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Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins

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Abstract

A series of transgenic poplars down-regulated for cinnamyl alcohol dehydrogenase (CAD) was analyzed by thioacidolysis. Among the lignin-derived monomers, the indene compounds that were recently shown to originate from sinapaldehyde incorporated into lignins through 8–O–4-cross-coupling, were found to increase as a function of CAD deficiency level. While these syringyl markers were recovered in substantial amounts in the most severely depressed lines, the markers for coniferaldehyde incorporation were recovered in only low amounts. In conjunction with these additional sinapaldehyde units and relative to the control samples, lignins in CAD-deficient poplar lines had less conventional syringyl-units and β–O–4-bonds and more free phenolic groups. We found that almost half of the polymers in the most deficient lines could be solubilized in alkali and at room temperature. This unusual behavior suggests that lignins in CAD-deficient poplars occur as small, alkali-leachable lignin domains. That mainly sinapaldehyde incorporates into the lignins of CAD-deficient poplars suggests that the recently identified sinapyl alcohol dehydrogenase (SAD), which is structurally distinct from the CAD enzyme targeted herein, does not play any substantial role in constitutive lignification in poplar.

Keywords: Poplar (*Populus deltoides*×*Populus nigra*; cv Ogy); Salicaceae; Lignin structure; Lignin biosynthesis; Cinnamyl alcohol dehydrogenase; Transgenic; Thioacidolysis; Sinapaldehyde; Coniferaldehyde

1. Introduction

Angiosperm lignins are composed mainly of guaiacyl (**G**) and syringyl (**S**) units linked by labile ether bonds and/or resistant carbon-carbon linkages. The formation of coniferyl and sinapyl alcohols, the immediate precursors of **G** and **S** lignin units, requires the reduction of coniferaldehyde and sinapaldehyde. For many years, this enzymatic step has been thought to be catalyzed by an enzyme with broad specificity, cinnamyl alcohol:NADP$^+$ dehydrogenase (CAD, EC 1.1.1.195), capable of reducing both hydroxycinnamaldehydes (Higuchi, 1997). Various CAD isoforms involved in monolignol biosynthesis have been implicated in a variety of species (reviewed in Higuchi, 1997 and Dixon et al., 2001). The role of CAD in the formation of sinapyl alcohol has been recently revised by the discovery, in *Populus tremuloides*, of a novel enzyme, sinapyl alcohol dehydrogenase (SAD), which was suggested to be specifically involved in the reduction of sinapaldehyde (Li et al., 2001). This hypothesis has lent support to a model in which coniferaldehyde is channeled to coniferyl alcohol and sinapyl alcohol via two metabolic pathways (Dixon et al., 2001; Humphrey et al., 1999; Humphreys and Chapple, 2002; Li et al., 2000; Osakabe et al., 1999).

In the last few years, down-regulating the various enzymes of the lignin biosynthetic pathway by genetic transformation has proven to be an efficient way to appraise their specific roles in controlling the lignification process (Grima-Pettenati and Goffner, 1999). This was made possible by in-depth structural analysis which

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revealed the structural peculiarities of the transformed lignins. The effect of CAD deficiency on angiosperm lignins has been investigated in brown-midrib sorghum (Pillonel et al., 1991), maize (Halpin et al., 1998; Marita et al., 2003) and arabidopsis (Sibout et al., 2003) mutants, and in poplar (Baucher et al., 1996; Lapierre et al., 1999), tobacco (Halpin et al., 1994; Higuchi et al., 1994; Stewart et al., 1997; Ralph et al., 1998; Yahiaoui et al., 1998; Kim et al., 2000) and alfalfa (Baucher et al., 1999) transgenic lines.

In order to clarify the respective role of CAD enzyme in poplar, we made a systematic and in-depth structural investigation of lignin in a series of transgenic poplar lines specifically down-regulated for CAD activity to a variable extent. We have recently identified thioacidolysis-derived syringyl indene derivatives which are diagnostic for the incorporation of sinapaldehyde into angiosperm lignins (Lapierre et al., 2001; Kim et al., 2002). By GC–MS monitoring of these indene derivatives, we demonstrate herein that the incorporation of sinapaldehyde into poplar lignins increases concomitantly with the CAD deficiency level. We also establish that the amount of coniferaldehyde units in poplar lignins is not substantially enhanced by CAD deficiency. Finally, we report on the specific structural traits of lignins in CAD-deficient poplars. In addition to an increased amount of sinapaldehyde units, we show that lignins of CAD-deficient poplar lines have fewer syringyl- and β–O–4-units and, more importantly, display an abnormally high level of free phenolic end groups responsible for their unusual solubility in alkali and at room temperature.

2. Results and discussion

2.1. Sinapaldehyde is preferentially incorporated into the lignins of CAD-deficient poplars

A series of primary transformants deficient in CAD activity was produced in the poplar clone Ogy (*Populus deltoides*×*Populus nigra*) by the antisense strategy (Pilate and Leplé, unpublished results). The Klason lignin content of the poplar lines with CAD activity ranging between 40 and 100% of the control level was found close to 20% of the extract-free dry wood (Table 1). In contrast, this value was reduced to 17–18% in all lines with severely down-regulated CAD activity (residual CAD activity lower than 10% of the control level). This result shows that down-regulating CAD activity in angiosperms provides a way to moderately reduce the plant lignin content. Such a moderate decrease is necessary when the objective is to simultaneously preserve the field performances of the plants and to obtain lignocellulosic material more susceptible to both industrial kraft pulping and cellulase hydrolysis.

Table 1
Lignification in control (Ogy clone, *Populus deltoides*×*Populus nigra*) and corresponding transgenic CAD-deficient poplar lines

Line	Wood coloration	Klason lignin (% extract-free wood)	Lignin composition: thioacidolysis monomers in μmole g^{-1} lignin					
			1S (β–O–4-linked S units)	**1G** (β–O–4-linked G units)	**2Sa + 2Sb** (8–O–4-linked sinapaldehydes)	**4S** (syringaldehyde end-groups)	**2Gb** (8–O–4-linked coniferaldehydes)	**3G** (coniferaldehyde end-groups)
Control	No	19.63±0.21	1502	963	0.9	2	0.13	2.7
ASCAD 8,3	No	20.64±0.04	1388	890	3.8	6.1	0.20	2.2
ASCAD 33,1	Bright Red	17.48±0.33	818	810	39	38	1.6	1.0
ASCAD 21,2	Bright Red	17.76±0.35	676	730	41	33	2.1	1.0
ASCAD 2,3	Bright Red	17.98±0.38	647	779	42	39	2.9	1.5
ASCAD 14,2	Bright Red	17.76±0.19	572	752	44	45	3.9	1.2

The transgenic lines correspond to independent insertion events varying in their residual CAD activity (Fig. 3). Lignin analyses are run on the extract-free wood of 7-month old greenhouse-grown poplar trees. The standard relative errors for thioacidolysis duplicates are lower than 5% of the reported mean values. The structures of thioacidolysis monomers are shown in Fig. 1

Thioacidolysis was used to explore the specific structural traits of lignins in CAD-deficient poplar wood. This analytical degradation mainly provides thioethylated phenylpropanoid compounds **1G** and **1S** from conventional β–O–4-ethers **L1** in lignins (Fig. 1). These lignin-derived monomers, recovered as a pair of *erythro*/*threo*-isomers (chromatographic pairs **1G** and **1S** in Fig. 2), were released in substantially lower amounts from lignins of the poplar lines displaying a severe CAD deficiency (Table 1). This result indicates that reduction in lignin levels in plants with down-regulated CAD activity is associated with a decrease in lignin units only involved in β-O-4-bonds, which are the parent structures of the main thioacidolysis monomers (structures **L1** in Fig. 1). This decrease was found to be much greater in **S**-units than in **G**-units (Table 1).

Relative to the control, the thioacidolysis reaction mixture recovered from CAD-deficient poplar samples not only provided about 60% less conventional **1S** compounds, but also contained two new isomeric syringyl indene monomers (**2Sa** and **2Sb** in Figs. 1 and 2). While the conventional β–O–4-ethers **L1** in lignins yield the main **1G** and **1S** monomers, we recently established that the source of the indene derivatives **2Sa** and **2Sb** are the sinapaldehyde 8–O–4-coupled units (structures **L2** in Fig. 1) (Kim et al., 2002). In the guaiacyl series, only one indene isomer **2Gb** (Figs. 1 and 2) could be observed as a trace component. While these indene compounds could be satisfyingly determined on the GC–MS trace obtained from a milled wood lignin fraction isolated from a CAD-deficient poplar (Fig. 2A), these peaks were obscured by peaks from hemicellulose-derived products (Fig. 2B, peaks annotated with an asterisk) when thioacidolysis was run on the corresponding extract-free wood. However, the **2S** and **2G** indene isomers could be monitored, without any interference from other compounds, on selected-ion chromatograms reconstructed at *m*/*z* 384 and 354, which respectively correspond to the base peaks of their trimethylsilylated derivatives. By so doing, we could determine that the levels of the indene syringyl compounds **2S**, relative to conventional syringyl monomers **1S**, increased together with the degree of CAD deficiency (Fig. 3). Moreover, this increase was observed before any wood phenotype (red coloration of the xylem) could be seen or before any other lignin structural alteration could be detected (eg line ASCAD8,3 with 44% residual CAD activity, Table 1 and Fig. 3). In poplar lines with residual CAD activity lower than 10% of the control level (Fig. 3 and other data not shown), these isomers averaged 5–8% of the conventional **S**-monomers while recovered in trace amounts in the lines with CAD activity ranging between 40 and 100% of the control level. These indene compounds can thereby be considered as a sensitive signature of CAD deficiency that can be used by researchers to monitor the CAD deficiency level in transgenic angiosperms, from a few milligrams of cell walls.

These results provide evidence that down-regulating CAD activity in poplar specifically causes the incorporation of sinapaldehyde into lignins. This incorporation primarily occurs through 8–O–4-cross-coupling, analogously with the conventional monomer, sinapyl alcohol. The incorporation of sinapaldehyde as end-groups (structure **L3** with R = OMe, Fig. 1) happens only to a negligible extent, as evidenced by the trace amount of the dithioketal derivative **3S** (Fig. 1). In contrast, this dithioketal derivative and its analogue methylated at C4 were recovered as the main monomers (50–80% recovery yield) from sinapaldehyde and from 3,4,5-trimethoxy-cinnamaldehyde that was used as a representative of sinapaldehyde end-groups.

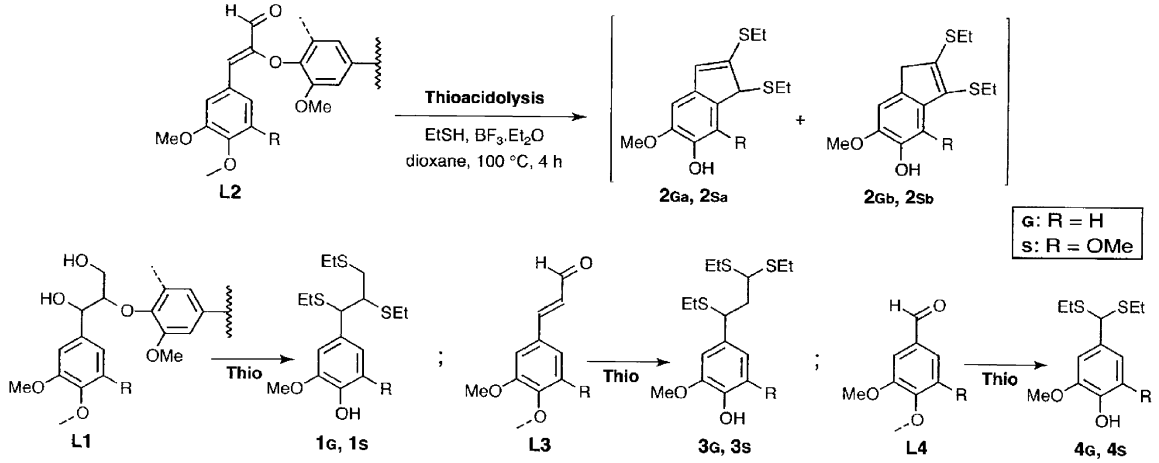

Fig. 1. Structures in lignins and their main thioacidolysis products. Conventional β-O-4-ethers **L1** in lignins yield the conventional thioacidolysis monomers **1G** and **1S** (as isomeric pairs, see Fig. 2). Hydroxycinnamaldehydes 8–O–4-coupled into lignins **L2** yield the diagnostic indene markers **2G** and **2S**. Hydroxycinnamaldehyde end-groups **L3** produce the dithioketal products **3G** and **3S**, whereas hydroxybenzaldehyde end-groups **L4** produce the dithioketals **4G** and **4S**. For all compounds, **G** is for R = H and **S** is for R = OMe.

While CAD deficiency induced the substantial incorporation of sinapaldehyde through 8–O–4-coupling in poplar lignins, the level of coniferaldehyde was not substantially increased, whatever its bonding mode. In the CAD deficient lines and relative to the control, the marker compound **2G** released from 8–O–4-linked coniferaldehyde units **L2** (Fig. 2) was observed to occur in much lower amount than the analogues **2S** (Table 1). The product **3G** originating from coniferaldehyde end-groups was recovered in similar amount in the transgenic and control lines (Table 1). As these end-groups characteristically stain with phloroglucinol–HCl (Adler et al., 1948), the phloroglucinol–HCl staining reaction proved to be ineffective to discriminate CAD-deficient and control lines; the reagent does not stain the hydroxycinnamaldehydes incorporated into 8–O–4-structures (Kim et al., 2002). In addition to the thioacidolysis compounds released from *p*-hydroxycinnamaldehyde units **L2** and **L3**, we estimated the relative importance of a dithioketal derivative **4S** (Fig. 1). This syringaldehyde-derived marker was found to be released in higher amount from the CAD deficient lines (Table 1), with recovery yields that approximate that of the sinapaldehyde-derived markers. The yield for the vanillin dithioketal derivative **4G** was not substantially increased by CAD deficiency (data not shown). As a model dimer for 8–O–4-linked sinapaldehyde almost quantitatively yielded the indene derivatives **2S** (Kim et al., 2002) and only trace amount of **4S,** this syringaldehyde dithioketal most likely originates from syringaldehyde end-groups (structure **L4** in Fig. 1, with R = OMe). Syringaldehyde might originate from the oxidative degradation of accumulated sinapaldehyde monomers under the conditions prevailing during lignin polymerization. This aldehyde would be incorporated into the lignin polymer as end-groups, a hypothesis supported by the recovery of deuterated syringyl C_6C_1 derivatives from the thioacidolysis of $NaBD_4$-reduced CAD-deficient samples (data not shown).

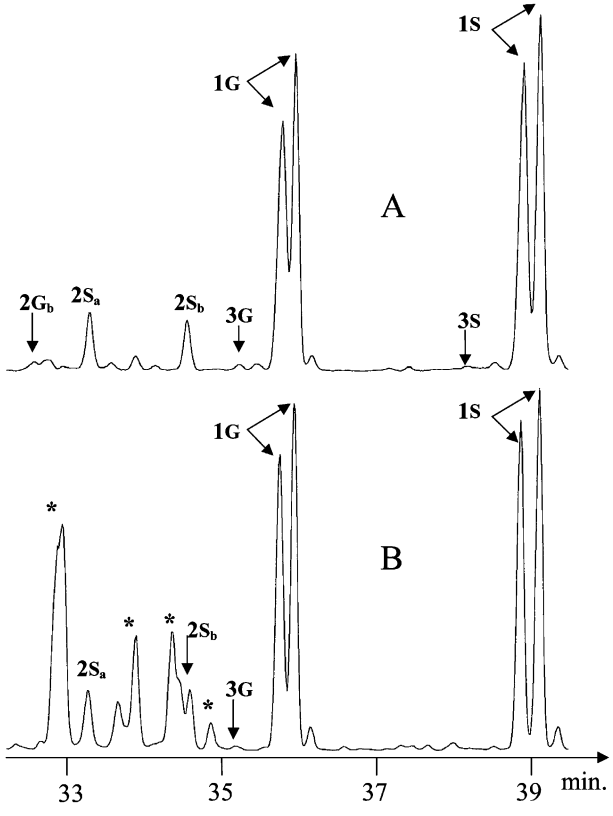

Fig. 2. Partial GC–MS trace showing the separation of the main thioacidolysis monomers **1G** and **1S** (analyzed as their TMS derivatives) recovered from A) a soluble lignin fraction isolated from a CAD deficient poplar wood and from B) the corresponding extract-free wood. Peaks labeled **1G** and **1S** correspond to the *erythro*/*threo* pairs of isomers **G**–CHR–CHR–CH_2R and **S**–CHR–CHR–CH_2R (R = –S–CH_2–CH_3), respectively. Peaks labeled **2Sa** and **2Sb** are two syringyl indene isomers specifically released from sinapaldehyde 8–O–4-linked units and peak **2Gb** is the guaiacyl analogue of **2Sb**. Peaks **3G** and **3S** correspond to the dithioketal derivatives released from coniferaldehyde and sinapaldehyde end-groups and are recovered in low and trace amounts, respectively. On the B trace, peaks with asterisks are degradation products released from hemicellulosic components. The determination of compounds **2S** and **2G,** which is not possible from the total ion chromatograms due to peak overlap, is therefore carried out on selected-ion chromatograms reconstructed at *m*/*z* 384 and 354. Compound structures are given in Fig. 1.

Overall, the data indicate that the CAD down-regulation event obtained herein in poplars more specifically impacts the formation of conventional **S**-lignin units while that of conventional **G**-lignin units is affected to a much lower extent. That mainly sinapaldehyde incorporates into the lignins of CAD-deficient poplars suggests that the recently identified sinapyl alcohol dehydrogenase (SAD) (Li et al., 2001), which is structurally distinct from the CAD enzyme targeted herein (Van Doorsselaere et al., 1995a), does not play any substantial role in constitutive lignification in poplar. The specific incorporation of sinapaldehyde into the lignins of CAD-deficient poplars may be the consequence of the F5H (Humphrey et al., 1999; Osakabe et al., 1999) and COMT (Osakabe et al., 1999; Parvathi et al., 2001) enzyme activities that channel coniferaldehyde to sinapaldehyde. From the coniferaldehyde pool which might transiently increase as a consequence of CAD deficiency, these F5H and COMT activities would lead to unusually high levels of sinapaldehyde. This sinapaldehyde could be stored and/or transported to the lignifying cell walls, possible as the glucoside, in a similar way as the corresponding alcohol (Steeves et al., 2001). It could be the substrate of peroxidases and thereby incorporated into lignins (Russell et al., 2000; Ralph et al., 2001; Ros Barcelo and Pomar, 2001). Another hypothesis to account for the preferred incorporation of sinapaldehyde over coniferaldehyde might be its higher oxidizability (Russell et al., 2000). The analysis of soluble phenolic compounds could be of

considerable interest in order to determine whether transgene expression might affect the pool of phenolic metabolites (Chen et al., 2003).

To further explore the lignin pathway, we subjected single and double poplar transformants, either down-regulated for COMT (ASCOMT2B) or CAD (ASCAD21) activity or down-regulated for both activities (ASCAD21×ASCOMT7), to the thioacidolysis procedure (Table 2). The COMT and CAD residual activities of the transformants were 10 and 30% of the control level, respectively. In agreement with previous results (Lapierre et al., 1999), the Klason lignin level of the control and ASCOMT2B lines were found to be similar while that of the ASCAD21 line was decreased. The double transformant ASCAD21×ASCOMT7, obtained by introducing an ASCOMT construct in the ASCAD21 background, displayed a reduced lignin level, similar to the parent ASCAD21 line. The effective down-regulation of COMT activity in the ASCAD21×ASCOMT7double transformant was ascertained by the recovery of 5-hydroxyguaiacyl (5-OH-G) monomers in substantial amounts via thioacidolysis (Lapierre et al., 1988, 1999), similar to the ASCOMT single transformant. Lignins of the double transformant also displayed an unusually high level of the marker **2S** compounds originating from 8–O–4-linked sinapaldehyde units, although this level did not reach the ASCAD21 level. The amount of conventional **S**-lignin units was found substantially lower in the ASCAD21×ASCOMT7 line, relative to the two single transformants, which may result from the cumulative detrimental effect of down-regulating both CAD and COMT activity on the biosynthesis of sinapyl alcohol and consequently **S**-units.

2.2. Sinapaldehyde-rich poplar lignins display an abnormally high alkaline solubility related to an increased level of free phenolic groups

The specific incorporation of sinapaldehyde into the lignins of CAD-deficient poplar lines had an important impact on the solubility of the lignin network. According to the pioneering work of Beckmann et al. (1923) and in contrast to grass lignins, wood lignins are not easily solubilized in alkali and at mild temperature. The lignin fraction that could be solubilized by a mild alkaline treatment (NaOH 2 mol.l^{-1}, 37 °C, overnight) was estimated by considering both the weight loss caused by this treatment and the Klason lignin contents of the extract-free wood and of the alkali-treated residue. The wood from control trees was delignified to a low extent by this mild treatment which solubilized only 17% of the total lignin (Table 3). In contrast, almost half of the total lignin could be solubilized from poplar lines with a residual CAD activity lower than 10%, a figure similar to that of grass lignins. The poplar lines moderately down-regulated for CAD activity (30% residual activity) displayed an intermediate behavior with about 30% of the total lignin solubilized in alkali. This unusual solubility of lignins in the severely CAD-deficient poplar lines could be correlated to their high content of free phenolic groups, which was estimated by thioacidolysis

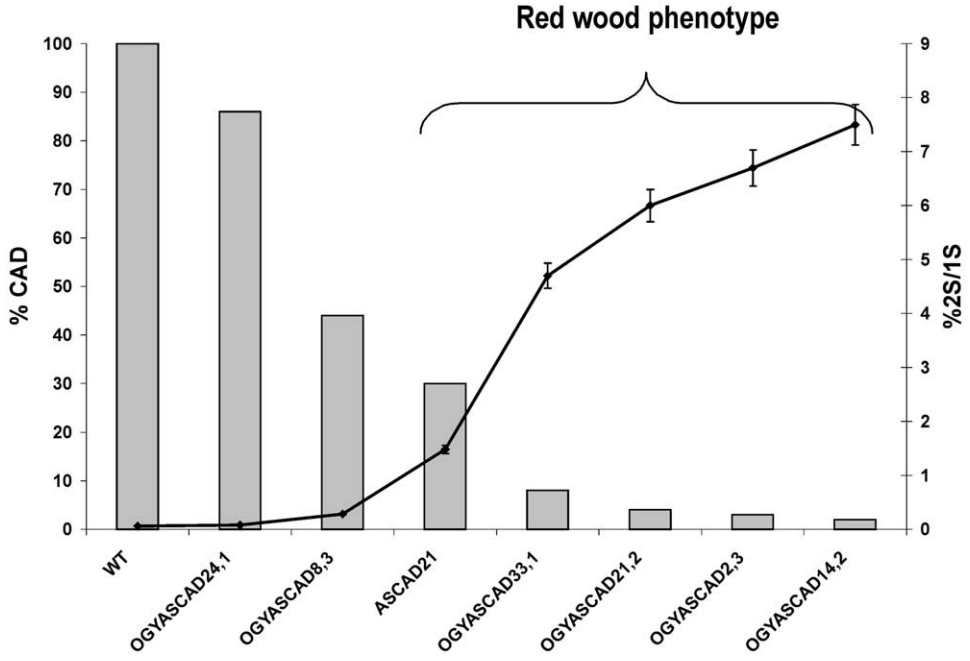

Fig. 3. Relative levels of the thioacidolysis markers to conventional monomeric products [%(**2Sa** + **2Sb**)/**1S**] depends on the residual CAD activity level (100% = control level). The sample with 44% residual activity does not show any red xylem phenotype. Samples with < 10% activity are different OGY70ASCAD lines.

Table 2
Lignification in control (7171B4 INRA clone, *Populus tremula*×*Populus alba*) and corresponding transgenic ASCAD21, ASCOMT2B and ASCAD21×ASCOMT7 poplar lines down-regulated for CAD or/and COMT activity

Line	Wood coloration	Klason lignin (% extract-free wood)	Lignin composition: thioacidolysis monomers in μmole g^{-1} lignin				
			1S (β–O–4-linked S units)	**1G** (β–O–4-linked G units)	[a] (β–O–4-linked 5-OH-G units)	**2Sa + 2Sb** (8–O–4-linked sinapaldehydes)	**3G** (coniferaldehyde end-groups)
Control	No	17.31±0.25	1826	765	5.5	0.5.	3.8
ASCOMT2B	No	18.17±0.08	721	1150	73.5	0.5	8.1
ASCAD21	Red	14.93±0.14	1360	630	6.4	13.1	3.3
ASCAD21xASCOMT7	Red	14.64±0.16	239	898	52.6	4.3	7.9

As previously reported (Baucher et al., 1996; Lapierre et al., 1999), the residual CAD activity of the ASCAD21 or ASCAD21×ASCOMT7 line is 30% of the control level whereas the residual COMT activity of the ASCCOMT2B or ASCAD21×ASCOMT7 line is 10% of the control level. Lignin analyses are run on the extract-free wood of 1 year-old greenhouse-grown poplar trees. The standard relative errors for duplicate thioacidolysis experiments are lower than 5% of the reported mean values. The structures of thioacidolysis monomers are shown in Fig. 1.

[a] The corresponding 5-OH-G monomer is the analogue of **1S** (Fig. 1) with R = OH.

Table 3
Percentage (by wt.) of lignins solubilized from extract-free poplar wood by a mild alkaline treatment (NaOH 2 mol.l^{-1}, 37 °C, 24 h) as a function of CAD deficiency level in various lines

No CAD deficiency		Moderate CAD deficiency[a] Single transformants ASCAD21		Moderate CAD deficiency[a] Double transformants ASCAD21×ASCOMT7		Severe CAD deficiency[b]	
WT OGY	17	ASCAD21	27.8	ASCAD21×ASCOMT7	28.1	OGYASCAD 4,3	42.2
WT OGY	17.2	ASCAD21	30.7	ASCAD21×ASCOMT7	28.9	OGYASCAD 27,1	48.5
WT 717 1B4	16.7	ASCAD21	30.4	ASCAD21×ASCOMT7	29.4	OGYASCAD 10,1	47.3
ASCOMT10B	18.2	ASCAD21	32.2	ASCAD21×ASCOMT7	28.3	–	–
Mean value (standard deviation)	17.3 (0.65)	Mean value (standard deviation)	30.3 (1.8)	Mean value (standard deviation)	28.7 (0.6)	Mean value (standard deviation)	46.0 (3.3)

The different values reported for the same line (ASCAD21 or ASCAD21xASCOMT7) corresponds to different trees (7 or 12 month-old). These percentages are calculated from the three following experimental data : Klason lignin content in the initial sample, Klason lignin content in the alkali-treated samples and recovery yield of alkali-treated residue relative to the initial sample. The standard error for one sample cumulates the three standard errors associated with these three gravimetric determinations and is in the 8–10% relative range.

[a] Residual CAD activity = 30% of the control level.

[b] Residual CAD activity lower than 10% of the control level (the three lines correspond to independent insertion events).

of permethylated wood. Among the β–O–4-linked **G**-units and in the control lines, 20% were found to be terminal units with free phenolic groups while this level systematically increased to up to 30% in the case of the severely CAD-deficient lines. The low-molecular weight phenolics released upon mild alkaline treatment were analyzed by GC-MS. In agreement with previous results (Baucher et al., 1996; Lapierre et al., 1999), we found that a mild alkaline treatment released 10 times more syringaldehyde from the CAD-deficient lines than from the control lines (0.25% of extract-free wood, versus 0.02%). Further model experiments are necessary to determine which are the parent structures of this alkali-released syringaldehyde, either the syringaldehyde end-groups **L4S** and/or the sinapaldehyde structures **L1S** with a free phenolic group at C4.

Sinapaldehyde is less easily oxidized than sinapyl alcohol (Deighton et al., 1999). In addition, phenolic structures are more susceptible to oxidation when occurring as free monomers than when attached to lignins as terminal end-groups (Brunow et al., 1998). During the polymerization step occurring in the cell walls of the CAD-deficient poplars, the available coniferyl and sinapyl alcohol would therefore be oxidized first to give rise to the conventional **G**- and **S**-units. The oxidation of sinapaldehyde which accumulates as a consequence of CAD deficiency would thereby follow through an 8–O–4-coupling mode. The resulting sinapaldehyde quinone methide 8–O–4-coupling product would re-aromatize by 8-proton elimination (Connors et al., 1970; Kim et al., 2003) to yield 8-O-4-linked sinapaldehyde end-groups, units **L2S**. Once incorporated into the lignin network, the lower oxidizability of sinapaldehyde end-groups would impede the further growth of the polymer from these terminal units. Such a scenario would lead to the formation of a disorganized lignin network with small lignin domains, a hypothesis which is strongly supported by the observation that lignins of CAD-deficient plants are enriched in free phenolic groups and more soluble in alkali than control lignins. Further support for this hypothesis derives from the substantially lower average molecular weight of lignin fractions isolated from CAD-deficient poplars, relative to the corresponding controls (Baumberger et al., 2002).

3. Conclusion

Thioacidolysis-derived indene marker compounds are valuable for ascertaining plant responses to various levels of CAD down-regulation. Beside these additional sinapaldehyde-derived units and relative to the control samples, lignins in CAD-deficient poplar lines had less conventional **S**-units and β–O–4-bonds and more free phenolic groups. We found that almost half of the polymer fraction in the most deficient lines could be solubilized in alkali at room temperature. This unusual behavior suggests that lignins in CAD-deficient poplars occur as small, alkali-leachable lignin domains.

4. Experimental

4.1. Plant material

The poplar clone Ogy (*Populus deltoides*×*Populus nigra*)was transformed using a desarmed *Agrobacterium tumefaciens* strain (C58pMP90 with a binary vector derived from pBIN+) to introduce the entire *cad* cDNA in antisense orientation under the control of the double 35S promoter from CaMV, according to a procedure optimized from the method of Noël et al. (2002). A series of 12 independent primary transformants was grown in the greenhouse for 7 months, together with control lines. Stems were collected and debarked. All lignin analyses were performed on extract-free wood, ground to pass a 0.5 mm sieve before solvent extraction (toluene-EtOH, 2:1, EtOH, and H_2O). Two soluble lignin fractions were isolated from the extract-free wood of a transformed and of a control line, by dioxane:H_2O (9:1) extraction and after ball milling and cellulase hydrolysis of the sample (Lapierre et al., 1999).

Single ASCAD21 and ASCOMT2B and double ASCAD21×ASCOMT7 transformants were obtained from the poplar clone INRA 717 1B-4 as previously described (Lapierre et al., 1999). A series of 1-year old transformants grown in the greenhouse was collected and treated as reported for the Ogy series, before lignin analyses.

Measurements of COMT and CAD activities were run as previously reported (Van Doorsselaere et al., 1995b; Baucher et al., 1996).

4.2. Lignin analyses

The Klason lignin determination of the extract-free wood was run by the standard procedure (Dence, 1992). Lignin structure was investigated using thioacidolysis as previously described (Lapierre et al., 1995, 1999). The lignin-derived compounds were identified by GC–MS of their TMS derivatives. Mild alkaline hydrolysis of the extract-free wood and GC–MS analyses of the low molecular weight phenolics were run according to Lapierre et al. (1999). The quantitative evaluation of indenes (**2Sa** + **2Sb**) and **2Gb** was carried out from ion chromatograms reconstructed at *m*/*z* 384 and 354, respectively, while that of the conventional **G**- and **S**-monomers, of the coniferaldehyde dithioketal **3G** and of the syringaldehyde dithioketal **4S** was done from their prominent benzylic ions at *m*/*z* 269 or 299. All the calculations were done with the response factors of the

main **1G** and **1S** monomers, relative to the C22 internal standard.

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