Essential Role of Caffeoyl Coenzyme A *O*-Methyltransferase in Lignin Biosynthesis in Woody Poplar Plants

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Caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) has recently been shown to participate in lignin biosynthesis in herbacious tobacco plants. Here, we demonstrate that CCoAOMT is essential in lignin biosynthesis in woody poplar (*Populus tremula × Populus alba*) plants. In poplar stems, CCoAOMT was found to be expressed in all lignifying cells including vessel elements and fibers as well as in xylem ray parenchyma cells. Repression of CCoAOMT expression by the antisense approach in transgenic poplar plants caused a significant decrease in total lignin content as detected by both Klason lignin assay and Fourier-transform infrared spectroscopy. The reduction in lignin content was the result of a decrease in both guaiacyl and syringyl lignins as determined by in-source pyrolysis mass spectrometry. Fourier-transform infrared spectroscopy indicated that the reduction in lignin content resulted in a less condensed and less cross-linked lignin structure in wood. Repression of CCoAOMT expression also led to coloration of wood and an elevation of wall-bound *p*-hydroxybenzoic acid. Taken together, these results indicate that CCoAOMT plays a dominant role in the methylation of the 3-hydroxyl group of caffeoyl CoA, and the CCoAOMT-mediated methylation reaction is essential to channel substrates for 5-methoxylation of hydroxycinnamates. They also suggest that antisense repression of CCoAOMT is an efficient means for genetic engineering of trees with low lignin content.

Lignin is a complex phenylpropanoid polymer mainly found in walls of xylem cells such as tracheary elements and xylary fibers. Lignin, which contributes up to 15% to 35% of the dry weight of wood, ranks the second most abundant biomass on earth after cellulose. Lignin is considered to be dehydrogenatively polymerized from the monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monolignols are synthesized through the phenylpropanoid pathway (Fig. 1). It is obvious that structurally these monolignols differ only by the methoxyl group at the 3C and 5C positions of the aromatic ring. Therefore, the enzymatic steps involved in the methoxylation of hydroxycinnamic acids are critical in the synthesis of different monolignols, thus influencing lignin composition. The important roles of methoxylation in determining lignin composition have been clearly demonstrated in transgenic plants with alterations in the expression of genes involved in methoxylation (Dwivedi et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Meyer et al., 1998; Tsai et al., 1998; Zhong et al., 1998).

Since the first elucidation of the phenylpropanoid biosynthetic pathway, the methylation step has been thought to be carried out by caffeic acid *O*-methyltransferase (COMT) using free acid forms of hydroxycinnamates as substrates (Fig. 1). COMTs from angiosperms have been shown to methylate both the 3-hydroxyl group of caffeic acid and the 5-hydroxyl group of 5-hydroxyferulic acid (Davin and Lewis, 1992). Because COMT was considered to be the sole enzyme involved in methylation, COMT had long been targeted for the reduction of lignin content in the hope of reducing pollution from pulping or improving digestibility of forages. However, transgenic plants with a reduction in COMT activity alone showed a dramatic reduction in syringyl lignin without an apparent decrease in total lignin content (Dwivedi et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Tsai et al., 1998). This contradicted with the early presumption of COMT as the sole *O*-methyltransferase (OMT) involved in monolignol biosynthesis. Instead, it suggested that there might be other OMTs involved in the methylation reactions in monolignol biosynthesis.

It was suggested that methylation might occur on the ester forms of hydroxycinnamic acids (Neish, 1968). However, this hypothesis was largely ignored probably due to the early demonstration that COMT used the acid forms of hydroxycinnamates as substrates. An OMT that uses CoA ester forms of hydroxycinnamates as substrates was first detected in parsley and carrot (Kühnl et al., 1989; Pakusch et al., 1989; Fig. 1). It was shown that the caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) activity in the cultured cells of parsley and carrot was rapidly induced in response to the challenge of fungal elicitors.

Figure 1. A scheme of methylation pathways in lignin biosynthesis. Because repression of CCoAOMT significantly reduces both guaiacyl and syringyl lignins, it indicates that CCoAOMT plays important roles in the methylation of the 3-hydroxyl group of caffeoyl CoA, as well as in the supply of substrates for further methoxylation at the 5-position of hydroxycinnamates. CAD, Cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-coenzyme A reductase; F5H, ferulate 5-hydroxylase.
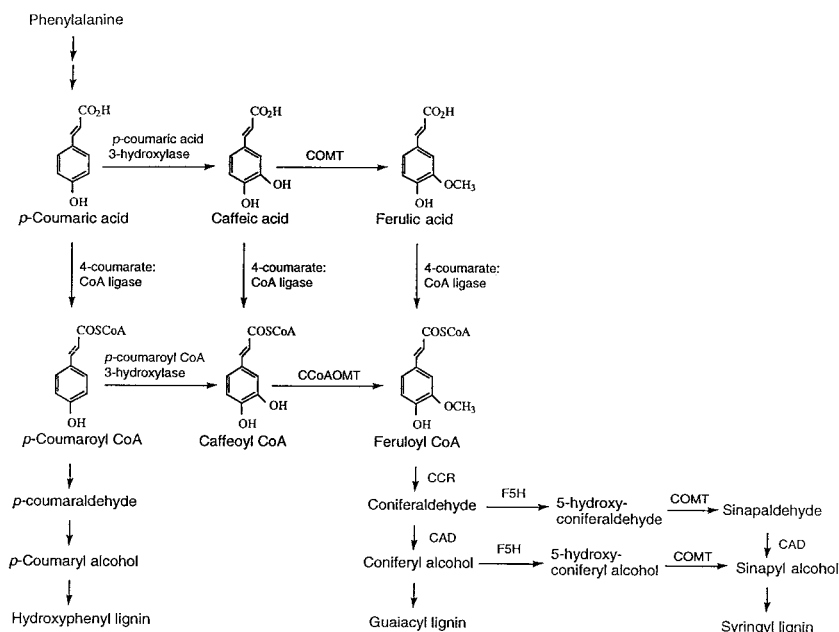
The CCoAOMT cDNA was subsequently isolated from parsley using partial peptide sequence deduced from purified parsley CCoAOMT protein (Schmitt et al., 1991). Because there was no lignin deposited in the walls of cultured cells challenged with fungal elicitors, it was suggested that CCoAOMT was involved in the formation of cell wall ferulic esters (Pakusch et al., 1991). The first evidence for a possible involvement of CCoAOMT in lignin biosynthesis came from the study of xylogenesis in the zinnia system (Ye et al., 1994). It was found that the activity of an OMT that uses both caffeoyl CoA and 5-hydroxyferuloyl CoA as substrates increased concomitantly with the timing of lignification during in vitro differentiation of tracheary elements. The expression of CCoAOMT gene was also shown to be induced during lignification in both in vitro tracheary elements and lignifying tissues of zinnia stems. Because of the close association of CCoAOMT expression with lignification, it was proposed that CCoAOMT was involved in an alternative methylation pathway in lignin biosynthesis (Ye et al., 1994).

In addition to the finding of its close association with lignification in zinnia and parsley (Ye et al., 1994; Ye and Varner, 1995), CCoAOMT has been shown to be expressed in lignifying tissues of a number of plants such as forsythia, tobacco, tomato, alfalfa, soybean, and pine (Ye, 1997; Inoue et al., 1998; Martz et al., 1998; Kersey et al., 1999; Li et al., 1999). In aspen, CCoAOMT activity was shown to be seasonally regulated during wood formation (Meng and Campbell, 1998). These studies further supported the hypothesis of the role of CCoAOMT in lignification.

The essential role of CCoAOMT in lignin biosynthesis was unequivocally demonstrated in transgenic studies (Zhong et al., 1998). Repression of CCoAOMT expression in transgenic tobacco plants caused a dramatic decrease in lignin content. The reduction in lignin content accompanied with a decrease in both guaiacyl and syringyl lignin. The effects of repression of CCoAOMT expression on lignin content and composition were in sharp contrast to those caused by repression of COMT expression, which only decreased syringyl lignin unit without reduction in lignin content. These transgenic studies on both COMT and CCoAOMT demonstrated that methylation reactions in lignin biosynthesis were carried out by both CCoAOMT and COMT in tobacco.

Because the bulk amount of lignin is produced in wood or secondary xylem in trees, it is important to investigate whether a similar effect on lignin production could be achieved by repression of CCoAOMT expression in woody species. In this paper, we present our transgenic analysis of CCoAOMT repression in poplar (*Populus tremula* × *Populus alba*) trees. Reduction of CCoAOMT resulted in alterations in both lignin content and composition in poplar wood, an effect similar to that obtained in transgenic tobacco plants. This demonstrates that CCoAOMT plays an essential role in lignin biosynthesis not only in herbacious plants but also in woody species.

RESULTS

Accumulation of CCoAOMT in Lignifying Tissues

To investigate the role of CCoAOMT in wood formation, we first examined whether CCoAOMT expression was closely associated with lignifying tissues in poplar. Young poplar stems were used for immunolocalization of CCoAOMT with antibodies against zinnia CCoAOMT (Ye, 1997). In the top part

of a stem in which the secondary growth was initiating, CCoAOMT signal was predominant in xylem parenchyma cells, especially those surrounding vessel elements (Fig. 2A). Low levels of CCoAOMT were also evident in differentiating phloem fibers and developing interfascicular xylem. In the lower part of a stem in which intensive secondary growth was undergoing, high levels of CCoAOMT were detected (Fig. 2, B and C). Noticeably, CCoAOMT signal was not only present in xylary fibers and phloem fibers but also abundant in xylem ray parenchyma (Fig. 2, B and C). The signal appeared to be present mainly in the newly formed xylem but absent in the early wood (Fig. 2C). It was noted that most vessel elements did not show high levels of signal except for a few of them (Fig. 2C). It was also evident that some phloem cells showed specific CCoAOMT signal (Fig. 2, A–C) compared with the control (Fig. 2D). These phloem cells were likely differentiating secondary phloem fibers that are common in woody species. The control
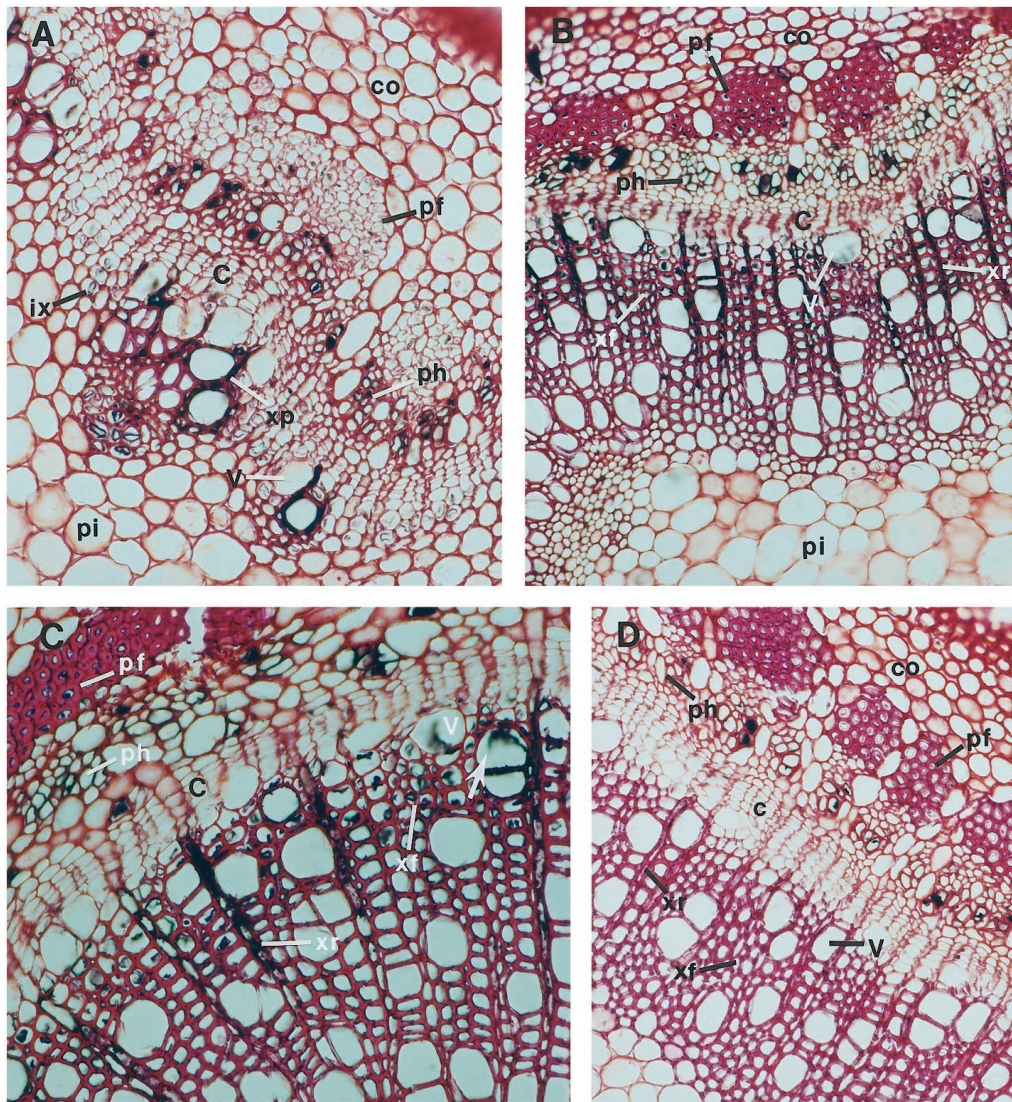

Figure 2. Immunolocalization of CCoAOMT in poplar. Stems from young poplar seedlings were sectioned, probed with polyclonal antibodies against zinnia CCoAOMT or the preimmune serum, and then detected with gold-conjugated secondary antibodies. Signals were amplified with silver enhancement and seen as black. Sections were counterstained with safranin O to show cell walls (red staining). A, Section from the top part of a stem probed with the CCoAOMT antibodies. The signal was mainly present in xylem parenchyma cells and phloem. Low levels of signal were also evident in vessel elements, differentiating phloem fibers, and differentiating interfascicular xylem cells. B and C, Sections from the lower part of a stem probed with the CCoAOMT antibodies. Intense signals were seen in xylary fibers, xylem ray parenchyma cells, phloem fibers, and phloem. The signal was also evident in a few of vessel elements (arrow). D, Section from the lower part of a stem probed with the preimmune serum. No signal was seen in xylem and phloem fibers except in a few scattered phloem cells. c, Cambium; co, cortex; ix, interfascicular xylem; ph, phloem; pf, phloem fiber; pi, pith; v, vessel; xf, xylary fiber; xp, xylem parenchyma; xr, xylem ray parenchyma. Magnifications, ×190 (A and C) and ×150 (B and D).

section incubated with the pre-immune serum did not show any signals in xylem and phloem fibers (Fig. 2D). The few scattered cells in phloem showing signals might be mucilage cells that non-specifically trapped antibodies (Fig. 2D). These results demonstrate that CCoAOMT expression is temporally and spatially regulated, and it is closely associated with lignification.

Generation of Transgenic Poplar Plants with a Reduction in CCoAOMT

To repress CCoAOMT expression in poplar, we used the antisense approach by using an endogenous CCoAOMT cDNA isolated from the poplar cDNA library. The amino acid sequence deduced from the coding region of the cDNA exhibited over 90% similarity with CCoAOMTs isolated from aspen (Meng and Campbell, 1995), *Populus trichocarpa* (Chen et al., 1998), tobacco (Martz et al., 1998), parsley (Schmitt et al., 1991), and zinnia (Ye et al., 1994). Because antisense expression of one CCoAOMT cDNA effectively repressed expression of all *CCoAOMT* genes in transgenic tobacco plants (Zhong et al., 1998), we reasoned that antisense expression of one CCoAOMT cDNA might also work in poplar. The poplar CCoAOMT cDNA was inserted in the antisense orientation downstream of the cauliflower mosaic virus 35S promoter in the pBI121 binary vector to create the expression construct pACoA. The pACoA construct was transformed into *Agrobacterium*, and *Agrobacterium* harboring the pACoA construct was used to infect poplar stem segments. Transgenic plants with resistance to the selection drug kanamycin were selected and confirmed for the presence of the antisense CCoAOMT cDNA.

To select plants with a reduction in CCoAOMT, we first assayed the CCoAOMT activity in stem extracts of the transgenic plants. Of eight independent transgenic plants analyzed, two plants (ACoA3 and ACoA8) showed a significant decrease in CCoAOMT activity compared with the wild-type plants transformed with the control pBI121 vector. The CCoAOMT activity in ACoA8 was reduced to approximately 30% of that in the wild type (Fig. 3). To confirm the specificity of the reduction in CCoAOMT activity, we examined the COMT activity in the stem extracts of transgenic plants. As expected, all the transgenic plants including ACoA8 had the similar levels of COMT activity as the wild type (Fig. 3).

We further used protein gel-blot analysis to confirm the activity assay results. The antibodies against zinnia CCoAOMT specifically recognized a protein band at an apparent molecular mass of 28 kD in the protein gel blot of poplar stem extracts (data not shown). Protein gel-blot analysis showed that the ACoA8 plant had a dramatic decrease in CCoAOMT protein level compared with the wild type (Fig. 4), which was consistent with the CCoAOMT activity

assay. In addition, ACoA3 also had a slight decrease in CCoAOMT protein level. When the stem extracts of transgenic plants were probed with COMT antibodies, a similar level of COMT was detected in all the transgenic plants and the wild type. These results clearly demonstrate that the reduction of CCoAOMT in ACoA8 is the result of antisense repression of CCoAOMT.

Reduction in CCoAOMT Decreases Lignin Content

To examine the effects of reduction in CCoAOMT on lignin synthesis, we collected stems of the transgenic plants for assay of Klason lignin. In the wild type, lignin constituted approximately 20% of total cell wall residues. Transgenic plants ACoA1 through ACoA7 had similar levels of lignin as the wild type, indicating that no significant alteration in lignin content occurred in these plants (Fig. 5). However, the ACoA8 plant, which had approximately 70% reduction in CCoAOMT, showed a significant decrease in Klason lignin content. The lignin content in ACoA8 was reduced to 60% that of the wild type (Fig. 5). We also assayed lignin content in the 9-month-old transgenic plants. At this stage, plants grew up to approximately 1.5 m tall, and basal parts of the stems were approximately 1.5 cm in diameter. The ACoA8 plant still had only 65% of wild-type lignin level. These results indicate that the repression of lignin synthesis in ACoA8 is maintained throughout the 9-month growth period.

To examine the effects of CCoAOMT repression on lignin composition, we determined both guaiacyl
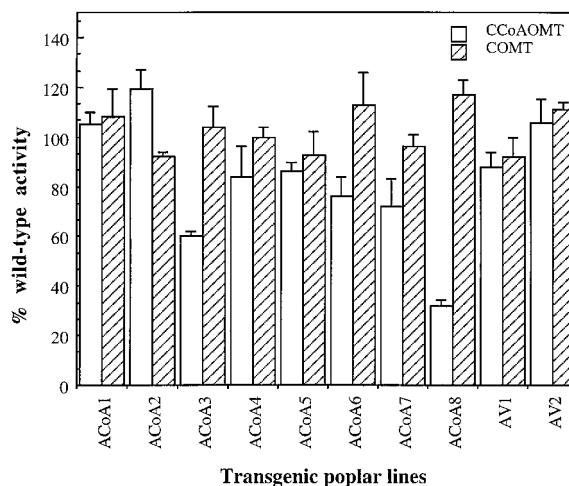

Figure 3. CCoAOMT and COMT activities in transgenic poplar plants. Stems of 6-month-old transgenic plants were used for assay of both CCoAOMT and COMT activities. *O*-methyltransferase activities in transgenic plants were expressed as percentage of activity in the wild type. The transgenic line ACoA8 had approximately 70% reduction in CCoAOMT activity, whereas it had the same level of COMT activity as the wild type. Data are the means ± SD of three assays. ACoA, Plants with antisense expression of CCoAOMT. AV, Wild type transformed with the binary vector pBI121.
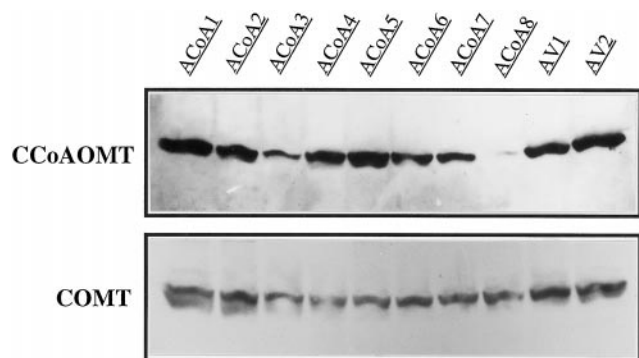
Figure 4. Protein gel-blot analysis of CCoAOMT and COMT in transgenic poplar plants. Protein extracts from stems of transgenic plants were separated on polyacrylamide gels and probed with either antibodies against zinnia CCoAOMT or antibodies against zinnia COMT. A reduction in CCoAOMT level was evident in the transgenic line ACoA8, which was consistent with the activity assay results. Abbreviations are as given for Figure 3.

and syringyl lignin levels in the ACoA8 plant by in-source pyrolysis mass spectrometry, a technique that is ideal for comparison of the relative amounts of cell wall macromolecules. The relative intensity of mass peaks for lignin over those for polysaccharides was significantly reduced in ACoA8 compared with that of the wild type (Fig. 6). For example, the relative ratio of mass peaks of *m/z* 180 (guaiacyl and syringyl lignin units) over *m/z* 126 (polysaccharides) in ACoA8 was reduced to 51% of that in ACoA4 (Fig. 6, A and B). This indicated that both guaiacyl and syringyl lignin levels relative to cell wall polysaccharides were lower in ACoA8 than in plants without lignin reduction. The decrease in monolignol levels was observed in both 6- and 9-month-old plants (Fig. 6). We further used principal component analysis of pyrolysis mass spectrometry data to evaluate the relationships between mass and samples (Morrison et al., 1999). Principal component analysis, based on masses for lignin, clearly separated ACoA8 away from the wild-type and other transgenic plants without lignin reduction (Fig. 7), confirming that the reduction in both guaiacyl and syringyl lignin levels in ACoA8 was significant. Taken together, these results clearly demonstrate that lignin biosynthesis was impaired as a result of reduction in CCoAOMT.

Fourier Transform Infrared Spectroscopy of Cell Walls in the Wild-Type and ACoA8 Plants

Fourier transform infrared spectroscopy (FT-IR) has been widely used to analyze plant cell wall structures and compositions (McCann et al., 1997). FT-IR spectrum is generated from radiation absorbed by molecules with polarized bonds or dipole bonds after excitation with infrared light. The ratio of absorbance intensities at different wavelengths is related to the concentration of different molecules in a cell wall sample. Diffuse reflectance infrared Fourier trans-

form spectroscopy (DRIFTS) of cell wall materials is non-destructive, which better represents the native state of wall structures and compositions (Himmelsbach et al., 1986; Pandey and Theagarajanm, 1997).

DRIFTS of the wild-type and ACoA8 samples gave almost identical spectra (Fig. 8B), which was expected because the only difference between the two was the lignin level. The absorbance at 1,504 cm^{-1}, due to the ring breathing mode of the aromatic rings in lignin (Hergert, 1971), was used for the measurement of lignin. As shown in the aspen lignin reference spectrum (Fig. 8A), this band has no interfering bands in cellulose. The band at 899 cm^{-1}, due to the anomeric C-O stretch in cellulose (Hergert, 1971), was chosen as the band to represent carbohydrate. This was chosen for two reasons: First, it is a weak band that is not subject to saturation when samples are run undiluted, and second, it has no interfering band from lignin (Fig. 8A). A comparison was made of the ratios of lignin to carbohydrate on the basis of the average intensities (five repetitions) of these two bands after the application of local baselines. The results (Table I) indicated that the lignin in ACoA8 was reduced to 56% from that of the wild type.

The DRIFTS analysis provided evidence that lignin in ACoA8 was also less cross linked. As shown in the spectrum of the standard aspen lignin (Fig. 8A), the absorbances at 1,504 cm^{-1} and 1,596 cm^{-1} are both characteristic of lignin. It has been reported that the absorbance at 1,504 cm^{-1} is more intense in more condensed, highly cross-linked lignin structure. The higher the 1,504:1,596 cm^{-1} ratio, the more condensed and cross-linked the lignin structure (Akin et al., 1993a; Stewart et al., 1997). Quantitative measurement of lignin absorbance clearly showed that the 1,504:1,596 cm^{-1} ratio in ACoA8 was reduced to 82% that of the wild type (Table II). This indicates that the reduction in lignin content in ACoA8 leads to a less
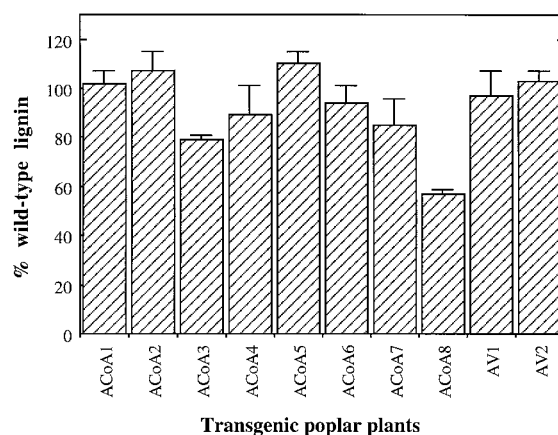

Figure 5. Lignin content in transgenic poplar plants. Stems of 6-month-old transgenic plants were collected and used for assay of Klason lignin. Klason lignin in transgenic plants is expressed as percentage of lignin in the wild type. The transgenic line ACoA8 showed approximately 40% reduction in lignin content. Data are the means ± SD of two assays. Abbreviations are as given for Figure 3.
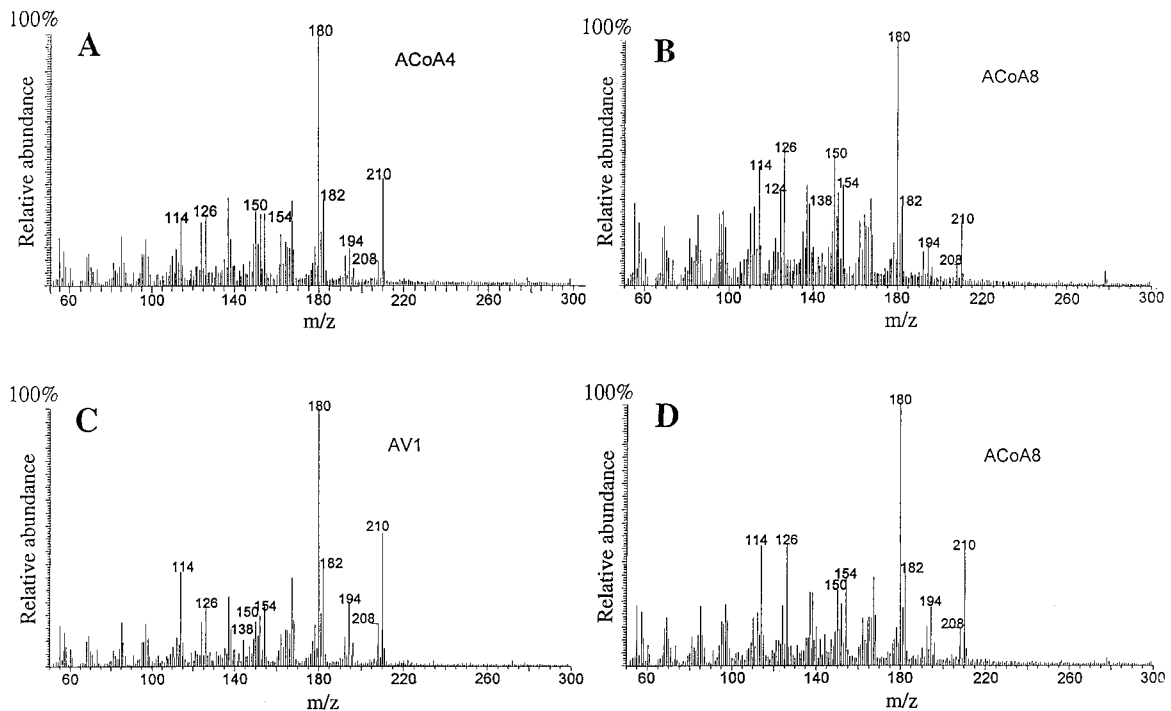
Figure 6. In-source pyrolysis mass spectrometry of cell walls of transgenic poplar plants. In-source pyrolysis mass spectrometry was performed using a Finnigan GCQ equipped with a direct exposure probe (rhenium loop), as described by Morrison and Archibald (1998). Mass peaks of guaiacyl lignin had m/z values of 124, 137, 138, 150, 152, 164, 166, 178, and 180. Mass peaks of syringyl lignin had m/z values of 154, 167, 168, 180,182, 194, 208, and 210. Mass peaks of cellulose and amylose had m/z values of 57, 60, 73, 85, 86, 96, 98, 100, 102, 110, 112, 126, and 144. Mass peaks of hemicellulose had m/z values of 58, 85, 86, and 114. A and B, Spectra of cell walls from 6-month-old plants. C and D, Spectra of cell walls from 9-month-old plants. It is evident that the relative intensity of mass peaks for lignin over those for cellulose, amylose, or hemicellulose in AcoA8 was significantly reduced compared with the controls. Abbreviations are as given for Figure 3.

condensed and less cross-linked lignin structure in wood.

Lignin Reduction Has No Significant Effect on Plant Growth and Morphology

Transgenic plants were grown in the greenhouse. During the 6- and 9-month growth periods, no abnormal growth patterns or lesions were observed in the ACoA8 plants (data not shown). This indicates that poplar plants could at least tolerate up to 40% reduction in lignin without major adverse effects on the normal plant growth and development in the greenhouse conditions.

Lignin provides mechanical strength to water-conducting vessel elements. To ascertain whether a 40% reduction in lignin could significantly weaken the wall strength, we examined the anatomy of vessels in wood. It appeared that although most vessel walls in the 6-month-old ACoA8 plant had regular shapes similar to the wild type, walls of a few vessels were slightly deformed (Fig. 9, A and B). It was also noted that the wood from ACoA8 had less intense lignin staining than the wild-type wood (Fig. 9, A and B), which was consistent with the data from lignin analysis. In the 9-month-old ACoA8 plant,

deformed walls were still found in only a few early-developed vessels. It was also evident that ACoA8 showed a wider zone of less lignin staining in the newly differentiated xylem region (Fig. 9D) compared with the wild type (Fig. 9C). These results indicate that a 40% reduction in lignin only causes a minor change in vessel wall shapes, and this mainly happened in the early stage of xylem development.

Repression of CCoAOMT Expression Causes a Slight Coloration in Wood

When the ACoA8 stems were cut, we noticed that the wood displayed a light orange color, whereas the wild-type wood did not (Fig. 10A). The light orange color was seen immediately after cut. Thus, it was not caused by wounding-induced reactions, which normally took a few minutes. The light orange color in ACoA8 wood was resistant to extraction with 50% (v/v) methanol (Fig. 10B), indicating that it was not caused by accumulation of free phenolics.

The observation of coloration in ACoA8 wood prompted us to investigate whether the coloration was attributed by an accumulation of phenolics. Because repression of CCoAOMT blocks the conversion of caffeoyl CoA into feruloyl CoA, it was predicted
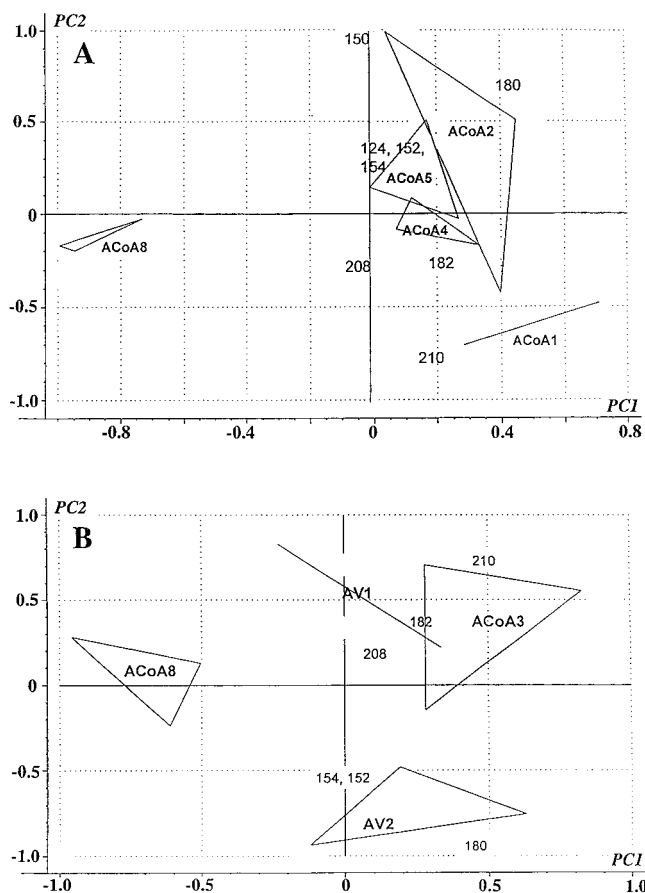
Figure 7. Principal component analysis of the pyrolysis mass spectrometry data. Each sample was run in triplicate with pyrolysis mass spectrometry. Data from three separate assays were used for principal component analysis based on masses for lignin (indicated with numbers). A, Cell wall samples from 6-month-old poplar plants. B, Cell wall samples from 9-month-old plants. It was evident that AcoA8 significantly separated from others based on masses for lignin. Abbreviations are as given for Figure 3.

way is a general one in lignin biosynthesis during normal plant growth and development (Ye et al., 1994; Ye and Varner, 1995).

## CCoAOMT Is Expressed in Lignifying Tissues in Poplar Stems

It has been shown that some lignin pathway enzymes are differentially expressed in different lignifying tissues and their differential expression may contribute to lignin heterogeneity (Ye and Varner, 1995). Thus, it is important to examine whether CCoAOMT is present in all lignified tissues in poplar. Immunolocalization results demonstrated that CCoAOMT was expressed in all lignifying tissues including tracheary elements, xylary fibers, and phloem fibers. These results are consistent with the CCoAOMT expression patterns found in zinnia, forsythia, tobacco, tomato, soybean, and alfalfa (Ye, 1997; Kersey et al., 1999). However, the vessel elements of poplar appear to have a much lower level of CCoAOMT than those of herbacious plants (Fig. 2; Ye, 1997), probably due to the rapid degradation of cellular contents in vessels of woody species.

A high level of CCoAOMT expression was also seen in xylem ray parenchyma cells (Fig. 2). Other lignin pathway enzymes such as Phe ammonia-lyase (Bevan et al., 1989) and cinnamyl alcohol dehydrogenase (Feuillet et al., 1995) have also been shown to be expressed in xylem ray parenchyma. Ray parenchyma cells are thought to provide lignin precursors to adjacent tracheary elements and fibers, which may be especially important for heartwood wood formation during which more lignin and lignans are deposited. Therefore, it is not surprising to see high-level expression of lignin pathway enzymes in xylem ray parenchyma in poplar wood.

that the substrate caffeoyl CoA might be accumulated. However, gas-liquid chromatography of the extracted phenolics did not show any significant increase of caffeate. Instead, it was found that a compound corresponding to *p*-hydroxybenzoic acid was elevated 2- to 3-folds in both free and wall-bound phenolic extracts of the ACoA8 plant (data not shown), indicating that repression of CCoAOMT resulted in an elevation of *p*-hydroxybenzoic acid in wood.

DISCUSSION

The essential role of CCoAOMT in lignin biosynthesis previously has been demonstrated in herbacious tobacco plants (Zhong et al., 1998). In this report, we have provided further evidence that CCoAOMT is essential in lignin biosynthesis in woody poplar plants. These results have unequivocally confirmed the hypothesis that CCoAOMT-mediated methylation path-

CCoAOMT Plays a Predominant Role in the Synthesis of Guaiacyl Lignin and It Is Essential in Providing Substrates for the Synthesis of Syringyl Lignin

It has been shown that, although repression of COMT led to an almost complete loss of syringyl lignin, the total lignin content was not altered (Atanassova et al., 1995; Doorsselaere et al., 1995). Results from both transgenic tobacco and transgenic poplar studies demonstrate that antisense repression of CCoAOMT results in a reduction in lignin content. This indicates that CCoAOMT plays a predominant role in the determination of lignin content. Furthermore, repression of CCoAOMT results in a decrease of both guaiacyl lignin and syringyl lignin units in both transgenic tobacco and transgenic poplar plants (Zhong et al., 1998). This suggests that CCoAOMT also plays important roles in the methylation of both 3- and 5-hydroxyl groups of hydroxycinnamates, which is consistent with its bifunctional activities

Figure 8. Diffuse reflectance infrared Fourier transform spectra of cell walls of poplar plants. A, Spectra of standard lignin and cellulose. B, Spectra of cell walls from transgenic poplar plants. Lignin absorbances at 1,504 and 1,596 cm^{-1}, and cellulose absorbance at 899 cm^{-1} were used for quantitative analysis. Abbreviations are as given for Figure 3.
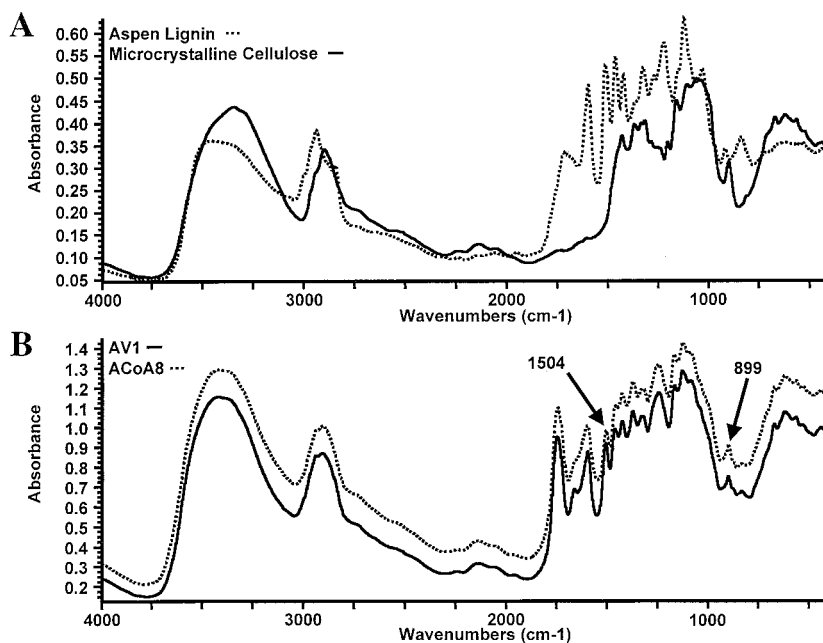
using both caffeoyl CoA and 5-hydroxyferuloyl CoA as substrates (Ye et al., 1994; Meng and Campbell, 1998).

Recent findings on the activities of ferulate 5-hydroxylase and COMT indicate that the roles of CCoAOMT and COMT in the methylation of 3- and 5-hydroxyl groups of hydroxycinnamates are much more complicated than previously expected. Biochemical analysis demonstrates that in the presence of ferulic acid, coniferaldehyde, or coniferyl alcohol, ferulate 5-hydroxylase almost exclusively hydroxylates the 5-position of coniferaldehyde or coniferyl alcohol (Humphreys et al., 1999; Osakabe et al., 1999). In addition, both 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol can be efficiently methylated by COMT (Humphreys et al., 1999; Maury et al., 1999; Osakabe et al., 1999). The possible use of coniferyl alcohol as a precursor for the synthesis of syringyl lignin was also revealed by feeding of labeled coniferyl alcohol precursors to plants (Matsui

et al., 1994; Chen et al., 1999). These new lines of evidence indicate that normally ferulic acid is not converted to 5-hydroxyferulic acid, thereby the CCoAOMT-mediated methylation of 5-hydroxyferuloyl CoA could not occur during lignin biosynthesis. In other words, the 5-methylation of hydroxycinnamates is solely mediated by COMT (Fig. 1).

This new route of 5-methoxylation of hydroxycinnamates places feruloyl CoA as the sole substrate for the synthesis of both guaiacyl and syringyl lignin units (Fig. 1). Therefore, a reduction in CCoAOMT could effectively affect the availability of the substrate feruloyl CoA. This can sufficiently explain the effective reduction in both guaiacyl and syringyl lignin in the antisense CCoAOMT plants. It also indicates that CCoAOMT plays a dominant role in the methylation of the 3-hydroxyl group of caffeoyl CoA, and the CCoAOMT-mediated methylation reaction is essential to channel substrates for 5-methoxylation of hydroxycinnamates.

Table I. *Measurement of lignin and carbohydrate absorbances from the DRIFTS spectra of the wild-type and transgenic plants*

Each data point is the means ± SD of absorbances from five separate DRIFTS.

Sample	Lignin Absorbance at 1,504 cm^{-1a}	Carbohydrate Absorbance at 899 cm^{-1b}	Absorbance Ratio between 1,504 and 899 cm^{-1}
Wild type	0.211 ± 0.020	0.054 ± 0.003	3.939 ± 0.354
ACoA8	0.138 ± 0.015	0.062 ± 0.004	2.237 ± 0.246

[a] The absorbance at 1,504 cm^{-1} was calculated using the baseline starting from the absorbance at 1,539 cm^{-1} to the absorbance at 1,481 cm^{-1}. [b] The absorbance at 899 cm^{-1} was calculated using the baseline starting from the absorbance at 914 cm^{-1} to the absorbance at 883 cm^{-1}.

Table II. *Measurement of lignin absorbances from the DRIFTS spectra of the wild-type and transgenic plants*

Each data point is the means ± SD of absorbances from five separate DRIFTS.

Sample	Lignin Absorbance at 1,504 cm^{-1a}	Lignin Absorbance at 1,596 cm^{-1b}	Absorbance Ratio between 1,504 and 1,596 cm^{-1}
Wild type	0.201 ± 0.019	0.266 ± 0.024	0.756 ± 0.014
ACoA8	0.133 ± 0.015	0.214 ± 0.016	0.621 ± 0.061

[a] The absorbance at 1,504 cm^{-1} was calculated using the baseline starting from the absorbance at 1,547 cm^{-1} to the absorbance at 1,485 cm^{-1}. [b] The absorbance at 1,596 cm^{-1} was calculated using the baseline starting from the absorbance at 1,643 cm^{-1} to the absorbance at 1,547 cm^{-1}.
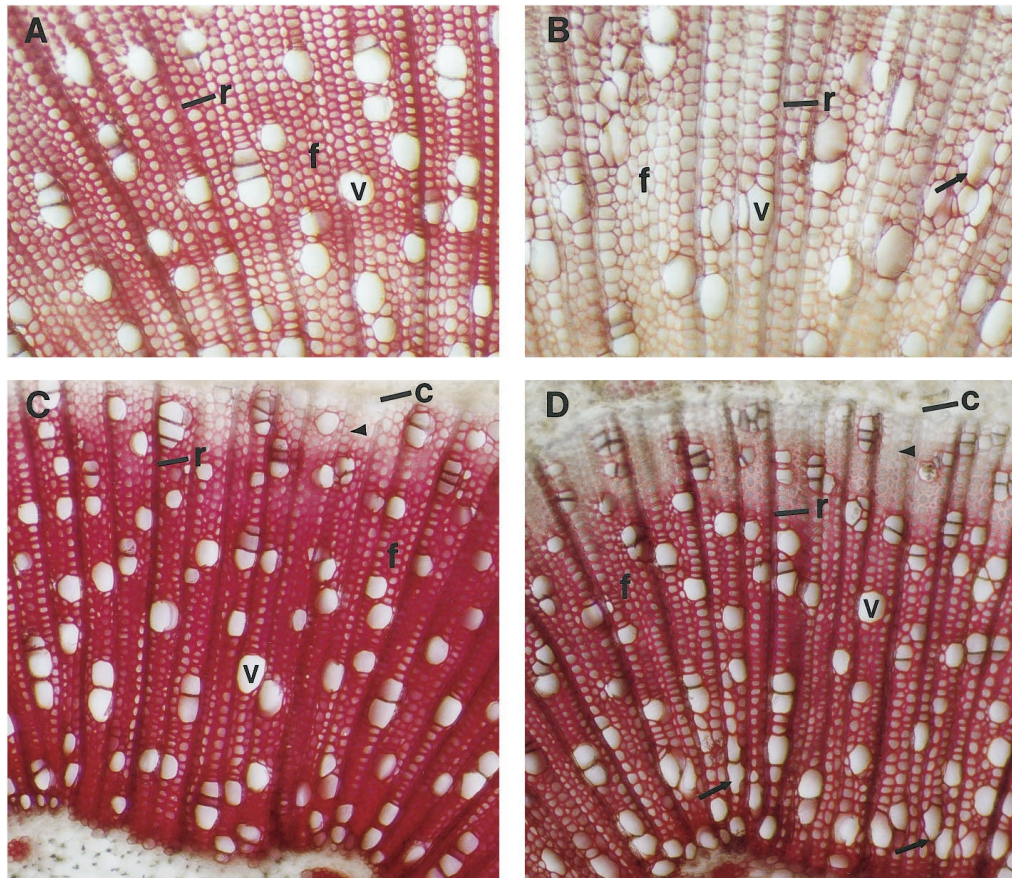
Figure 9. Wood anatomy of transgenic poplar plants. Wood from 6-month-old (A and B) and 9-month-old (C and D) plants was sectioned and stained for lignin with phloroglucinol-HCl. A, Wood from wild-type AV1 showing intense lignin staining and regular vessel shapes. B, Wood from transgenic line ACoA8 showing less lignin staining and a few deformed vessels (arrow). C, Wood from AV1 showing regular vessel shapes. D, Wood from ACoA8 showing less lignin staining in the newly-differentiated xylem (arrowhead) and a few deformed vessels (arrow) in the early wood. f, Fiber; r, ray parenchyma; v, vessel. Magnifications, ×220 (A and B) and ×110 (C and D).

It is evident from the transgenic analyses that CCoAOMT plays a predominant role in supplying feruloyl CoA for the synthesis of both guaiacyl lignin and syringyl lignin units, whereas COMT has no significant effect on the synthesis of guaiacyl lignin. However, results from biochemical characterizations of recombinant COMTs appear not to be in agreement with the transgenic studies. It has been shown that recombinant COMTs from alfalfa (Inoue et al., 1998), pine (Li et al., 1997), aspen (Meng and Campbell, 1998), and tobacco (Maury et al., 1999) can efficiently methylate both caffeoyl CoA and caffeic acid. This implies that repression of CCoAOMT could be compensated by COMT. Then, how could one explain the drastic difference between antisense repression of CCoAOMT and COMT? One possible explanation is that CCoAOMT might be present in a much larger quantity than COMT in lignifying cells, so that CCoAOMT could compensate for the loss of COMT, whereas COMT could not sufficiently compensate for the loss of CCoAOMT. It is also possible that in vivo COMT either might not have access to the substrate

caffeoyl CoA or it might not use caffeoyl CoA as a substrate.

Moderate Repression of CCoAOMT Shows Little Effect on Tree Growth

We previously have shown that transgenic tobacco plants with repression of CCoAOMT grew normally under greenhouse conditions (Zhong et al., 1998). In a similar manner, the transgenic poplar plants with a 70% repression of CCoAOMT showed normal growth patterns. These observations are in contrary to some reports from transgenic studies of some other genes involved in lignin biosynthesis. This may be because repression of CCoAOMT does not affect the biosynthesis of phenylpropanoid compounds such as chlorogenic acids and salicylic acid, which are important for plant defense. In addition, the remaining CCoAOMT activity is probably sufficient for the biosynthesis of monolignol derivatives, which have been shown to be important for plant growth (Lynn et al., 1987; Savidge, 1987). Therefore, it is not surprising
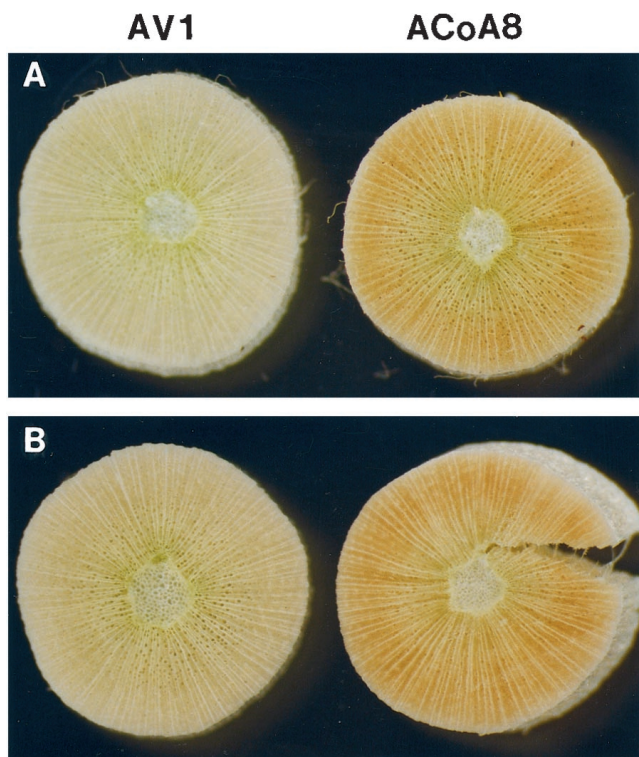
AV1   ACoA8

Figure 10. Fresh wood color of transgenic poplar plants. Fresh wood from 9-month-old poplar was sectioned and photographed immediately (A) or treated with 50% (v/v) methanol before photography. It was evident that the ACoA8 wood displayed light orange color, and the coloration remained after methanol extraction. Abbreviations are as given for Figure 3.

that a 70% repression of CCoAOMT does not have significant adverse impact on plant growth.

Lignin provides mechanical strength to wood. It is predicted that a complete loss of lignin would greatly reduce wood strength and adversely affect plant growth and morphology. The critical question is to what extent lignin reduction plant can tolerate without major adverse effects on plant growth. It appears that both tobacco and poplar transgenic plants with repression of CCoAOMT can tolerate a significant amount of lignin reduction without major visible defects in plant growth under greenhouse conditions. Similar observations were also found in transgenic Arabidopsis and aspen plants with repression of 4-coumarate:coenzyme A ligase (Lee et al., 1997; Hu et al., 1999). These observations are in agreement with the natural variation of lignin levels in woods. It has been reported that the lignin level varies from 15% to 35% of the dry weight of most woods (Sederoff et al., 1994), indicating that a moderate reduction of lignin in wood may not significantly alter plant growth and form. Further examination of the growth and survival of transgenic plants with reduction of lignin in field conditions will be necessary to validate these observations.

Coloration of Wood with Repression of CCoAOMT

It is interesting to note that the wood of transgenic poplar with repression of CCoAOMT displays a slight orange color. However, transgenic tobacco plants with repression of CCoAOMT did not show any coloration in xylem region (Zhong et al., 1998). This indicates that herbacious tobacco and woody poplar plants respond differently to the repression of CCoAOMT. It is possible that the phenylpropanoid pathway is more active in woody plants than in herbacious plants. This might lead to more accumulation of phenylpropanoid intermediates in the wood of transgenic plants, which might result in the coloration of wood. Analysis of wall-bound phenolics in the transgenic plant did reveal a significant accumulation of p-hydroxybenzoic acid. However, it appears this may not be the cause of the slight coloration in the ACoA8 wood.

Coloration of xylem or wood has been reported in transgenic plants or mutants with repression of other lignin pathway genes such as COMT, 4-coumarate: coenzyme A ligase, and cinnamyl alcohol dehydrogenase (Halpin et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Campbell and Sederoff, 1996; Kajita et al., 1997; Ralph et al., 1997; Tsai et al., 1998). Xylem or wood in plants with repression of COMT or cinnamyl alcohol dehydrogenase often shows strong red coloration, which has been attributed to the accumulation of hydroxycinnamyl aldehydes (Halpin et al., 1994; Higuchi et al., 1994; Ralph et al., 1997; Stewart et al., 1997; Tsai et al., 1998). In plants with repression of 4-coumarate-coenzyme A ligase, xylem displayed a brownish color, probably due to accumulation of hydroxycinnamic acids (Kajita et al., 1997). It is obvious that the coloration of wood with repression of CCoAOMT could not be caused by hydroxycinnamyl aldehydes because the CCoAOMT-mediated reaction is at the early step of the phenylpropanoid pathway. It will be interesting to figure out the cause of coloration in the wood of antisense CCoAOMT poplar plants.

CCoAOMT Is an Ideal Target for Genetic Manipulation of Lignin Content

Because the presence of lignin in forages decreases digestibility of animal feeding and because removal of lignin during pulping results in hazardous waste, it has long been proposed that alteration in lignin content or composition in forages and trees could lead to an improvement of digestibility of forages and a reduction of pollutants derived from pulping. To manipulate lignin content or composition, many attempts have been made to alter the expression of genes in the phenylpropanoid pathway or genes involved in the regulation of the phenylpropanoid pathway. Most of the transgenic works have been done in tobacco plants because of the easiness of transformation and the presence of significant amount of second-

ary xylem in stems. These studies have demonstrated that not only the lignin content but also the lignin composition can be altered in transgenic plants (Whetten et al., 1998). It has also been shown that alteration of lignin content or composition in transgenic plants could present benefits to reduce pollutions from pulping or increase digestibility of forages (Baucher et al., 1996, 1999).

It appears that only a few genes in the phenylpropanoid pathway have been shown to be ideal for lignin reduction. One obvious criterium would be that alteration of a target gene expression only results in a reduction in lignin content without adverse effects on plant growth and development. Several genes including Phe ammonia-lyase, cinnamoyl-coenzyme A reductase, and Myb proteins have been genetically manipulated, and reduction of lignin content in transgenic plants with alteration of expression of these genes has been achieved. However, the transgenic plants displayed abnormal plant growth and development (Elkind et al., 1990; Piquemal et al., 1998; Tamagnone et al., 1998), indicating that Phe ammonia-lyase, cinnamoyl-coenzyme A reductase, and Myb proteins are not ideal targets for genetic manipulation of lignin in forages and trees. In contrast, transgenic plants with repression of genes such as 4-coumarate:coenzyme A ligase (Kajita et al., 1996; Lee et al., 1998; Hu et al., 1999) and CCoAOMT showed normal growth patterns, suggesting that these genes are ideal targets for genetic manipulation of lignin in forages and trees.

Genetic manipulation of lignin has been attempted in poplar trees. Down-regulation of COMT or cinnamyl alcohol dehydrogenase in poplar led to an alteration of lignin composition without an apparent change in lignin content in the wood. It has been shown recently that repression of 4-coumarate:coenzyme A ligase leads to a decrease in lignin content in aspen trees (Hu et al., 1999). Our demonstration of reduction in lignin content in the CCoAOMT-repressed transgenic poplar trees represents another step toward the long-sought goals of reduction of lignin content in wood in the hope of reducing pollutants generated from pulping.

MATERIALS AND METHODS

Plant Materials

Poplar (*Populus tremula* × *Populus alba*; a gift from S.H. Strauss and C. Ma) seedlings were esthetically propagated on the modified Murashige and Skoog medium (Murashige and Skoog, 1962) as described by Leple et al. (1992) and used for transformation.

Immunolocalization

Poplar stem segments were fixed in 2% (v/v) glutaraldehyde overnight at 4°C. After fixation, segments were dehydrated through a gradient series of ethanol, cleared with xylene, and finally embedded in paraffin. Fifteen-

micrometer-thick sections were cut with a microtome and transferred onto glass slides coated with poly-Lys. Sections were deparaffinized with xylene and rehydrated through a gradient series of ethanol before used for immunolocalization. After being blocked in 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) solution (10 mM sodium phosphate, pH 7.2, 138 mM NaCl, and 3 mM KCl), sections were incubated with antibodies against zinnia CCoAOMT (1:1,000 dilution in PBS solution containing 0.1% [w/v] BSA; Ye, 1997). After washing, sections were probed with gold-conjugated goat anti-rabbit polyclonal antibodies (AuroProbe LM GAR, Amersham-Pharmacia Biotech, Uppsala; 1:40 dilution in PBS solution containing 0.1% [w/v] BSA). Specific bindings were revealed with a silver enhancement kit (IntenSe, Amersham-Pharmacia Biotech). Sections were further counter-stained with safranin O to show anatomy.

Construction of Antisense CCoAOMT Cassette

Stems from poplar seedlings were used to isolate mRNAs that were used to construct a poplar stem cDNA library. The library was used for immunoscreening of caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) cDNA with antibodies against zinnia CCoAOMT as described by Sambrook et al. (1989). Positive clones were selected for sequencing, and the CCoAOMT cDNA sequences were identified based on comparison with those in the public databases using the BLAST network service from the National Center for Biotechnology Information (Bethesda, MD).

To construct the antisense CCoAOMT cassette, the poplar CCoAOMT cDNA was re-amplified from the CCoAOMT clone by polymerase chain reactions with two primers (5'-ATGGCGACGAATGGGGAAGAGCAGCA-3' and 5'-CTGCTGCATAGGGTGACTCCGTATCC-3') and ligated into pGEM-T EASY vector (Promega, Madison, WI) to create the first shuttle vector pGEM-CoA. The cDNA insert in pGEM-CoA was cut out with *Eco*RI and ligated into the *Eco*RI site of pBluescript KS+ vector (Stratagene, La Jolla, CA) to create the second shuttle vector pBS-CoA. The insert in pBS-CoA was then cut out with *Bam*HI and *Eco*RV and cloned in the antisense orientation into *Bam*HI and *Eco*ICRI sites of the binary vector pBI121 (CLONTECH Laboratories, Palo Alto, CA) to create the antisense expression construct pACoA.

Poplar Transformation and Regeneration

The pACoA plasmid was transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation. Stem segments from poplar seedlings were used for transformation with *A. tumefaciens* containing the pACoA plasmid as described by Leple et al. (1992). After incubation with *A. tumefaciens*, stem segments were first cultured on Murashige and Skoog medium containing 500 mg/L carbenicillin for 2 weeks and then transferred onto the medium containing 50 mg/L kanamycin and 500 mg/L carbenicillin for selection of transformants. After rooting, transgenic

poplar seedlings were transferred to soil and grown in the greenhouse.

Enzyme Activity Assay

Poplar stems were frozen in liquid nitrogen and ground into powder in a mortar and pestle. The ground stem tissues were extracted with extraction buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 7.5, 0.2 mM MgCl₂, 2 mM dithiothreitol, 10% [v/v] glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/L aprotinin). Extracts were cleared by centrifugation and the supernatants were saved for assays of both CCoAOMT and COMT activities (Pakusch et al., 1989; Ye et al., 1994). Total protein concentration was determined using the Bradford method (Bradford, 1976) with BSA as the standard protein.

Protein Gel-Blot Analysis

Proteins extracted from poplar stems were separated on 4% to 20% (w/v) gradient SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Sambrook et al., 1989). The membranes were probed with polyclonal antibodies against zinnia CCoAOMT or COMT and then with peroxidase-conjugated goat anti-rabbit polyclonal antibodies. Signals were detected with the chemiluminescent reaction reagents (Amersham-Pharmacia Biotech) according to the manufacturer's protocol.

Klason Lignin Assay

Poplar stems were ground into powders by a mill. After being extracted with methanol and air-dried, the samples were assayed for Klason lignin according to Kirk and Obst (1988). Lignin content was expressed as percentage of the original weight of cell wall residues.

In-Source Pyrolysis Mass Spectrometry

In-source pyrolysis mass spectrometry was performed on a Finngan GCQ mass spectrometer equipped with a direct exposure probe (rhenium loop) (Thermoquest, San Jose, CA), as described by Morrison and Archibald (1998). Poplar stem samples were suspended in distilled water by using a glass mortar and pestle. A small amount of the suspension was placed on the loop, and the water was evaporated under vacuum. Analysis conditions were as follows: ionization energy of 20 eV; mass range of 50 to 500; scan time of 1 s; temperature rise of approximately 10°C s^{-1} to 700°C; and ion source temperature of 175°C. All samples were run in triplicate. The pyrolysis mass spectrometry data were further evaluated with principal component analysis using the Unscrambler 6.1 software (Coma AS, Trondheim, Norway).

DRIFTS

DRIFTS was performed using a Magna 850 FT-IR bench (Nicolet Instruments Corporation, Madison, WI). The bench was configured with globar source, KBr beamsplitter, MCT/B detector, and a Spectra-Tech Collector sampling accessory. Samples were packed into 13-mm DRIFTS macro sample cups. Spectra were collected in the range of 4,000 to 400 cm^{-1} at a resolution of 8 cm^{-1} with 512 scans, a mirror physical velocity of 0.95 cm/s with an aperture setting of 75 and a gain of 8. The spectra were then apodized with a Happ-Genzel function, ratioed against the background spectrum of infrared grade KBr, and displayed in the absorbance mode.

The aspen lignin reference (National Renewable Energy Laboratory, Golden, CO) and the microcrystalline cellulose reference (FMC Corporation, Princeton) samples were diluted to 5% (w/w) in infrared grade KBr (Aldrich, Milwaukee, WI). The diluted samples were placed into a stainless steel grinding vial with a 0.25-inch stainless ball and ground for 2 min in a Spex 5100 mill (Spex, Metuchen, NJ). Samples were packed into 13-mm DRIFTS macro sample cups and dried in a vacuum oven at 60°C for 1 h. Samples were then transferred to the nitrogen purged spectrometer sample compartment and allowed to equilibrate for 30 min prior to data collection.

Histology

Thin stem sections were stained for lignin with phloroglucinol-HCl (1% [w/v] phloroglucinol in 6 N HCl) and observed under a dissection microscope.

Analysis of Cell Wall Phenolics

Poplar stem materials were sequentially extracted with 50% (v/v) methanol for free phenolics, 1 N NaOH for wall bound phenolics, and 4 N NaOH for ether-linked cell wall phenolics. Phenolic composition was determined as described by Akin et al. (1993b) and Morrison et al. (1996). The extracted residues were dissolved in pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide and analyzed for phenolics by gas-liquid chromatography. Compounds were identified by comparison of their mass spectra with published spectra or those of the authentic compounds. All samples were run in duplicate.

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