine De Cock for help in preparing the manuscript.

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