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Structural Alterations of Lignins in Transgenic Poplars with Depressed Cinnamyl Alcohol Dehydrogenase or Caffeic Acid *O*-Methyltransferase Activity Have an Opposite Impact on the Efficiency of Industrial Kraft Pulping[1]

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We evaluated lignin profiles and pulping performances of 2-year-old transgenic poplar (*Populus tremula* × *Populus alba*) lines severely altered in the expression of caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) or cinnamyl alcohol dehydrogenase (CAD). Transgenic poplars with *CAD* or *COMT* antisense constructs showed growth similar to control trees. CAD down-regulated poplars displayed a red coloration mainly in the outer xylem. A 90% lower COMT activity did not change lignin content but dramatically increased the frequency of guaiacyl units and resistant biphenyl linkages in lignin. This alteration severely lowered the efficiency of kraft pulping. The Klason lignin level of *CAD*-transformed poplars was slightly lower than that of the control. Whereas CAD down-regulation did not change the frequency of labile ether bonds or guaiacyl units in lignin, it increased the proportion of syringaldehyde and diarylpropane structures and, more importantly with regard to kraft pulping, of free phenolic groups in lignin. In the most depressed line, ASCAD21, a substantially higher content in free phenolic units facilitated lignin solubilization and fragmentation during kraft pulping. These results point the way to genetic modification of lignin structure to improve wood quality for the pulp industry.

Lignins are wall polymers that are essential for mechanical support, water transport, and disease resistance in higher terrestrial plants. Their removal from wood is the basis of kraft pulping, the major process used for the production of pulp and paper. Because of the insoluble and cross-linked nature of lignins, delignification of cellulosic fibers requires harsh pulping conditions and large amounts of polluting chemicals. Currently, there is considerable interest in lignin genetic engineering as a means of improving the efficiency of the pulping process and lowering its economic and environmental costs.

Wood lignins are composed of S and/or G units linked by a series of ether and carbon-carbon bonds (Adler, 1977). The ether β-*O*-4 linkages (Higuchi, 1990) are both frequent and labile, which makes them the target of the delignification process. In contrast, the carbon-carbon linkages are resistant, especially the biphenyl 5–5 bonds involving the aromatic C-5 position, which is available for interunit linkages only in G units (Adler, 1977). From this rationale, it can be understood why conifer wood lignins essentially made of G units are less susceptible to kraft delignification than deciduous wood lignins comprising both G and S units (Chiang et al., 1988). Tailoring plants more amenable to industrial delignification may be achieved by lowering lignin concentration and/or altering lignin structure to an extent that does not affect plant development and defense.

We recently produced transgenic poplar (*Populus tremula* × *Populus alba*) trees with down-regulated enzymes of the lignin biosynthetic pathway by introducing antisense and sense gene constructs in the plant (Van Doorsselaere et al., 1995; Baucher et al., 1996). The selected target enzymes were the bispecific COMT and CAD. The first analyses carried out on juvenile (3-month-old) poplars revealed a severe reduction of COMT and CAD activities of some of the transgenic lines. Both *COMT*- and *CAD*-transformed lines showed lignin content similar to the control. We failed to detect a substantial alteration of lignin structure in CAD-down-regulated poplar trees, which displayed a striking red coloration of the xylem. However, a noticeable fraction (approximately 5% by weight) of the lignin of the CAD-depressed samples was found to be

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA-*O*-methyltransferase; COMT, caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase; G, guaiacyl; 5-OH G, 5-hydroxyguaiacyl; S, syringyl.

solubilized upon mild alkaline hydrolysis. More important, these samples showed improved performances toward alkaline kraft delignification relative to the control (Baucher et al., 1996). However, the molecular basis of their altered reactivity toward alkaline treatment was not addressed. Similar to the *bm3* maize mutant with depressed COMT activity (Lapierre et al., 1988), COMT-down-regulated poplars displayed a severe alteration of lignin structure, mainly revealed by a decrease in S units (Van Doorsselaere et al., 1995).

In this paper we report the in-depth evaluation of both lignin structure and pulping characteristics of 2-year-old poplars with down-regulated CAD and COMT activity. This evaluation is of fundamental importance to assess the stability of transgene expression with age and to comprehensively evaluate the pulping characteristics of the transgenic lines in relation to specific structural traits of the lignin. The lignin amount of 2-year-old *COMT*-transformed poplars was found to be similar to that of the control, whereas it was slightly reduced for some of the *CAD*-transformed lines. We show that depressed CAD activity does not change the proportions of labile β-*O*-4 bonds and S units. However, we provide the first evidence, to our knowledge, that this transformation substantially increases the frequency of free phenolic units in lignin, an essential trait that could account for the specific reactivity of *CAD*-transformed samples toward alkaline treatment. COMT down-regulation dramatically affects lignin structure: relative to the control, the content in labile β-*O*-4 bonds was lower, whereas the proportion of G units and resistant biphenyl structures was dramatically enhanced. In view of these alterations, the efficiency of kraft pulping toward *CAD*-transformed poplars was improved, especially for the ASCAD21 line showing the highest level in lignin-free phenolic groups. In contrast, *COMT*-transformed poplar lines displayed a higher resistance to kraft delignification, consistent with the reported structural changes of the lignin.

MATERIALS AND METHODS

Plant Material

We used 2-year-old greenhouse-grown or field-grown poplars (*Populus tremula* × *Populus alba*) multiplied from the control line (clone INRA 717-1-B4) from two transgenic lines with depressed COMT activity caused by antisense constructs (ASOMT2B and ASOMT10B) or from three transgenic lines with depressed CAD activity caused by antisense (ASCAD21 and ASCAD52) or sense (SCAD1) constructs. For each line, the main stems of five plants were harvested and debarked. These wood samples were cut into chips for laboratory scale-pulping experiments (see "Pulping Experiments"). Lignin analyses were carried out on dry, extract-free wood, ground to pass a 0.5-mm sieve before solvent extraction (toluene:ethanol, 2:1 [v/v], ethanol, and water).

In addition, a large series of younger plants (greenhouse-grown) were individually sampled to monitor the lignin content and structure as the plant aged and to evaluate the variability of lignin profiles within the same line. A few experiments were performed on plantlets regenerated from shoot nodal explants of control and ASOMT2B poplars.

To obtain a transgenic line underexpressing both CAD and COMT, line ASCAD21 was retransformed with a construct containing the antisense *COMT* gene associated with a gene (*hpt* [h̲ygromycin p̲hosphot̲ransferase]; L. Jouanin, unpublished data) conferring resistance to hygromycin. The transformation protocol reported by Leplé et al. (1992) was used. Hygromycin (20 mg L^{-1}) was added to the culture medium to select the transformed cells. Line ASCAD21 × ASOMT7 showed a reduction of COMT activity in xylem tissues of about 90%, as observed for ASOMT2B and ASOMT10B. Lines ASCAD21 and ASCAD21 × ASOMT7 displayed a similarly reduced CAD activity. Measurements of the COMT activity toward caffeic acid and of the CAD activity in the xylem were as previously reported (Van Doorsselaere et al., 1995; Baucher et al., 1996).

Determination of Lignin Content

The lignin content of the extract-free samples was determined by the Klason method from 300 mg of the sample, according to the standard procedure (Dence, 1992). The Klason lignin content was calculated as weight percentage of the extract-free wood and reported as the average of at least three independent determinations on the same sample.

Analysis by Thioacidolysis

All of the subsequent analyses were done in duplicate. SEs between duplicate analyses were in the 3% to 5% range for the lignin-derived monomers and in the 5% to 10% range for the lignin-derived dimers.

Thioacidolysis reagent was prepared by introducing 2.5 mL of BF_3 etherate (Aldrich) and 10 mL of ethane thiol EtSH (Aldrich) into a 100-mL flask and adjusting the final volume to 100 mL with dioxane (pestipur grade). The extract-free wood (10 mg) was added to 10 mL of reagent and 1 mL of a solution of GC internal standard (docosane, 0.25 mg mL^{-1} in CH_2Cl_2) in a glass tube closed with a Teflon-lined screwcap. Thioacidolysis was performed at 100°C (oil bath) for 4 h. The cooled reaction mixture was diluted with 30 mL of water and the pH was adjusted to 3.0 to 4.0 (aqueous 0.4 M $NaHCO_3$). The reaction mixture was extracted with CH_2Cl_2 (three times with 30 mL). Combined organic extracts were dried over Na_2SO_4 and then evaporated under reduced pressure at 40°C. The final residue was redissolved in approximately 1 mL of CH_2Cl_2 before silylation and GC or GC-MS analyses according to the method of Lapierre et al. (1995).

The determination of lignin-derived dimers was done after desulfurization according to the method of Lapierre et al. (1995). The analyses of lignin-derived monomers released from exhaustively CH_2N_2-methylated samples were run as previously described (Lapierre and Rolando, 1988).

Analysis of Low-M_r Phenolics Released by Mild Alkaline Hydrolysis

The extract-free wood (50 mg) was treated with 20 mL of 2 N aqueous NaOH at 37°C, overnight, with magnetic stirring and under argon. After acidification to pH 2.0 (6 N HCl) and addition of the GC internal standard (0.2 mg of docosane), the low-M_r phenolics were extracted with 90 mL of a $CH_3COOC_2H_5/CH_2Cl_2$ mixture (1:1, v/v). The organic extracts were dried over Na_2SO_4 and evaporated to dryness. The final residue was redissolved in 1 mL of CH_2Cl_2 before silylation. Benzoic acids and aldehydes released by mild alkaline treatment of the wall were quantified by GC-MS of their trimethylsilyl derivatives, by reference to a calibration curve prepared from authentic compounds. SES between duplicate analyses were in the 5% to 10% range.

Wiesner Reaction Performed on Isolated Lignin Fractions

Soluble lignin fractions were isolated from extract-free wood (1-year-old stems from greenhouse-grown poplars) by dioxane:water (9:1, v/v) extraction and after ball milling and cellulase hydrolysis of the sample (Lapierre et al., 1986). The extract was concentrated by rotoevaporation, and the extracted lignin was purified by precipitation into water. The water-insoluble lignin fraction was recovered by centrifugation and freeze-dried. The lignin fractions isolated from control, ASCAD21, ASOMT2B, and ASCAD21 × ASOMT7 samples were contaminated with polysaccharide components to a very low extent, as shown by ^{13}C NMR spectra (D. Robert and C. Lapierre, unpublished results).

The Wiesner reaction was applied to these lignin fractions according to the method of Nakano and Meshitsuka (1992). The molar absorptivity (ϵ; liters per mole per centimeter) of the coniferaldehyde reaction product was determined with pure coniferaldehyde (ϵ at 552 nm = 63,000). This absorptivity was used to calculate, from the A_{552} of the pink solution obtained when the Wiesner reaction was applied to isolated lignins, the equivalent amount of coniferaldehyde per gram of lignin sample. The Wiesner reaction applied to sinapaldehyde led to similar results (ϵ at 563 nm = 89,000), whereas benzaldehyde-condensation products showed weak absorptivity at a lower wavelength (503 nm for the syringaldehyde reaction product).

Pulping Experiments

Two series of pulping experiments were independently run at laboratory scale for the field-grown and greenhouse-grown poplars. This was carried out on 200 g of wood chips put together with the kraft liquor into small, pressurized reactors (field-grown poplars) or into microdigesters (greenhouse-grown poplars, Haato, Vantaa, Finland). The conditions were as follows: 15% to 20% active alkali, 25% sulfidity, liquor-to-wood ratio of 4, temperature raised to 170°C during 90 min and then maintained for 60 min. After cooking the pulp was washed, screened to pass a 0.15-mm sieve to retain uncooked particles, and bleached using an elementary chlorine-free sequence, as previously described, or a totally free chlorine sequence (Biermann, 1993). After the sample was pulped and bleached, several characteristics of the unbleached or bleached pulp were measured according to standard procedures (Valette and de Choudens, 1992; Biermann, 1993): (a) the pulp yield; (b) the κ number, which is an indication of the pulp content in residual lignin; and (c) the degree of polymerization of cellulose, which is a measure of the average chain length of cellulose and is therefore inversely related to cellulose degradation. The mechanical properties and brightness were finally evaluated on paper handsheets according to international standards (Valette and de Choudens, 1992).

RESULTS

COMT or CAD Activity and Phenotype of Transgenic Poplar Trees

COMT and CAD activities were monitored in the xylem of 4- to 18-month-old poplars grown in an open greenhouse. In the growing season, from May to September 1996, and when control lines showed the highest CAD activity, this activity in the lines ASCAD21 and SCAD1 was down-regulated by approximately 70% (Pilate et al., 1997). The reduction in CAD activity was less pronounced in line ASCAD52. In the xylem of *COMT*-transformed poplars and relative to the control line, COMT activity toward caffeic acid was reduced by about 90% to 95% in the single (ASOMT2B and ASOMT10B) and double (ASCAD21 × ASOMT7) transformants.

No phenotypic differences in development or growth were observed between control and transgenic poplars with antisense *CAD* or *COMT* constructs whether grown in the greenhouse or in the field. When the bark was removed during the growing season, wood of *COMT*-transformed poplars displayed a pale-rose coloration, whereas that of the *CAD*-transformed lines appeared red, particularly intense for line ASCAD21. This red coloration was essentially located in the outer part of the xylem. The red, fresh xylem turned brown after drying. This brown resisted solvent extraction. One-year-old ASCAD21 × ASOMT7 double transformants displayed the same coloration pattern as the ASCAD21 trees.

Lignin Content in Transgenic Poplars

The lignin content of transgenic poplars was monitored as the plants aged (Table I). Statistical tests (Student's *t* test) showed that the Klason lignin content of ASCAD52, ASOMT2B, and ASOMT10B trees did not significantly differ from that of the controls. In contrast, the Klason lignin content of ASCAD21 and SCAD1 appeared to be slightly lower.

To evaluate the variations in the lignin level within the same line, a series of 1-year-old poplars grown together in the greenhouse were individually analyzed. Whereas some variations in lignin content could be observed between trees from the same line (up to 10%), this experiment confirmed that ASCAD21 poplars have less Klason lignin

Table I. *Lignin content of extract-free wood from transgenic and control poplars*

Data are means ± SD from 3 to 18 independent measurements performed on the same sample. Student's *t* tests carried out on the whole series showed that ASCAD21 and SCAD1 stems had less lignin than the control.

Age or Growth Environment	ASCAD52	ASCAD21	SCAD1	Control	ASOMT2B	ASOMT10B
month			*% wt*			
4[a]	16.7 ± 0.3	17.4 ± 0.4	17.0 ± 0.05	17.8 ± 0.2	17.1 ± 0.1	16.7 ± 0.2
5[a]	18.8 ± 0.1	18.4 ± 0.1	17.6 ± 0.1	19.2 ± 0.2	19.0 ± 0.1	18.6 ± 0.1
6[a]	18.3 ± 0.2	18.3 ± 0.1	18.0 ± 0.1	19.0 ± 0.1	19.5 ± 0.2	19.3 ± 0.1
7[a]	18.5 ± 0.7	18.1 ± 0.6	17.5 ± 0.2	19.4 ± 0.6	18.1 ± 0.3	18.8 ± 0.3
Greenhouse[b]	19.4 ± 0.3	18.7 ± 0.2	17.7 ± 0.3	19.4 ± 0.2	21.1 ± 0.3	20.3 ± 0.2
Field[b]	18.9 ± 0.1	18.3 ± 0.1	18.5 ± 0.2	19.1 ± 0.1	19.6 ± 0.3	18.5 ± 0.2

[a] One plant per line was grown in an open greenhouse. [b] Five plants per line were gathered for analysis.

than the control. This trend was observed not only in ASCAD21 (Klason lignin = 15.60 ± 0.5, mean value and SD for eight stems individually analyzed) but also in ASCAD21 × ASOMT7 (Klason lignin = 14.5 ± 0.5, nine stems individually analyzed), relative to the control (Klason lignin = 17.2 ± 0.7, five stems individually analyzed).

Analysis of Lignin-Derived Monomers Released by Thioacidolysis

Analysis by thioacidolysis was performed to evaluate the lignin structure in transgenic and control poplars. The key reaction of thioacidolysis (lignin depolymerization using BF_3 etherate in ethane thiol-dioxane) is the cleavage of labile β-*O*-4 ether bonds, which are the major interunit linkages in native lignins, as well as the target of industrial delignification processes. The G and S units exclusively involved in β-*O*-4 linkages specifically give rise to thioethylated G and S monomers. On this basis, the recovery of these diagnostic monomers is a close reflection of the lignin content in β-*O*-4 bonds and, therefore, of the lignin susceptibility toward ether-cleaving processes such as kraft cooking.

The total yield in thioacidolysis main monomers ArCHSEt-CHSEt-CH_2SEt was monitored as a function of poplar age and line (Table II). For the sake of clarity, the data for 4- to 7-month-old poplars are not reported but are strictly consistent with the following comments. Within each line, the lowest yields calculated on the basis of the lignin content were observed in younger trees, which confirms that lignins deposited at the early stage of lignification have fewer β-*O*-4 bonds (Terashima et al., 1993). Whatever the age, lower yields were obtained for ASOMT2B and ASOMT10B lignins, which points to a lower frequency of β-*O*-4 bonds relative to control lignins. In contrast, lignins in *CAD*-transformed poplars showed a thioacidolysis yield similar to control lignins.

The relative importance (percent molar) of S and G monomers was measured to evaluate the distribution of S and G units involved only in β-*O*-4 bonds. The proportion of S units in the lignin of 2-year-old poplars was found to be 2-fold reduced by COMT down-regulation (Table II). In contrast, this proportion remained at a high and stable level (approximately in the 50%–60% range) in the control and *CAD*- transformed lines, the lower level corresponding to younger plants, which is in agreement with previously published data (Terashima et al., 1993).

The first stage of lignification was studied in ASOMT2B plantlets obtained from poplar nodal explants. The decrease in the S units induced by the antisense effect could be observed very early in 4-week-old stems. In contrast, the relative frequencies of the S units in root lignin were similar in ASOMT2B and control plantlets. It is interesting that, when sections from a few-week-old ASOMT2B stems were subjected to the histochemical Wiesner stain specific for lignin, the primary xylem appeared not to be lignified, in contrast to the secondary xylem (data not shown). Taken together, these results suggest that the transformation does not affect lignification in the various plant tissues to the same extent.

Table II. *Analysis of the main lignin-derived monomers obtained by thioacidolysis of extract-free wood from transgenic and control poplars: total yield and relative frequency of S, G, and 5-OH G monomers*

For each line, measurements were done from one (3 and 6 months old) or five plants (2 years old). The 5-OH G monomer occurred as a trace amount in control and *CAD* lines. KL, Klason lignin.

Line	Total Yield	S/G/5-OH G
	μmol g^{-1} KL	*% molar*
Three months old		
ASCAD52	1670	57/43/−
ASCAD21	1700	53/47/−
SCAD1	1920	58/42/−
Control	1500	59/41/−
ASOMT2B	1300	18/75/7
ASOMT10B	1280	25/70/5
Six months old		
ASCAD52	2060	67/33/−
ASCAD21	1930	67/33/−
SCAD1	2030	69/31/−
Control	2150	67/33/−
ASOMT2B	1470	29/65/6
ASOMT10B	1610	31/64/5
Two years old		
ASCAD52	2360	66/34/−
ASCAD21	2235	65/35/−
SCAD1	2580	66/34/−
Control	2580	64/36/−
ASOMT2B	1570	41/53/6
ASOMT10B	1610	30/62/8

Similar to maize *bm3* mutants with depressed COMT activity (Lapierre et al., 1988), a substantial amount of 5-OH G units occurred in the lignin from the ASOMT lines, whereas trace amounts were found in wood of control poplars (Table II).

The increased incorporation of cinnamaldehyde units into lignin has been repeatedly reported in plants with depressed CAD activity, in sorghum (Bucholtz et al., 1980; Pillonel et al., 1991) and pine (MacKay et al., 1997) mutants, and in transgenic tobacco (Halpin et al., 1994; Higuchi et al., 1994). With this in mind, the dithioketal compounds G-CHSEt-CH_2-$CH(SEt)_2$ and S-CHSEt-CH_2-$CH(SEt)_2$, respectively, formed upon thioacidolysis of coniferaldehyde and sinapaldehyde, were quantified. Relative to the main monomers Ar-CHSEt-CHSEt-CH_2SEt (with Ar = G or S ring), these dithioketal derivatives were recovered in similar low amounts from the control and *CAD*-transformed lines (i.e. in the range of 5–10 μmol g^{-1} lignin, which is less than 0.5% of the main monomers).

To ascertain further that lignins from CAD-down-regulated poplars did not contain increased levels of cinnamaldehyde units, soluble lignin fractions isolated by mild procedures (see "Materials and Methods") were subjected to the Wiesner reaction according to the standard protocol (Bucholtz et al., 1980; Nakano and Meshitsuka, 1992). We found that the intensity of the pink color developed by cinnamaldehyde units was similar in samples from *CAD*-transformed and control lignin samples (Table III). When this coloration was expressed as equivalent to coniferaldehyde units per gram of lignin fraction, the coniferaldehyde level was found to be lower than 0.5% in the control and in line ASCAD21 (Table III). In contrast, in lignins isolated from lines ASOMT2B and ASCAD21 × ASOMT7, this level was higher, about 1% by weight. In agreement with the literature (Lai and Sarkanen, 1973), these values are lower than those obtained for conifer G lignins, which are reported to be in the 2% to 3% range (Chen, 1992).

In contrast to a recent study (Tsai et al., 1998), the level of coniferaldehyde units in these poplar lignin samples was not related to the wood coloration but rather seemed to be associated with the enrichment in G units induced by the antisense *COMT* construct (Table III). Upon thioacidolysis, ASCAD21 xylem-released S-$CH(SEt)_2$, which represents the dithioketal derivative of syringaldehyde, was recovered in a 2% yield relative to the major monomer S-CHSEt-CHSEt-CH_2SEt, versus 0.2% in the case of control sample. From the outer part of the xylem displaying the red coloration, the relative recovery of syringaldehyde dithioketal was still more pronounced (6% relative to the major S monomers). This result suggests that the heterogeneous pattern of the red coloration of ASCAD21 xylem might coincide with an increased incorporation of syringaldehyde units in the lignin.

Lignin characterization in a series of poplars individually analyzed revealed a fairly stable level of β-*O*-4 bonds in control and ASCAD21 trees, as evidenced by the similar thioacidolysis yield (Fig. 1). In these lines, the relative proportion of S units involved in β-*O*-4 bonds was found to be remarkably constant (Fig. 2). In contrast, lignins of the double transformants ASCAD21 × ASOMT7 displayed pronounced structural variations, both in β-*O*-4 content (Fig. 1) and in S level (Fig. 2). In spite of this variability between individual plants, the antisense *COMT* construct introduced either in the ASCAD21 or in the wild-type background caused a similar alteration of the lignin profile, namely a reduction in β-*O*-4 bonds, a decrease in S units, and the appearance of substantial amounts of 5-OH G units.

Evaluation of Free Phenolic Groups in β-*O*-4-Linked Lignin Units

Comprehensive studies of the mechanisms that govern lignin depolymerization during kraft pulping (for review, see Gierer, 1982) have shown that β-*O*-4-ether bonds in units with free phenolic groups are more easily cleaved than β-*O*-4-ether bonds in units with etherified phenolic groups. The relative proportion of free phenolic groups in β-*O*-4-linked units is thus an important structural trait influencing lignin solubilization and/or fragmentation in the cooking liquor. When thioacidolysis is performed from exhaustively CH_2N_2-methylated lignins, additional monomers are recovered with methylated phenolic groups. Their relative content, compared with the overall monomer yield, indicates the proportion of free and methylatable

Table III. *Determination of coniferaldehyde units in soluble lignin fractions by the Wiesner reaction*

These lignin fractions were isolated by solvent extraction (dioxane:water, 9:1, v/v) of 1-year-old extract-free stems after ball milling and cellulase hydrolysis, which were then purified by precipitation in water. The isolation yield of the recovered lignin fractions is expressed as a weight percentage of total lignin. KL, Klason lignin. The percentage (molar) of G units in β-*O*-4 lignin structures was determined by thioacidolysis.

Sample		Lignin Fraction		Coniferaldehyde Units in Lignin Fraction	
Line	Wood coloration	Isolation yield	G in β-O-4	*wt (%)*	μmol g^{-1}
		% KL	*%*		
Control	White	37	26	0.45	25.3
ASCAD21	Red	50	27	0.30	16.8
ASOMT2B	Pale pink	43	56	1.13	63.5
ASCAD21 × ASOMT7	Red	59	63	1.08	60.6

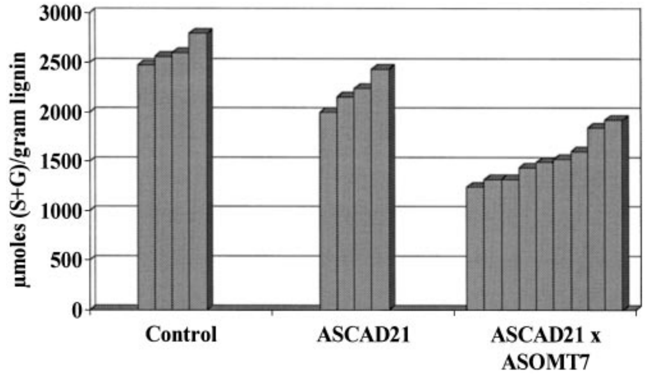

Figure 1. Yield in main lignin-derived thioacidolysis monomers recovered from extract-free wood of 1-year-old transgenic and control poplars grown together in the greenhouse and individually analyzed. For each tree, the main stem was collected, debarked, ground, and solvent extracted. Data are means of duplicate experiments (SE 3% to 5%) and expressed in micromoles of G, S, and 5-OH G monomers recovered per gram of lignin (evaluated as Klason lignin). This yield is a close reflection of the frequency of units involved only in labile *β-O*-4 bonds in the lignin.

phenolic groups within the *β-O*-4-linked G or S units (Lapierre and Rolando, 1988). In control lines, approximately 25% of the G units involved in *β-O*-4 linkages had free phenolic groups (Table IV). In agreement with past results (Lapierre, 1993), 3% was observed for the S units in the control line. Relative to the control, ASOMT10B displayed a lower level of free phenolic G units. In contrast to COMT, CAD down-regulation induced a substantial increase in free phenolic units, both in the G and in the S series (Table IV). ASCAD21 showed the highest content in free phenolic groups, whereas the double transformant ASCAD21 × ASOMT7 displayed an intermediate behavior relative to the parent lines.

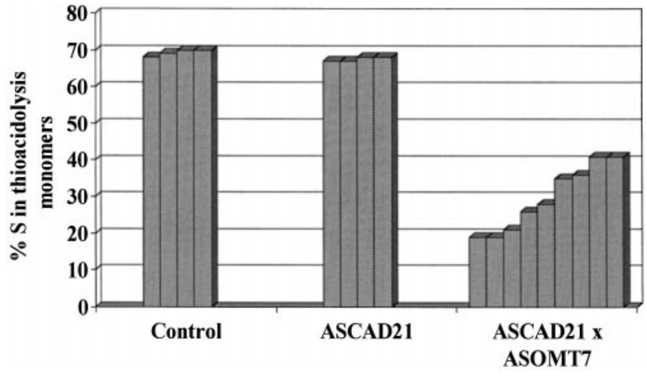

Figure 2. Relative proportion (percent molar) of S thioacidolysis monomers recovered from 1-year-old transgenic and control poplars grown together in the greenhouse and individually analyzed. For each tree, the main stem was collected, debarked, ground, and solvent extracted. Data are means of duplicate experiments (SE 3% to 5%) and expressed as a molar percentage of the main lignin-derived thioacidolysis monomers.

Table IV. *Frequency of free phenolic groups in G or S units involved in β-O-4 bonds, as shown from thioacidolyses of methylated extract-free poplars*

For each line, measurements were done from one (6-month-old poplars) or three to five (older poplars gathered for analyses) plants.

Line	Age	G with Free Phenol	S with Free Phenol
		% molar	
ASCAD52	2 years	30.5	3.5
ASCAD21	2 years	33.6	4.5
	6 months	32	4.5
SCAD1	2 years	29.8	3.6
	6 months	29.4	3.6
Control	2 years	26.4	3.0
	6 months	25.7	2.8
ASOMT10B	1 year	21.5	2.6
ASCAD21 × ASOMT7	6 months	25.9	2.6

Relative Frequencies of Condensed Bonds in Lignins

The GC analysis of dimeric structures from thioacidolysis was performed after Raney nickel desulfurization. This procedure allows the determination of lignin-derived dimers that have retained resistant carbon-carbon and diarylether bonds from the polymer. The main dimers shown in Figure 3 include representatives from the common interunit linkages in angiosperm lignins, namely the 4-*O*-5, 5–5, *β*-5, *β*-1, and *β*-*β* linkages. Model experiments showed that the *β*-*β* S dimers originate from syringaresinol structures.

The determination of thioacidolysis dimers provided unique information concerning the relative frequencies of

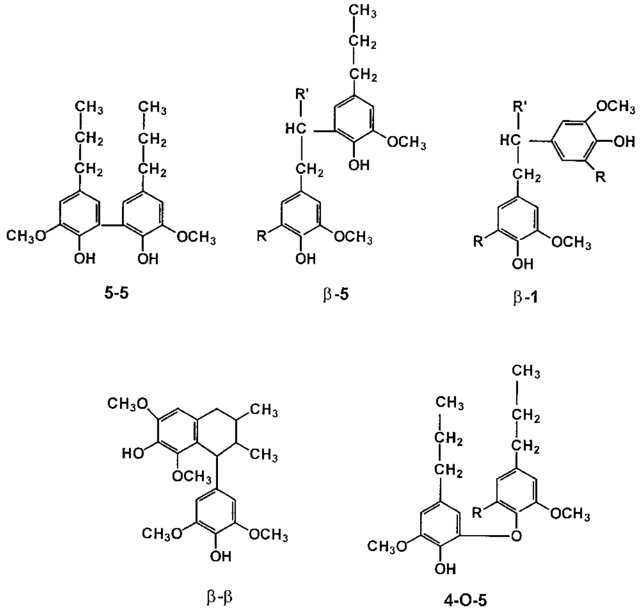

Figure 3. Main dimers recovered from thioacidolysis followed by Raney nickel desulfurization of poplar lignins (R = H or O methyl; R′ = H or CH_2OH). The S-S *β*-*β* dimers originate from syringaresinol structures. These dimers are representatives of resistant interunit linkages occurring in angiosperm lignins.

Table V. *Relative frequencies of the main dimers obtained by thioacidolysis of poplar samples (Fig. 3) that represent the lignin-condensed interunit linkages*

For each line, measurements were done from one (6-month-old poplars) or three to five (older poplars gathered for analysis) plants.

Age and Line	Bond Type				
	4-O-5	5-5	β-5	β-β	β-1
			% molar		
Two years old					
ASCAD52	8.9	10.6	25.3	24.2	31.0
ASCAD21	9.6	10.4	23.7	21.8	34.5
SCAD1	8.0	9.6	23.3	23.0	36.1
Control	8.2	9.8	26.5	26.0	29.5
One year old					
Control	7.5	10.3	28.1	24.4	29.7
ASOMT10B	9.5	19.8	34.2	10.8	25.7
Six months old					
ASCAD21	9.8	8.6	19.1	25.7	36.8
ASCAD21 × ASOMT7	6.9	35.3	32.9	3.4	21.5

resistant interunit bonds in control and transgenic poplar trees (Table V). The main effect of depressed CAD activity in poplar lignins was the enrichment in β-1 diarylpropane structures (+15%). The most remarkable effect of depressed COMT activity was the increase in 5–5 biphenyl structures, together with the severe decrease in β-β syringaresinol structures. In the double transformant ASCAD21 × ASOMT7, the profile for interunit bonds was similar to that of ASOMT10B single transformants.

Analysis of Low-M_r Phenolics Released by Mild Alkaline Hydrolysis

Poplar wood specifically contains *p*-hydroxybenzoic esters linked to lignin units (Smith, 1955). Alkaline hydrolysis of extract-free samples was performed to determine the impact of the transformation on this acylation. Relative to the control and calculated on the basis of lignin content, *CAD*-transformed poplars showed similar ranges of *p*-hydroxybenzoic esters: The data of Table VI indicate that about 2% of the poplar lignin units are *p*-hydroxybenzoylated, and in ASOMT plants this acylation degree was found to be slightly lower.

In addition to *p*-hydroxybenzoic acid, vanillin and syringaldehyde were released upon alkaline hydrolysis of the samples (Table VI). With the exception of line ASOMT10B, which is particularly enriched in G units, syringaldehyde was obtained in substantially higher amounts than vanillin. Past studies have shown that small amounts of benzaldehydes are released by alkaline hydrolysis of lignified walls (Hartley and Keene, 1972). In agreement with a previous study (Baucher et al., 1996), higher levels of vanillin and, more markedly, of syringaldehyde were obtained from *CAD*-transformed poplars, especially from line ASCAD21. From the data of Table VI, we could estimate that 1.2% of the lignin units in line ASCAD21 gave rise to syringaldehyde upon alkaline hydrolysis, whereas the figure obtained for the control lignin was 10-fold lower. Taken together with the increased recovery of syringaldehyde dithioketal from thioacidolysis of line ASCAD21, this result suggests that CAD down-regulation increased the incorporation of syringaldehyde into lignin, although the overall level remained low.

Pulp Production from Transgenic and Control Poplars

Laboratory-scale kraft-pulping experiments were performed on 2-year-old transgenic and control poplars. These experiments were aimed at examining, for the first time to our knowledge, the pulping characteristics of COMT-down-regulated poplars. In addition, the promising pulping performances of 3-month-old *CAD*-transformed poplars (Baucher et al., 1996) had to be confirmed with older trees, because 3-month-old samples have short fiber cells and their cooking behavior may not adequately represent that of mature fiber cells.

The greenhouse-grown series was subjected to cooking experiments with various alkali charges (Table VII). At a given charge, no difference in cellulose degree of polymerization (data not shown) could be observed between transgenic and control samples, which means that cellulose degradation was similar. COMT-down-regulated poplars displayed a markedly higher resistance toward kraft delignification, as evidenced by higher κ numbers relative to other lines. In contrast and at a low charge of active alkali (15%), ASCAD21 poplars were more extensively deligni-

Table VI. *Yields of phenolic compounds released by alkaline hydrolysis of 2-year-old, extract-free poplar wood*

For each line, measurements were made from the stems of three to five plants grown in the greenhouse and gathered for analysis. KL, Klason lignin.

Line	*p*-Hydroxybenzoic Acid	Vanillin	Syringaldehyde
	$\mu mol\ g^{-1}$ *KL*		
ASCAD52	82	7	20
ASCAD21	89	12	62
SCAD1	102	12	37
Control	101	5	6
ASOMT10B	68	14	6

Table VII. *Kraft-cooking characteristics of 2-year-old transgenic poplars grown in the greenhouse at three different levels (%) of active alkali*

Line	15%		17%		20%	
	Pulp yield	κ no.	Pulp yield	κ no.	Pulp yield	κ no.
	%		%		%	
ASOMT2B	51.5	30.5	49.3	19.6	47.2	14.3
ASOMT10B	51.5	28.7	49.1	19.5	47.6	14.8
Control	49.7	20.5	48.2	15.1	46.9	11.9
ASCAD52	49.2	19.4	48.0	14.7	46.9	12.5
ASCAD21	50.1	16.1	49.1	15.0	47.5	12.4

fied, whereas ASCAD52 did not display improved delignification. The measurements of mechanical properties of sheets made from the pulps did not reveal differences between transgenic and control samples. When pulps with similar κ numbers from the various lines were subjected to a totally chlorine free bleaching sequence, no difference in bleachability could be evidenced (data not shown).

Evaluation of the field-grown series essentially confirmed the previous results. ASOMT2B and ASOMT10B lines proved to be substantially more resistant toward kraft delignification, as shown by their higher κ number (+60% relative to the control). By contrast, the ASCAD21 line was more extensively delignified than the control. The other *CAD*-transformed lines, ASCAD52 and SCAD1, were not better delignified than the control. Only slight variations in bleachability and brightness of the pulps prepared from the various lines could be observed after an elementary chlorine-free or a totally free chlorine-bleaching sequence. The mechanical properties of the pulps from the various field-grown lines were similar, which means that the genetic transformation did not detrimentally affect the bonding potential of cellulosic fibers.

DISCUSSION

Lignin Levels in Poplar Trees Are Not Altered by COMT Down-Regulation but Are Slightly Reduced by CAD Down-Regulation

In recent years several studies of transgenic plants with CAD- or COMT-down-regulated activity have shown that the lignin level of the transformed plant was maintained at a value similar to the control (Dwivedi et al., 1994; Halpin et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995; Baucher et al., 1996; Tsai et al., 1998). In the present study we confirmed (as applied to 2-year-old poplar trees grown in the greenhouse or in the field) that a reduction of COMT activity to approximately 5% to 10% of control activity is not enough to reduce the overall Klason lignin. Recently, an alternative methylation pathway was evidenced in the lignin biosynthetic pathway. In this pathway CCoAOMT converts caffeoyl CoA into feruloyl CoA (Ye et al., 1994). The close association of *CCoAOMT* expression with lignification has been demonstrated in several dicot plants (Ye, 1997). Therefore, in ASOMT poplar lines, CCoAOMT activity is likely to compensate for depressed COMT activity and to maintain a constant flux of lignin precursors. Moreover, several lines of evidence indicate that, in addition to COMT and CCoAOMT activities, other *O*-methyltransferase activities may occur in the lignin biosynthetic pathway with various substrate specificities (Matsui et al., 1994; Daubresse et al., 1995; Li et al., 1997).

Contrary to the above-mentioned studies, a moderate decrease in lignin content has been evidenced in transgenic tobacco lines (Ni et al., 1994; Sewalt et al., 1997b) and in the maize *bm3* mutant (Barrière and Argillier, 1994) with depressed COMT activity. The occurrence of several *O*-methyltransferase activities with various substrate specificities and spatial or temporal expression patterns makes it difficult to predict the effect of selectively targeting bifunctional COMT. That primary xylem of COMT-down-regulated poplar plantlets did not display any Wiesner staining in contrast to secondary xylem suggests that the lignin level is dramatically reduced in the early stages of lignification but is restored in secondary xylem because of *O*-methyltransferase-compensating activities and/or of spatiotemporal variations in the effect of the antisense *COMT* construct.

In CAD-down-regulated poplars, our findings suggest a slightly lower Klason lignin level in the more depressed lines (ASCAD21 and SCAD1) relative to the control. This trend should be confirmed with older trees. Even though the maintenance of normal growth and lignin level in transgenic poplars with antisense *CAD* or *COMT* construct does not seem remarkable in itself, it is positive for agronomical use of the transgenic plants.

In contrast to our present findings, recent studies have shown that modifying the expression of enzymes located at an earlier stage of the lignin biosynthetic pathway may induce substantial decreases in lignin content. For instance, down-regulating cinnamate-4-hydroxylase in tobacco (Sewalt et al., 1997a) and 4-coumarate:CoA ligase in tobacco (Kajita et al., 1997) or in poplar (Hu et al., 1998) have been reported to noticeably reduce the lignin content. In cinnamoyl-CoA reductase down-regulated tobacco, an approximately 2-fold decrease in lignin content has been evidenced in the most severely depressed line, which displayed altered growth and development together with collapsed vessels (Piquemal et al., 1998).

Even though a change in lignin distribution at the tissue or cell level cannot be ruled out, the present study suggests that changes in lignin structure may be primarily responsible for the higher and lower pulping characteristics of *CAD*- and *COMT*-transformed poplar trees, respectively.

COMT-Down-Regulated Poplar Trees Display Severe Alteration in Lignin Structure and the Pulping Characteristics Are Lower

Whereas the obtained level of COMT down-regulation did not affect the lignin content of poplar trees, this transformation induced severe alterations in the lignin structure. Whatever the age, the lignin content in β-*O*-4 linkages was found to be reduced relative to the control plants. In agreement with these data, we observed a 2-fold increase in resistant 5–5 biphenyl structures and in the G units. Taken together, these results are consistent with what can be expected from our current knowledge of the lignin biosynthetic pathway. A shortage of S precursors occurs as a consequence of the severely depressed COMT activity. In contrast, the alternative CCoAOMT methylation route appears as a compensating pathway capable of redirecting caffeic intermediates toward G precursors. The overall increased flux of the G precursors thereby induces the lignin enrichment in G units observed not only in mature plants but also in the stems of poplar plantlets. When the *COMT* antisense construct was introduced in *CAD*-transformed trees, the double transformant displayed a lignin pattern very similar to that of the single COMT transformant. The occurrence of 5-OH G units in substantial proportions seems to be another specific structural trait of the lignin from plants with depressed COMT activity (Lapierre et al., 1988; Atanassova et al., 1995; Van Doorsselaere et al., 1995; Tsai et al., 1998).

The dramatic alterations induced by COMT down-regulation gave poplar lignins some similarities with softwood lignins. Because the overall lignin level is maintained in *COMT*-transformed poplars, the reduction in S units, β-*O*-4 linkages, and free phenolic units, together with the increase in resistant interunit linkages, would result in a lignin less amenable to kraft degradation. This proposition was repeatedly confirmed by our kraft cooking assays. Wood of the line ASOMT was found to be more resistant to kraft delignification, as shown by higher κ numbers relative to the control. In other words, the 90% decrease in COMT activity seems detrimental for kraft pulping.

The Subtle Alteration of Lignin Structure Induced by CAD Down-Regulation Makes Poplar Lignin More Amenable to Kraft Delignification

Unlike transgenic tobacco plants with reduced CAD activity (Halpin et al., 1994; Higuchi et al., 1994), the red coloration of the xylem tissues in CAD-down-regulated poplar trees could not be assigned to any increased incorporation of cinnamaldehyde units into lignin. This result was obtained by two different methods: thioacidolysis and the Wiesner reaction. The former method is applied to native lignin without isolation, but it characterizes only the so-called noncondensed lignin that represents about 50% of total lignin. The latter method allows the determination of the total content in cinnamaldehyde units, when applied to soluble lignin fractions isolated with a 30% to 50% isolation yield. In contrast to earlier studies in which thioacidolysis (Higuchi et al., 1994; Jacquet, 1997; Tsai et al., 1998) and/or the Wiesner reaction (Bucholtz et al., 1980; Tsai et al., 1998) provided evidence for the incorporation of cinnamaldehyde units in the lignin, lignins from *CAD*-transformed poplars neither provided high amounts of cinnamaldehyde dithioketal upon thioacidolysis nor showed an increased Wiesner reaction. Whatever the line, the lignin content in cinnamaldehyde units remained at a normally low level (i.e. 0.5% to 1% of lignin units). This level displayed some variations according to the poplar line, which were not related to the wood coloration but to the frequency of G units. The only carbonyl compound recovered in a higher amount from the ASCAD21 sample and relative to the control was syringaldehyde, accounting for approximately 2% of the lignin units (versus 0.2% in control). It is interesting that this carbonyl compound was found in higher amounts in the red xylem area. Taken together, these results suggest that an abnormal red coloration of the xylem is not necessarily related to the pronounced incorporation of cinnamaldehydes in lignin.

The alteration of lignin structure induced by CAD down-regulation in poplar could be evidenced only by using the most recent developments of thioacidolysis. Whereas the proportion of G and S units and the frequency of β-*O*-4 linkages did not appear to be affected by the transformation, we provide here the first evidence to our knowledge that CAD down-regulation induced a substantial enrichment of the lignin in free phenolic units, as well as in diarylpropane structures. That both diarylpropane and free phenolic groups increase is consistent with our current knowledge of lignin, because the former structures have been shown to be distributed mainly in lignin as end groups with free phenolic functions (Gellerstedt and Zhang, 1991). Some physical and/or chemical parameters that influence the polymerization mechanisms may be affected by the transformation. Whatever the biochemical basis of the phenomenon, the effect that can be expected from an enrichment in free phenolic units is an easier kraft delignification. Two-year-old poplar trees of the ASCAD21 line, which displayed the most severely depressed CAD activity, the brightest red coloration of the xylem, and the highest lignin content in free phenolic units, proved to be more easily delignified in two independent pulping assays. This moderate yet significant improvement in delignification efficiency can be of very high value because of the large scale of the pulping industries. In contrast, line ASCAD52, which displayed less-pronounced enzymic, phenotypic, and structural changes relative to the control, did not show any improvement in kraft-pulping characteristics. These results suggest a threshold level in free phenolic groups to be reached to beneficially affect the kraft-pulping efficiency.

Our present data were obtained from 2-year-old poplar trees regenerated from CAD- and COMT-down-regulated primary transformants. The stability of the transgene expression has therefore been demonstrated for a large series of mature plants grown in the greenhouse or in the field. It still needs to be shown that this effect is maintained for 10 years, the age at which poplars are harvested for pulping. From the present results, down-regulation of CAD activity in poplars appears to be an efficient route for improving the kraft-pulping characteristics of the trees, whereas the 90% down-regulation of COMT activity has a dramatically adverse effect on the efficiency of the kraft process. These effects may be accounted for by the changes in lignin structure induced by the transformation. In addition to the industrial interest of the transformants, the down-regulation of the CAD and COMT enzymes allowed us to obtain new insights in the complex biosynthetic pathway leading to lignin precursors and its regulatory mechanisms.

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