Cell-Specific and Conditional Expression of Caffeoyl-Coenzyme A-3-*O*-Methyltransferase in Poplar[1]

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Caffeoyl coenzyme A-3-*O*-methyltransferase (CCoAOMT) plays an important role in lignin biosynthesis and is encoded by two genes in poplar (*Populus trichocarpa*). Here, we describe the expression pattern conferred by the two *CCoAOMT* promoters when fused to the *gus*-coding sequence in transgenic poplar (*Populus tremula* × *Populus alba*). Both genes were expressed similarly in xylem and differentially in phloem. In xylem, expression was preferentially observed in vessels and contact rays, whereas expression was barely detectable in storage rays and fibers, suggesting different routes to monolignol biosynthesis in the different xylem types. Furthermore, after wounding, fungal infection, and bending, the expression of both genes was induced concomitantly with de novo lignin deposition. Importantly, upon bending and leaning of the stem, the cell-specific expression pattern was lost, and both genes were expressed in all cell types of the xylem. *CCoAOMT* promoter activity correlated well with the presence of the CCoAOMT protein, as shown by immunolocalization. These expression data may explain, at least in part, the heterogeneity in lignin composition that is observed between cell types and upon different environmental conditions.

Lignin is a major structural component of plant secondary cell walls and is, after cellulose, the most abundant organic polymer on earth. In vascular plants, lignin provides rigidity to the cell walls, confers impermeability to xylem vessels, and forms a physicochemical barrier against microbial attack (Monties, 1989; Northcote, 1989; Moerschbacher et al., 1990). Lignin is mainly derived from the dehydrogenative polymerization of three different hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohol, which give rise to the *p*-hydroxyphenyl, guaiacyl (G), and syringyl (S) units of the lignin polymer, respectively. These units differ by the degree of methoxylation at the 3 and/or 5 positions of the aromatic ring (Fig. 1). Therefore, the hydroxylation and methylation reactions are important in determining lignin composition.

Although the overall biochemical route to lignin has been studied for many years, it is still largely unknown which reactions control the amount and composition of lignin (Boudet et al., 1995; Whetten and Sederoff, 1995; Dixon et al., 1996; Douglas, 1996; Baucher et al., 1998; Boudet, 1998; Whetten et al., 1998). The lignin polymer is complex and heterogenous with respect to the relative proportions of the three monolignol units and the different types of interunit linkages (for review, see Campbell and Sederoff, 1996). For example, lignin from gymnosperms consists mainly of G units, whereas lignin from angiosperms is predominantly made up of both G and S units. Lignin from grasses incorporates also considerable amounts of *p*-hydroxyphenyl units. The content and composition of lignin do not only differ among taxa but also between different cell types of a single tissue. Even within a single cell wall, lignin structure and/or composition can show considerable variation (Joseleau and Ruel, 1997). Furthermore, lignin heterogeneity is influenced by environmental stress. This heterogeneity is probably caused by differences in the spatiotemporal expression of certain enzymes of the lignin biosynthetic pathway and by differences in their substrate specificities (Campbell and Sederoff, 1996).

Both lignin content and composition are known to have an impact on several agro-industrial uses of plants. For instance, the amount of lignin and the S/G ratio are of critical importance in paper pulping and forage crop digestibility (for review, see Dixon et al., 1996; Baucher et al., 1998). A better understanding of the biosynthesis of lignin would provide opportu-
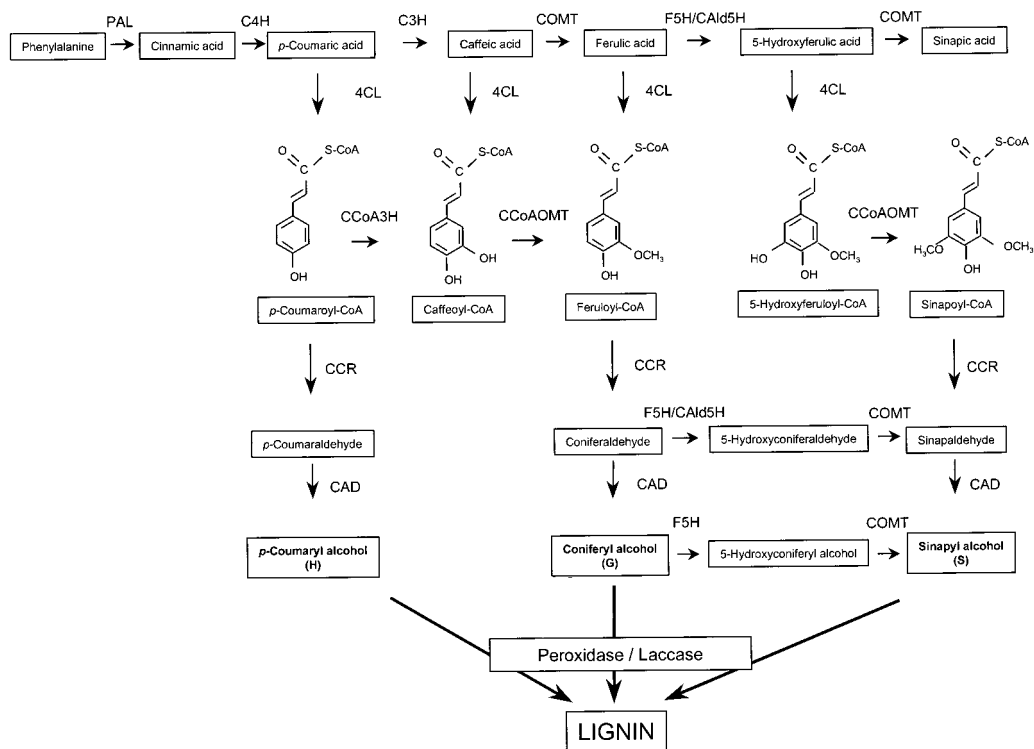
Figure 1. Phenylpropanoid and monolignol biosynthesis pathways. C3H, Coumarate 3-hydroxylase; CCoA3H, coumaroyl-CoA 3-hydroxylase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; CAld5H, coniferaldehyde 5-hydroxylase.

nities to develop strategies that allow a more economic use of raw materials in the agro-industry. Therefore, there is considerable interest in genetic engineering of lignin levels and/or composition to improve digestibility of forage crops and pulping properties of trees (Baucher et al., 1998).

The biosynthesis pathway of the lignin precursors proceeds through the common phenylpropanoid pathway starting from Phe and leading to the synthesis of cinnamoyl-coenzyme A (CoA) esters. Subsequently, the cinnamoyl-CoA esters are channeled into the monolignol branch pathway to produce cinnamyl alcohols. Caffeic acid/5-hydroxyferulic acid O-methyl-transferase (COMT; EC 2.1.1.68) has been characterized in a number of species (Baucher et al., 1998; Whetten et al., 1998) and has long been considered as the only methylating enzyme involved in lignification. However, it has been shown that the O-methylation of the lignin precursors can also occur at the level of the hydroxycinnamoyl-CoA esters (Ye et al., 1994; Inoue et al., 1998; Martz et al., 1998; Meng and Campbell, 1998; Li et al., 1999). A specific O-methyltransferase, caffeoyl-CoA-3-O-methyltransferase (CCoAOMT; EC 2.1.1.104), which catalyzes the methylation of caffeoyl-CoA to feruloyl-CoA, was initially characterized in cell suspensions of parsley and carrot challenged with a fungal elicitor (Kneusel et al., 1989; Pakusch et al., 1989; Kühnl et al., 1989) and hence was believed to play a role in disease resistance. Later, based on the observation that *CCoAOMT* was mark-

edly induced during lignification of in vitro differentiating tracheary elements of zinnia, Ye et al. (1994) suggested that this gene could also have an important function in lignification. Immunolocalization, tissue printing studies, and promoter β-glucuronidase (GUS) fusion analyses in various plant species have shown that expression of CCoAOMT is highly correlated with lignifying tissues (Ye and Varner, 1995; Ye, 1997; Inoue et al., 1998; Li et al., 1999). The possible involvement of CCoAOMT in lignification was further supported by studies in which COMT had been downregulated in transgenic tobacco and poplar (*Populus tremula × Populus alba*). In these transgenic plants, thioacidolysis experiments revealed that the S to G ratio had decreased because of a drop in the number of S units, whereas the number of G units had remained the same (Atanassova et al., 1995) or had even increased (Van Doorsselaere et al., 1995; Tsai et al., 1998; Lapierre et al., 1999). Because inhibition of the activity of COMT did not reduce the production of G units, CCoAOMT was hypothesized to play a role in bypassing the COMT-mediated methylation of the lignin precursors at the cinnamoyl-CoA ester level (Van Doorsselaere et al., 1995; Lapierre et al., 1999). Finally, the involvement of CCoAOMT in lignification was recently demonstrated in transgenic tobacco, downregulated for CCoAOMT (Zhong et al., 1998). Lignin analysis of these plants showed an increased S to G ratio and a decreased lignin content because of an overall decrease in both G and S units, indicating a

role for CCoAOMT in the pathway toward G and S lignin.

We have recently isolated two genes encoding CCoAOMT from poplar (Chen et al., 1998, 1999). Here, we have focused on the spatiotemporal expression directed by the two poplar *CCoAOMT* promoters in transgenic poplar. Consistent with a predominant role for CCoAOMT in lignification, the expression driven by both promoters was closely associated with ligni-fying tissues, both during normal development and upon biotic (fungal infection) and abiotic (wounding and bending) stress, which is known to influence lig-nin deposition. In non-stressed xylem tissue both pro-moters were shown to confer expression preferentially in vessel elements and in contact rays, whereas ex-pression was barely detectable in storage rays and fibers. However, upon bending of the stem, cell spec-ificity in the xylem was lost and the expression of the chimeric genes was observed in all cell types of the xylem. The *CCoAOMT* promoter activity corre-lated well with the presence of CCoAOMT protein, as shown by immunolocalization, indicating that CCoAOMT production is transcriptionally regulated. This is the first report demonstrating differential gene expression between different ray cell types and cell-specific alterations in *CCoAOMT* expression upon mechanical stress in plants. Our data further-more underscore differential and conditional expres-sion of lignin biosynthesis genes as a molecular mechanism to explain lignin heterogeneity.

RESULTS

The Two *CCoAOMT* Promoters Confer Expression in Lignifying Cells

We have previously demonstrated that CCoAOMT is encoded by two genes in poplar (Chen et al., 1999). To analyze the spatiotemporal expression pattern conferred by the two *CCoAOMT* promoters, the 2.0-kb and the 1.4-kb upstream regions of g*PtC-CoAOMT1* and of g*PtCCoAOMT2*, respectively, were fused to the coding sequence of the *gus* gene. Poplar was transformed with a T-DNA containing either the chimeric P*CCoAOMT1-GUS* or the P*CCoAOMT2-GUS* gene. For each construct, 20 independent pri-mary transformants were isolated. Histochemical GUS assays were carried out on 3-month-old greenhouse-grown transgenic poplars (60 cm height, 25 internodes). All transformants harboring the same construct exhibited a similar qualitative pattern of GUS localization. At the macroscopic level, GUS staining revealed that the two chimeric genes were expressed in vascular tissue (leaves, petioles, stems, and roots). Seven representative transgenic lines for each construct were chosen for further detailed analyses.

In agreement with the results from Osakabe et al. (1996), in transversal sections of top internodes (inter-node 2–4), only the primary xylem but not the phloem

tissue was lignified as indicated by phloroglucinol-HCl (P-HCl) staining (data not shown). GUS activity driven by both promoters was detected in the pri-mary xylem region (Fig. 2A). No staining was ob-served in the phloem, cambium, and pith. Observa-tions under higher magnification revealed GUS activity in the vessels and in the primary xylem pa-renchyma cells between and surrounding the vessels (Fig. 2B). In internodes located at middle positions of the stem (internodes 8–15), in addition to the primary xylem, secondary xylem and phloem fibers had de-veloped and were lignified, as revealed by P-HCl staining (data not shown). GUS staining driven by both promoters was mainly localized in young dif-ferentiating xylem and in the phloem region. Under higher magnification, we observed that both promot-ers directed a similar expression pattern in xylem, whereas they conferred differential expression in phloem tissue.

In xylem, GUS activity was preferentially localized in developing vessel cells and in the ray cells adjacent to these vessels (Fig. 2C). A longitudinal section shows that only those ray cells that are connected to the adjacent vessel by pits (contact ray cells) stained blue but not the storage (isolation) rays (Fig. 2D). No GUS activity was detected in xylem fibers except for some fibers that were adjacent to the vessels that showed GUS activity. In phloem tissue of the top one-half of the plant, P*CCoAOMT1-GUS* was ex-pressed in companion cells, whereas expression in phloem fibers was barely detectable (Fig. 2E). However, expression was observed in a limited number of cells surrounding the phloem fibers (data not shown). In contrast, GUS activity directed by P*CCoAOMT2-GUS* was located preferentially in dif-ferentiating phloem fibers and the adjacent cells but was undetectable in companion cells. No expression was detected in mature phloem fibers (Fig. 2F). In the lower part of the stem (internode 22–25) however, GUS activity conferred by P*CCoAOMT1-GUS* shifted from companion cells to the phloem fibers. There-fore, in the lower part of the stem, both genes were similarly expressed in young differentiating phloem fibers. The cell-specific expression in xylem remained the same throughout the whole stem. Both chimeric genes were also expressed in the epidermis and in the phellem of the periderm (Fig. 2G).

Poplars transformed with a P*CaMV35S-GUS* con-struct were used as controls (Fig. 2H). In agreement with the data of Nilsson et al. (1996), in cross sections of either young or older stems of the plants, GUS activity was localized in the cortex, the phloem tis-sue, the cambial zone, and the pith. Less-intense staining was observed in differentiating xylem and was not restricted to the contact ray cells. Therefore, we believe that the cell-specific expression of both chimeric genes truly reflects *CCoAOMT* promoter activity.
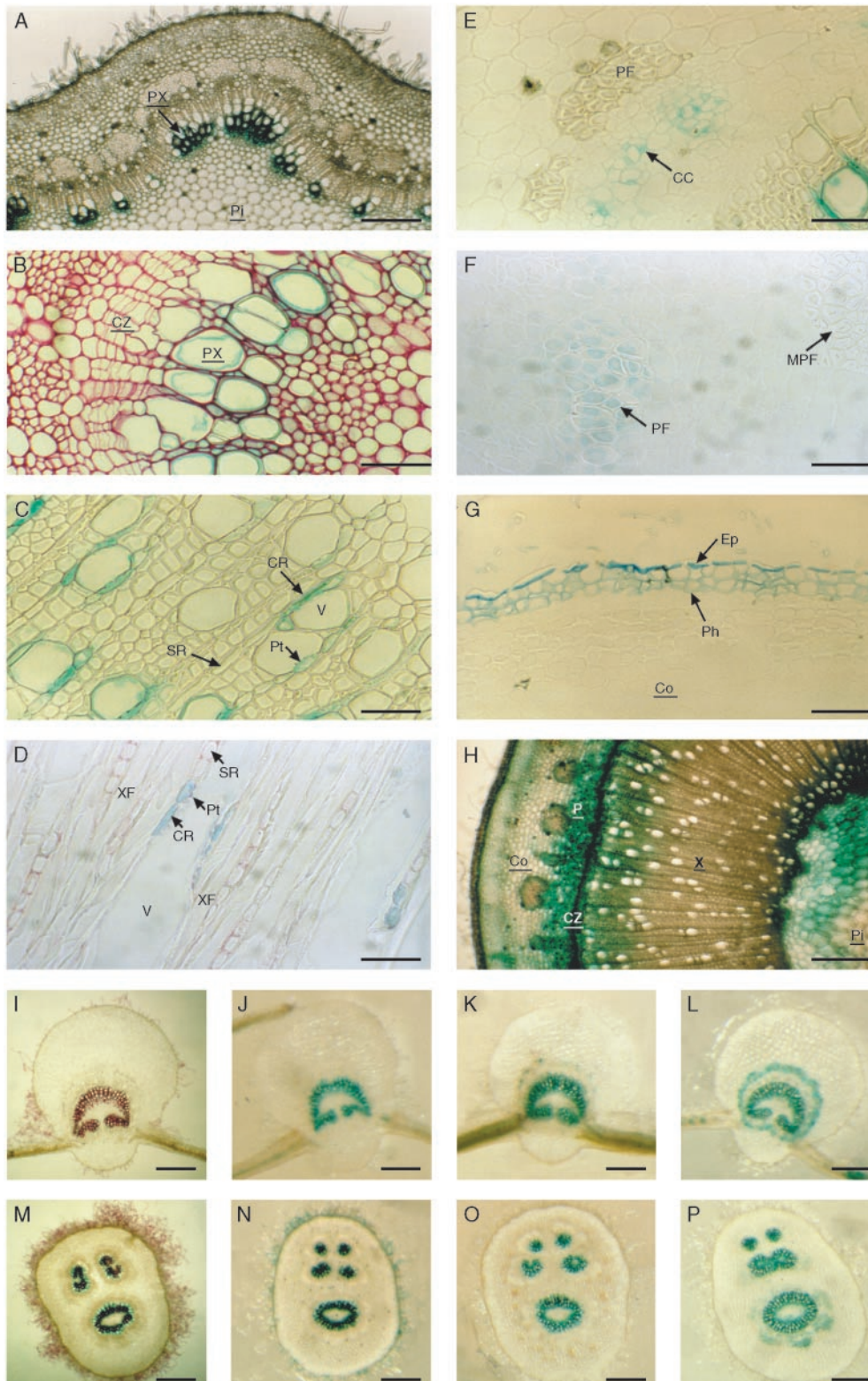
Figure 2. Histochemical analysis in transgenic poplar showing GUS activity or lignin deposition. A Thick transversal section of young stem of poplar transformed with P*BINPOP1*. B, Enlargement of A showing GUS activity in young xylem cells. C, Thin transversal section of the xylem in the middle part of the stem (P*BINPOP1*). D, Longitudinal section of the same stem as shown in C. E, Thin transversal section of the bark of the middle part of the stem (P*BINPOP1*). F, Thin section of the bark of the middle part of the stem (P*BINPOP2*). G, Thin transversal section of the bark showing GUS staining (P*BINPOP1*). H,

(Legend continues on facing page.)

A similar temporal and cell-specific expression pattern was observed in leaves and petioles. In the leaves and petioles from the top part of the plant, expression of both chimeric genes was located in the primary xylem where lignification takes place (Fig. 2, I, J, M, and N). In the leaves and petioles from the middle part of the plant, both phloem fibers and xylem cells were highly lignified as indicated by P-HCl staining (data not shown). GUS staining revealed that both chimeric genes were highly expressed in the xylem and PCCoAOMT2-GUS was additionally expressed in phloem fibers (Fig. 2, K, L, O, and P). In the leaves and petioles from the basal part of the plant, staining in the vascular tissue was less intense, presumably because xylem and phloem fibers were fully lignified at this stage. Quantitative GUS analyses indicated a GUS activity gradient in the leaves, with 2- to 3-fold higher levels in the youngest leaves than in the oldest leaves (data not shown).

In addition to the expression in xylem, phloem, and periderm, expression conferred by both chimeric genes was often detected in hair cells of young stems, petioles, and leaves. In agreement with the function of CCoAOMT, these cells stained positively for phenolic compounds when treated with P-HCl (Fig. 2, I and M). Additional expression was observed in the meristem of apical and axillary buds (data not shown). In root tissue, both chimeric genes were expressed in phloem fibers, vessels, and contact rays, as in the stem (data not shown).

Both *CCoAOMT* Promoters Are Responsive to Biotic and Abiotic Stress

In addition to the developmentally regulated deposition of lignin, de novo formation of lignin can be induced at sites of wounding or pathogen attack. At these sites, lignin deposition plays a major role in plant defense by strengthening the cell wall to prevent the spread of the invading pathogen (Vance et al., 1980; Borg-Olivier and Monties, 1993; Hawkins and Boudet, 1996). To investigate whether the expression of the chimeric genes is associated with lignin deposition upon wounding, leaves and petioles of 2-month-old in vitro as well as 3-month-old greenhouse-grown transgenic poplar were wounded (see "Materials and Methods"). Regardless the tissue type (leaves or petioles), the developmental state (young or mature leaves), or the growth conditions (in vitro or greenhouse-grown), de novo lignin dep-

osition occurred at the site of injury between 4 and 5 d after wounding, as visualized by P-HCl staining (Fig. 3A). GUS activity driven by both promoters was induced at the wounded site (Fig. 3B) and in the cells adjacent to those staining for lignin 4 d after wounding (Fig. 3A). GUS staining was barely detectable at the wounded sites until 3 d after wounding (data not shown). The induction of CCoAOMT production upon wounding of leaves was investigated by protein gel blotting as well. CCoAOMT was induced 4 d after wounding compared to the control (Fig. 4).

Detached leaves from transgenic and wild-type poplar were sprayed with spores of the fungus *Melampsora pinitorca*, a natural pathogen for poplars belonging to the *Leuce* section. Seven days after infection, the orange uredosores became visible, and necrotic lesions developed at the site of infection. At this stage, both promoters were induced in the cells surrounding the uredosores and necrotic lesions, as shown by the intense GUS staining, whereas no GUS staining was observed before the formation of the lesions. Figure 3C shows that the blue precipitate was closely associated with the lignified region at the infection site, indicating a close correlation between the induced expression of both chimeric genes and lignification at the site of necrosis.

To study the expression of both chimeric genes upon mechanical stress, histochemical GUS analyses and quantitative fluorimetric assays were conducted on stems of 3-month-old greenhouse-grown poplar of which the stem had been gently fixed to an angle of 90° (see "Materials and Methods"). Quantitative GUS assays showed that after 9 d of bending, GUS activity had increased almost 4-fold in xylem and 5.5-fold in phloem tissue (Fig. 5). GUS activity was induced at similar levels in the tensed and the opposite part of the xylem and phloem. GUS activity in the pith, which was undetectable in the non-bent condition, increased to approximately 70% of the non-bent xylem value. Transversal sections were made after 9 d of bending. For both chimeric genes, a more intensive GUS staining was observed in xylem and phloem, compared with that of the non-bent condition, as well as in the pith, where no GUS activity was detected in the non-bent condition (Fig. 3, D and E). In agreement with a role for CCoAOMT in lignification, P-HCL staining was observed in the pith (Fig. 3D). Interestingly, in contrast to the non-bent condition, GUS staining was detected in all xylem cell types (Fig. 3F), although not uniformly over the entire xylem. The expression of PCCoAOMT1-GUS was

Figure 2. (Legend continued from facing page.)
Thick transversal section of a poplar stem transformed with a P*CaMV35S-GUS* construct. I and M, Thick transversal sections of young leaf and petiole stained with P-HCl. J and N, Thick transversal sections of young leaf and petiole (P*BINPOP1*). K and O, Thick transversal section of old leaf and petiole (P*BINPOP1*). L and P, Thick transversal section of old leaf and petiole (P*BINPOP2*) at the same developmental stage as sections in K and O. CC, Companion cell; Co, cortex; CR, contact ray cell; CZ, cambial zone; Ep, epidermis; MPF, mature phloem fibers; P, phloem; PF, phloem fibers; Ph, phellem; Pi, pith; Pt, pit; PX, primary xylem; SR, storage ray cell; V, vessel; X, xylem; XF, xylem fiber. Bars = 200 μm (in A and H–P) and 50 μm (in B–G).
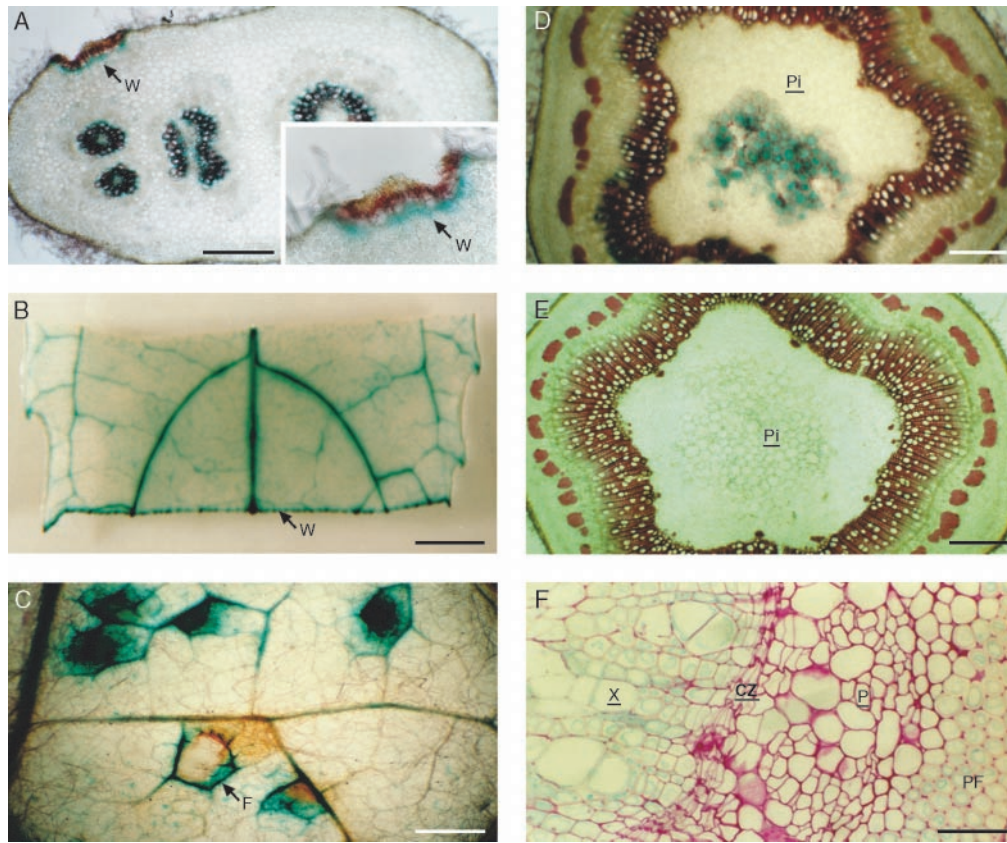
Figure 3. *CCoAOMT* promoter activity in transgenic poplar upon biotic and abiotic stress conditions. A, Transversal section of a petiole stained for both lignin and GUS. B, GUS activity in a mechanically wounded leaf. C, *M. pinitorca*-infected leaf stained for both lignin deposition and GUS activity. D, Double staining of a thick transversal section of a bent stem for lignin and GUS activity. E, Thick transversal section of a non-bent stem stained by P-HCl as control. F, Enlargement of a thin transversal section of the bent stem, transformed with P*BINPOP1*. CZ, Cambial zone; F, site of fungal infection; P, phloem; PF, phloem fibers; Pi, pith; W, wound; X, xylem. Bars = 200 μm (in A, B, D, and E), 100 μm (in C), and 50 μm (in F).

also up-regulated in phloem fibers upon bending (Fig. 3F), whereas its expression was barely detectable in this tissue in the non-bent condition (Fig. 2E). However, bending did not induce GUS activity in phloem companion cells in P*CCoAOMT2-GUS*-transformed poplars. Thus, bending stress resulted in the loss of the cell-specific expression pattern in the vascular tissue and in a patchy expression of both genes in all lignifying cells of the xylem and phloem.

Immunolocalization of CCoAOMT in Poplar Stem

To verify whether the cell-specific and conditional expression driven by the *CCoAOMT* promoters coincided with the localization of the CCoAOMT protein, immunolocalization experiments were carried out on similar stem sections as those used for the GUS assays with antiserum against alfalfa CCoAOMT (Kersey et al., 1999). With these antibodies, a single 28-kD protein band was detected in the protein extracts from wild-type poplar stem by protein gel-blot analysis, whereas no signal was detected in protein extracts from transgenic poplars down-regulated for CCoAOMT (H. Meyermans and W. Boerjan, unpub-

lished results). This observation indicates that the alfalfa CCoAOMT antibodies can specifically recognize CCoAOMT in poplar. Poplar CCoAOMT was localized by immunogold complexes with silver enhancement and examined by light microscopy. In general, labeling was present in differentiating phloem and xylem. Virtually no labeling appeared in the pith, cambium, and cortex. In sections through the top part of the stem, CCoAOMT was present in primary xylem bundles (Fig. 6A), which is in agreement with the result from promoter-GUS assays (Fig.
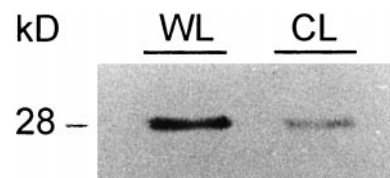

Figure 4. Protein gel-blot analysis of poplar protein extracts. Crude protein extracts from poplar control leaf (CL) and wounded leaf (WL) separated by SDS-PAGE, immobilized on a nitrocellulose membrane, and incubated with anti-CCoAOMT antibodies. The protein molecular mass is shown at the left in kD.
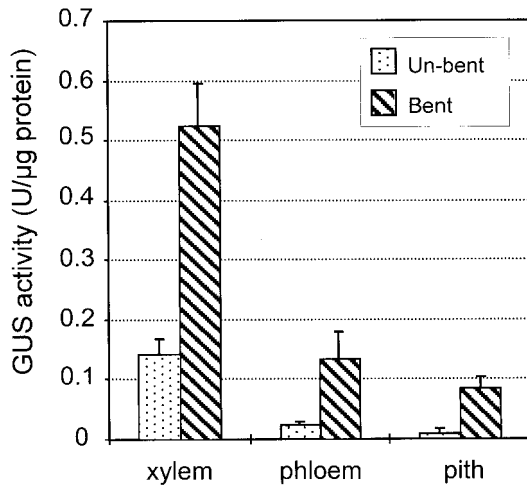
Figure 5. Induction of GUS activity in transgenic poplar stems as a consequence of mechanical bending. GUS activity was measured in xylem, phloem, and pith tissues. The data represent the average of three independent experiments. The SE is shown.

2B). In the middle and lower part of stem, CCoAOMT was detected in differentiating xylem (Fig. 6B), in differentiating phloem fibers but not in mature ones (Fig. 6C), as well as in the epidermis (Fig. 6D). Very faint labeling was observed in the companion cells (data not shown). In xylem tissue, as shown in Figure 6B, CCoAOMT was preferentially localized in contact ray cells associated with vessels and in the vessels themselves. This cell-specific localization of CCoAOMT in secondary xylem is similar to that of GUS directed by both *CCoAOMT* promoters (Fig. 2, A–G). No signal could be observed in control sections of the same stem treated with the preimmune serum (Fig. 6H) nor in the sections of the transgenic poplar down-regulated for CCoAOMT (Fig. 6E).

To confirm that upon mechanical stress the cell-specific expression of *CCoAOMT* was altered, immunolocalization experiments were carried out on bent stems. As shown in Figure 6F, CCoAOMT was significantly induced in xylem tissue of stems that were bent for 9 d. In agreement with the induction of *CCoAOMT* promoter activity upon bending, immunolabeling was detected in all cell types of the xylem tissue and most intensely in the rays cells. In addition to mechanical bending, poplars were leaned at an angle of 45°, and sections were made through the curved part of the stem (see "Materials and Methods"). Also under this condition, the expression of *CCoAOMT* was significantly induced in all cell types of the xylem (Fig. 6G) and revealed an expression pattern similar to that after bending.

Subcellular Localization of CCoAOMT

It is still largely unclear where monolignol biosynthesis occurs at the subcellular level. Several studies have suggested the existence of multienzyme com-

plexes, other studies have shown the association of lignin biosynthesis enzymes with cellular organelles (Smith et al., 1994; Nakashima et al., 1997; Šamaj et al., 1998; Rasmussen and Dixon, 1999). To investigate whether CCoAOMT is entirely cytosolic or fractionally associated with membranes, we have localized CCoAOMT by electron microscopy on sections similar to those used for light microscopy and found a similar cellular distribution of CCoAOMT. Along the differentiating xylem, immunogold particles appeared mostly in the contact rays and in the vessels, although in the vessels only a small layer of cytoplasm remained prior to autolysis. Interestingly, at more advanced stages of xylem development, after autolysis of the vessels, no immunogold particles could be detected anymore neither in mature vessels nor in the contact rays adjacent to those mature vessels. Furthermore, labeling was observed in differentiating but not in mature phloem fibers, in the companion cells, in the epidermis, and in the phellem of the periderm, consistent with the promoter-GUS data and the immunolocalizations carried out by light microscopy. No signal was found in the cambium, pith, or cortex region (data not shown). A more detailed observation showed that the silver-enhanced particles were randomly localized in the cytoplasm and were not located on the cell wall or associated with any organelles (Fig. 7A). When similar sections were incubated with preimmune serum, no specific binding of gold particles was observed (Fig. 7B).

DISCUSSION

Here, we have shown that the two *CCoAOMT* promoters direct expression in lignifying cells and in cells closely associated with lignifying cells, both during normal development and upon stress conditions known to influence lignin deposition (wounding, pathogen attack, and mechanical bending). These data are consistent with a role for CCoAOMT in lignification. Furthermore, both chimeric *CCoAOMT* genes were expressed in the epidermis (including hair cells) of young stems, leaves, petioles, and in the shoot apex. The g*PtCCoAOMT1* promoter additionally directed expression in phloem companion cells, which are generally accepted to be not lignified or only poorly lignified. The deposition of phenolic compounds in non-lignified cells or tissues may serve important protective functions. In these cells, CCoAOMT may for example be involved in the biosynthesis of lignans, which share the same monolignol precursors as lignin. Lignans are an abundant class of phytochemicals in plants that play a role in defense, but little is known about their biosynthesis and their site of accumulation (Davin et al., 1992; Higuchi, 1997). The promoter of the eucalyptus cinnamyl alcohol dehydrogenase (*CAD2*) gene also conferred expression in poorly lignified cells, such as the periderm of stems, cortical cells containing crystals,
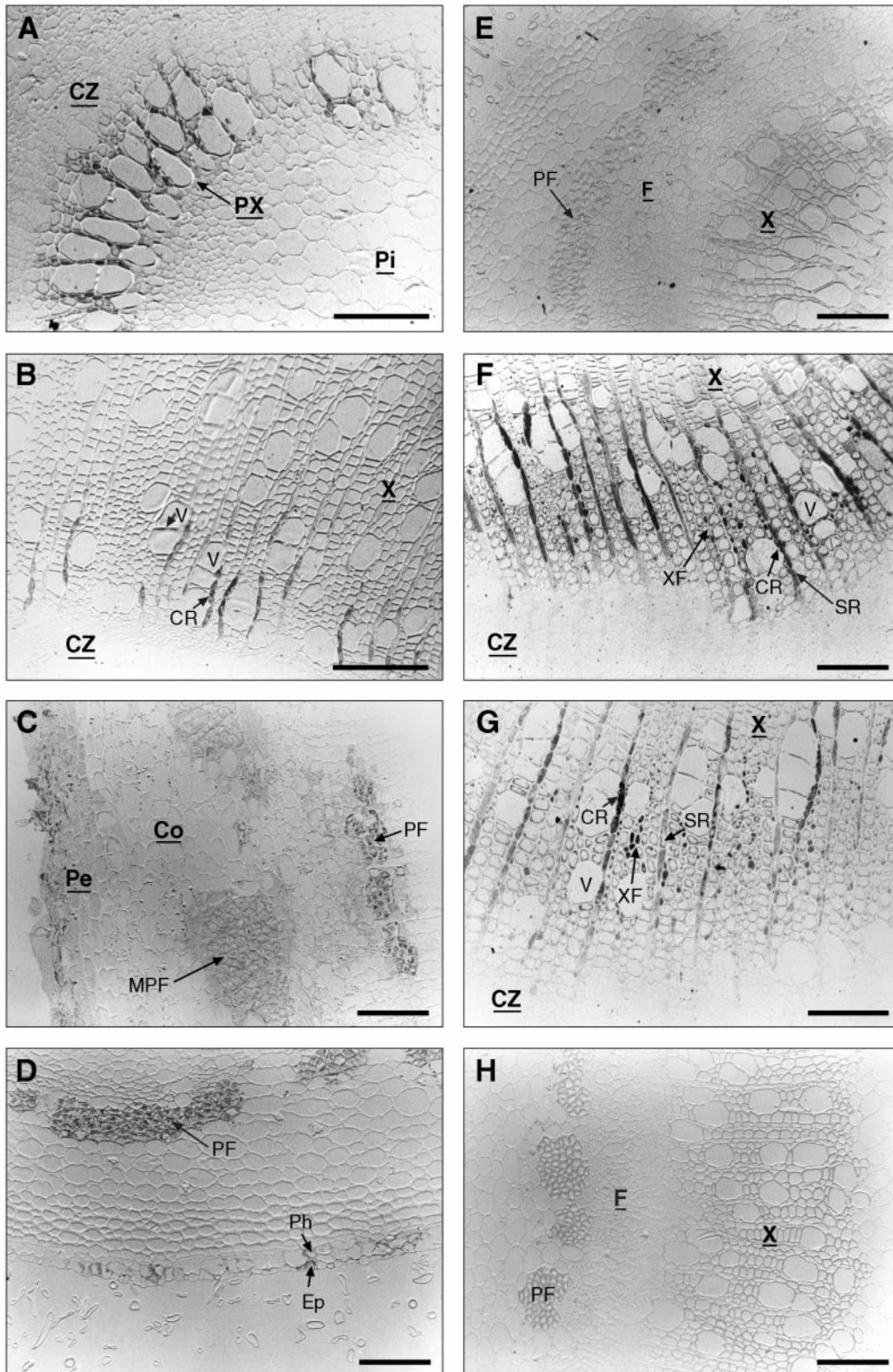
Figure 6. Immunolocalization of CCoAOMT in transversal sections of poplar stem by light microscopy. A, Young stem of 3-month-old greenhouse-grown poplar. B, Xylem tissue of the middle part of a 3-month-old greenhouse-grown poplar stem. C and D, Bark tissue of old and young stem. E, Stem of transgenic poplar down-regulated for CCoAOMT. F, Bent stem. G, Leaned stem. H, Control section treated with preimmune serum. Co, Cortex; CR, contact ray cell; CZ, cambial zone; Ep, epidermis; F, phloem; MPF, mature phloem fibers; Pe, periderm; PF, phloem fibers; Ph, phellem; Pi, pith; PX, primary xylem; SR, storage ray cell; V, vessel; X, xylem; XF, xylem fiber. Bars = 100 μm.
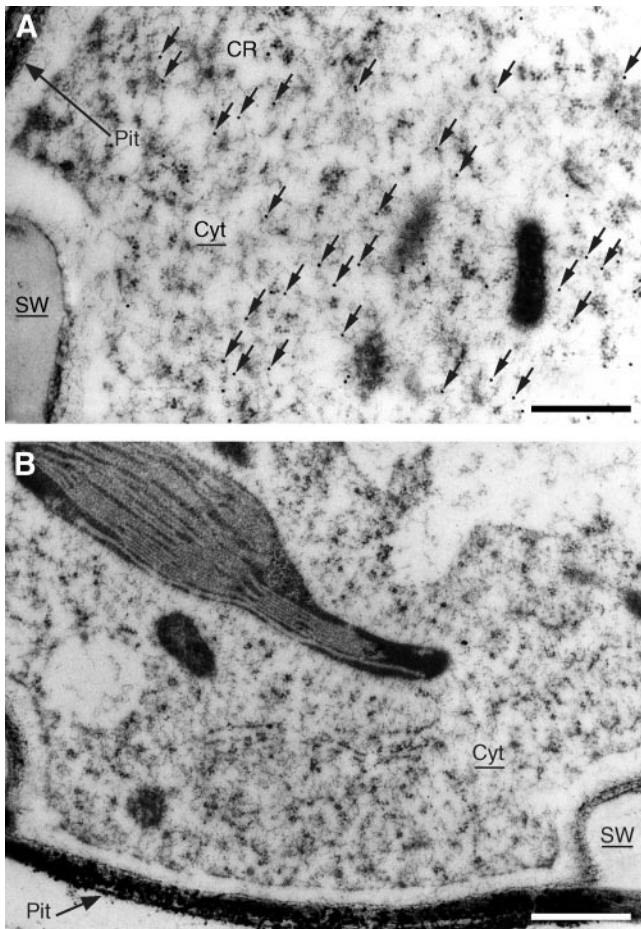
Figure 7. Subcellular localization of CCoAOMT in poplar stem by electron microscopy. A, Contact ray cell from a stem section of the middle part of a 3-month-old greenhouse-grown poplar. The labeling with alfalfa CCoAOMT antibodies is concentrated in the cytosol. B, control section treated with preimmune serum. CR, Contact ray cell; Cyt, cytoplasm; SW, secondary wall. Arrows indicate gold particles. Bars = 1 μm.

companion cells, and cambial cells in transgenic poplar (Feuillet et al., 1995; Hawkins et al., 1997; Šamaj et al., 1998). CAD2 catalyzes the conversion of cinnamaldehydes to cinnamyl alcohols (Fig. 1) and may also be involved in lignan synthesis in these cells (Hawkins et al., 1997). The expression data additionally show that both promoters are multifunctional rather than diverged where each promoter has adopted separate functions. This result is in contrast to the 4-coumarate:CoA ligase (*4CL*) genes of poplar: *Pt4CL1* is expressed in cells that participate in the developmentally regulated lignification process (mainly in xylem), whereas the *Pt4CL2* confers expression in the epidermis of stem and leaf where it functions in the biosynthesis of phenylpropanoids other than lignin (Hu et al., 1998). Because immunolocalization of CCoAOMT showed the same tissue and cell-specific localization as revealed by GUS assays, we can conclude that the expression of CCoAOMT is mainly regulated at the transcriptional level.

CCoAOMT Is Expressed in Vessels and Contact Ray Cells

For several genes involved in lignin biosynthesis, the tissue-specific expression has been analyzed. For example, a bean Phe ammonia-lyase (*PAL*)-*GUS* fusion in tobacco and potato was expressed in all developing xylem cells including ray cells (Bevan et al., 1989). Hauffe et al. (1991) reported that P*4CL-GUS* expression was localized preferentially in differentiating xylem tissue and in xylem ray cells between highly lignified vessels and fibers in transgenic tobacco. The promoter of the *CAD2* gene from eucalyptus conferred expression in phloem fibers, in the rays of differentiating xylem, and in the vascular cambium in transgenic poplar (Feuillet et al., 1995; Hawkins et al., 1997).

In contrast to the expression pattern conferred by the *PAL*, *4CL*, and *CAD2* promoters, the promoter of *CCoAOMT* was differentially expressed in different ray cell types. Under normal, non-stressed growth conditions, P*CCoAOMT-GUS* expression was preferentially observed in the contact ray cells that are connected to vessels through pits and not in the storage ray cells (isolation rays). These two types of ray cells have first been reported by Czaninski (1977), and they differ with respect to their contacts, through pits, with vessels. In dicotyledonous plants, pits are formed in the secondary xylem between a parenchyma cell and a conducting cell (vessel). Such pits are considered to play an important role during xylem cell differentiation, providing channels through which not only wall precursors and other metabolites may be transferred into the developing cell, but also signals that control their differentiation (for review, see Mezitt and Lucas, 1996). Our electron microscopy studies furthermore showed that at the stage that vessels are still alive, as judged by the presence of cytoplasm, CCoAOMT was present both in vessels and in the adjacent contact rays. However, upon autolysis of the vessel, expression of CCoAOMT was coincidentally switched off both in the vessels and in the contact rays. The similarity in timing of *CCoAOMT* expression in vessels and contact rays suggests that the vessels and contact rays share a common signal that triggers expression of the *CCoAOMT* genes in both cell types and that may be transported through the connecting pits. This hypothesis is in agreement with recent data of Murakami et al. (1999), who have shown by UV absorption of cell walls that lignification of the cell wall of contact rays and vessels in *Populus maximowiczii* occurs simultaneously and prior to that of the storage rays and fibers.

The observation that the *CCoAOMT* promoters drive expression in vessels and contact rays further raises the question whether the lignin composition of these cells differs from that of xylem fibers and storage rays. Higuchi (1997) has reported the predominant presence of G units in the lignin of vessel cell

walls in angiosperm trees. The analysis of transgenic plants down-regulated for CCoAOMT and in vitro enzymatic assays support the idea that the difference in lignin composition is indeed due to differential expression of *CCoAOMT*. Zhong et al. (1998) have shown that lignin from transgenic tobacco plants down-regulated for CCoAOMT, apart from having a lower total Klason lignin content, is more depressed in G than in S units. Similar results were obtained in transgenic poplar, down-regulated for CCoAOMT (H. Meyermans, unpublished data).

Based on in vitro enzymatic assays, Osakabe et al. (1999) have recently proposed that the lignin precursors are predominantly synthesized via coniferaldehyde and not via ferulic acid, 5-hydroxyferulic acid, and sinapic acid. These data are based on the observation that coniferaldehyde inhibits the coniferaldehyde 5-hydroxylase/ferulic acid 5-hydroxylase reaction from ferulate to 5-hydroxyferulate in vitro. The cell-specific expression of *CCoAOMT* suggests that in vessels and contact rays, G and S units may be formed predominantly via caffeoyl-CoA and feruloyl-CoA, whereas in fibers and storage ray cells G and S units may be formed preferentially via caffeic acid, ferulic acid, and feruloyl-CoA. Based on the fact that COMT plays an important role in controlling S-unit biosynthesis (Atanassova et al., 1995; Van Doorsselaere et al., 1995; Tsai et al., 1998; Lapierre et al., 1999) by converting 5–hydroxyconiferaldehyde and 5–hydroxyconiferyl alcohol to sinapaldehyde and sinapyl alcohol, respectively (Humphreys et al., 1999; Osakabe et al., 1999), the conversion of caffeic acid to ferulic acid may also be less efficient in cells that make less S units. In vessels, where the S to G ratio is low compared with fibers (Saka and Goring, 1985), CCoAOMT may effectively bypass the COMT-mediated conversion of caffeic acid to ferulic acid. Together, the cell-specific expression of *CCoAOMT* and the different lignin composition in these cells suggest that the metabolic flux through the phenylpropanoid and lignin biosynthesis pathway is different in different cell types.

CCoAOMT Promoters Are Responsive to Wounding and Fungal Attack

The responses of plants to mechanical wounding and pathogen attack are often very similar and include the rapid accumulation of phenolic compounds, the production of phytoalexins, the synthesis of hydrolytic enzymes, and the reinforcement of cells with lignin and/or suberin at the site of injury (Blanchette and Biggs, 1992). In most of the cases examined, the appearance of such defense substances is the result of increased gene expression either within the affected tissue or throughout the plant. Most of the genes that code for enzymes of the lignin biosynthesis pathway, such as *PAL*, *4CL*, *COMT*, and cinnamate 4-hydroxylase (*C4H*), have been reported

to be induced by wounding, elicitors, and/or fungal infection (Baucher et al., 1998). CCoAOMT activity has been extensively studied in response to elicitors in cell suspension culture (Kneusel et al., 1989; Kühnl et al., 1989; Schmitt et al., 1991; Ni et al., 1996; Busam et al., 1997). Transient expression assays performed with *PCCoAOMT-GUS* constructs in parsley protoplasts showed a high expression level in response to elicitation (Grimmig and Matern, 1997). In tobacco, RNA gel-blot analyses showed an increased steady-state *CCoAOMT* mRNA level upon infection with tobacco mosaic virus or fungal elicitor (Martz et al., 1998). In our study the expression of both chimeric *CCoAOMT* genes was strongly induced at the wound site and at the infection site of leaves, concomitantly with the deposition of lignin or lignin-like material. That GUS activity was observed in the cell layer adjacent to the lignifying cell layer suggests that these cells provide monolignols to their neighboring cells for lignification or the production of lignin-like material. These results show that the induced formation of lignin or lignin-like material is closely correlated with the cellular localization of *CCoAOMT* gene expression in response to both wounding and fungal attack in plants.

CCoAOMT Promoters Are Responsive to Mechanical Stress

Mechanical stress caused by leaning stems results in compression wood in gymnosperms and tension wood in angiosperms (Timell, 1986; Castéra et al., 1994). Both compression and tension wood have been shown to have an altered lignin content and composition compared with normal non-stressed wood (Timell, 1986; Rolando et al., 1992). In contrast to the intensive study of lignin biosynthesis enzymes, such as PAL, 4CL, COMT, CAD, and cinnamoyl-CoA reductase in compression wood (Kutsuki and Higuchi, 1981; Zhang and Chiang, 1997), little information is available on gene expression in tension wood. Here, we report on a gene that is induced upon bending in a hardwood species. The expression of both chimeric *CCoAOMT* genes was up-regulated in all lignifying tissues of the stem 9 d after mechanical bending. There was a pronounced increase in GUS activity in xylem and phloem and in the pith cells concomitantly with the deposition of lignin or lignin-like material in the pith. It is noteworthy that in response to mechanical bending, the cell-specific expression pattern directed by both *CCoAOMT* promoters in the stem was lost.

Mechanical stress may also be the reason why *PCCoAOMT1-GUS* expression was observed in phloem fibers at the base of the stem and in the root, whereas no activity was observed in phloem fibers in the top one-half of the stem. Immunolocalization of CCoAOMT on sections of stems that were bent or leaned were in agreement with the promoter-GUS

analyses and show that the induction of *CCoAOMT* expression upon mechanical stress is regulated at the transcriptional level. Considering the involvement of CCoAOMT in lignification, our observations suggest that the up-regulation of CCoAOMT in a non-cell-type-specific manner may alter lignin composition or content. However, Vander Mijnsbrugge et al. (2000) have recently reported that a poplar phenylcouma-ran benzylic ether reductase, which is the most abun-dant protein in poplar xylem and involved in the biosynthesis of lignans (Gang et al., 1999), is up-regulated in xylem, phloem, and pith tissues of poplar stem in response to mechanical bending. Therefore, up-regulation of CCoAOMT in response to bending may also be involved in the synthesis of lignans apart from lignin. The analysis of lignin amount and composition as well as soluble pheno-lics from transgenic poplar, down-regulated for CCoAOMT, may shed further light on the fate of the produced monolignol intermediates upon mechani-cal stress.

CCoAOMT Is a Cytosolic Protein

The phenylpropanoid pathway is involved in the biosynthesis of a wide variety of natural products from plants. Little is known about the mechanism the cell uses to regulate the flux into the different end products of the pathway. Metabolic labeling experi-ments have suggested the existence of multienzyme complexes that channel intermediates of phenylpro-panoid synthesis without their release into general metabolic pools (Stafford, 1981; Hrazdina, 1992; Ras-mussen and Dixon, 1999). Several of the enzymes of the phenylpropanoid and lignin-specific pathways have been studied for their subcellular distribution by electron microscopy. In French bean, PAL has been localized in the cytosol whereas C4H was asso-ciated with the endoplasmic reticulum membrane and Golgi bodies (Smith et al., 1994). In poplar, CAD2 has been localized in the cytosol, on endoplasmic reticulum membranes and on Golgi-derived vesicles (Šamaj et al., 1998). In tracheary elements derived from zinnia mesophyll cells, two different types of PAL and CAD exist. For both enzymes, one isoform was shown to be cytosolic and the other associated to Golgi-derived vesicles and secondary cell walls (Na-kashima et al., 1997). Recently, Rasmussen and Dixon (1999) have presented evidence for metabolic chan-neling, involving coupling of PAL and C4H, based on in vivo and in vitro labeling. These authors demon-strated that one form of PAL (PAL1) was associated with tobacco microsomes and was involved in chan-neling, and suggested that this isoform is in close physical association with C4H on microsomal mem-branes. Our results show that CCoAOMT is randomly distributed in the cytoplasm and is not directly in-volved in multienzyme complexes that are present at the membrane.

In summary, our data show that the *CCoAOMT* promoters are responsive to signals that control lig-nin deposition throughout plant development and adjust lignin quality according to environmental con-ditions. Taken together with the differential *CCoAOMT* promoter activity in the different xylem cell types, our observations may explain, at least part of, the large heterogeneity in lignin amount and com-position that is observed between different cell types and within individual cell walls (Joseleau and Ruel, 1997; Baucher et al., 1998). Extensive promoter com-parisons and deletion analyses are necessary to iden-tify the cis elements that are involved in the re-sponses to the different signals that induce *CCoAOMT* promoter activity.

MATERIALS AND METHODS

Plasmid Constructions

The characterization of g*PtCCoAOMT1* (accession no. AJ223620) and g*PtCCoAOMT2* (accession no. AJ223621) has been described previously (Chen et al., 1998, 1999). To fuse the 5′-untranslated region sequences of both *CCoAOMT* genes to the coding sequence of the *GUS* gene, an *Nco*I site was generated by PCR three codons downstream of the *CCoAOMT* start codon by using an oligonucleotide com-plementary to the 5′ flanking vector DNA in combination with the 21-mer oligonucleotide 5′-CTCTC<u>CCATGG</u>TGGC**CAT**TAT-3′ and 5′-CTCTC<u>CCATGG</u>CGGC**CAT**TAT-3′ for g*PtCCoAOMT1* and g*PtCCoAOMT2*, respectively (a single line indicates the *Nco*I site and the reversed original start codon is bold and underlined. By using these oligonucle-otides, the 1,994-bp and 1,363-bp promoter fragments of g*PtCCoAOMT1* and g*PtCCoAOMT2* were generated by PCR, respectively. Subsequently, both PCR products were digested with *Nco*I and *Sac*I and cloned into the *Nco*I/*Sac*I site of pGUS1 (Peleman et al., 1989), yielding the plasmids P*GUSPOP1* and P*GUSPOP2*. Both chimeric P*CCoAOMT1*-*GUS* and P*CCoAOMT2*-*GUS* genes were subsequently iso-lated from P*GUSPOP1* and P*GUSPOP2*, respectively, by an *Xba*I digest, and cloned into the *Xba*I site of the binary vector P*BIN19* (Bevan, 1984), resulting in the plasmids P*BINPOP1* and P*BINPOP2*, respectively.

Plant Material and Transformation

P*BINPOP1*, P*BINPOP2*, and P*CaMV35-GUS* (Nilsson et al., 1996) were mobilized to *Agrobacterium tumefaciens* strain C58C1Rif harboring the plasmid pMP90 by the freeze-thaw method described by Zahm et al. (1984). Poplar (*Populus tremula × Populus alba* Institut National de la Recherche Agronomique clone 717-1B4) was stably transformed with all three constructs according to Leplé et al. (1992). Each primary transformant was derived from a different ex-plant. The plants were maintained and micropropagated in vitro on one-half Murashige and Skoog medium at 24°C with a 16-h light/8-h dark cycle. Five-week-old plants were transferred to soil and further grown in a greenhouse at 21°C with the same light/dark cycle.

Histochemical GUS Assays

Histochemical staining for GUS activity was performed essentially as described by Jefferson et al. (1987) and Hawkins et al. (1997). Small pieces of stems, roots, leaves, and petioles were excised and pretreated with 95% (v/v) acetone for 30 min at room temperature to prevent wound induction and rinsed three times with 100 mM potassium phosphate buffer (pH 7.0). GUS staining was carried out by incubating sections with 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.1 mM potassium ferricyanide, and 0.1 mM potassium ferrocyanide in 100 mM potassium phosphate buffer (pH 7.0). Staining was allowed to proceed at 37°C until blue stain developed in the samples (1–4 h). Subsequently, samples were fixed in 3% (v/v) glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) overnight at 4°C, washed in the same buffer, and embedded in Histoform according to the manufacturer's protocol (Historesin Embedding Kit, Heraeus Kulzer, Wehrheim, Germany). Thin sections (8 μm) were cut on a microtome (Reichert-Jung, Nussloch, Germany). For thick slices, small pieces of stems, leaves, and petioles were pretreated with 95% (v/v) acetone, embedded in 7% (w/v) agarose, and sectioned (75–150 μm) with a vibroslicer (Laborimpex, Brussels). GUS staining was carried out as described above. All the sections were mounted on microscope slides for photography (Diaplan, Leitz, Wetzlar, Germany).

Quantitative GUS Assays

Quantitative GUS analyses were performed as described by Jefferson et al. (1987). Protein concentrations were measured with the method of Bradford (1976). GUS activity was assayed by enzymatic conversion of 4-methylumbelliferyl-β-D-glucuronide to 4-methylumbelliferone which was quantified with a fluorimeter (Labsystems Fluoreskan II, Helsinki). GUS activity was expressed in units per microgram of protein.

Lignin Staining

Lignin and/or phenolic compounds were revealed by P-HCl staining according to Speer (1987). Sections and samples were incubated for 2 min in phloroglucinol solution (1% in ethanol:water, 92:8 [v/v]) and then mounted in 25% (v/v) HCl, prior to microscopic examination.

Wounding, Bending, Leaning, and Pathogen Infection

For the wounding experiments, petioles of 3-month-old, greenhouse-grown plants were wounded by making a 0.5-cm-long slit with a scalpel. Wounding of the leaves was done on plants that were grown axenically in glass jars for 2 months. From each of six comparable plants, one-half of a leaf was removed using a sterile scalpel, and a strip of leaf tissue of approximately 2 mm bordering the cutting site was harvested after 0, 1, 2, 3, 4, and 5 d after wounding. The harvested samples were analyzed by histochemistry for GUS activity and lignin deposition as well as by protein gel-blot analysis.

For the bending experiments, stems of 3-month-old greenhouse-grown wild-type and transgenic poplars were gently fixed at a 90° angle at internode 3 to 4 counted from the top of the stem for various periods. Non-bent stems of the same, vegetatively propagated transgenic line were used as control. The bent part of the stem was collected for GUS assays and immunolocalization experiments. For quantitative GUS assays, the xylem, phloem, and pith tissues from the bent stems were collected separately and immediately frozen in liquid nitrogen. The harvested samples were stored at −70°C until analysis.

Alternatively, 3-month-old greenhouse-grown poplars were leaned at 45° for 9 d. The upper part of the stem (approximately 15–25 cm) grows vertically after leaning the plant. The samples for immunolocalization were taken from the curved part of the plant, i.e. between the oblique and the vertical part of the stem.

Spores of the fungus *Melampsora pinitorca* were collected from leaves of outside-grown poplar trees and determined according to Pinon (1973). For fungal infection, detached leaves were put upside down on wet filter paper in 9-cm Petri dishes. Subsequently, these leaves were sprayed for 2 s with spores of the fungus, in a concentration of 200,000 spores mL^{-1}, using a spray machine (Aku-Sprühpistole W50, Wagner, Markdorf, Germany). The infected leaves were subsequently floated upside down on water in 9-cm Petri dishes and incubated at 22°C in a greenhouse for various periods.

Protein Gel Blotting

Proteins were extracted with 100 mM Tris (tris-[hydroxymethyl]aminomethane)-HCl (pH 7.5), 2 mM EDTA, 20% (v/v) glycerol, and 1 mM dithiothreitol. Protein (5 μg) from leaves was separated by SDS-PAGE. The protein gel was subsequently blotted on Hybond-C super membrane according to the manufacturer's instructions (Amersham, Aylesbury, UK). After incubation with antiserum raised against alfalfa CCoAOMT (Kersey et al., 1999), filters were incubated with anti-rabbit IgG alkaline phosphatase conjugate (Roche Diagnostics, Brussels) at a dilution of 1/2,500. The immunodetection was performed using an alkaline phosphate *p*-toluidine salt (Duchefa, Haarlem, The Netherlands) and *p*-nitroblue tetrazolium chloride (Duchefa) in the detection reaction.

Sample Preparation for Light and Electron Microscopy

Small pieces of tissue harvested from non-stressed stem, wounded stem, and stems that had been bent or leaned were cut into pieces of approximately 1 mm³. These pieces were immersed in fixation solution (2.5% [v/v] paraformaldehyde and 0.3% [v/v] glutaraldehyde in 0.1 M sodium-cacodylate buffer [21.4 g $Na(CH_3)_2AsO_2 \cdot 3H_2O_2$ in 1,000 mL of distilled water], pH 7.2) under vacuum for 4 h at room temperature and then incubated for 14 h at 4°C under rotation. Following three washes for 2 h in 0.1 M sodium-cacodylate buffer (pH 7.2) at 4°C, the samples were dehydrated through a graded ethanol series under rotation

at 4°C as follows: 30% (v/v) ethanol for 2 h, 50% (v/v) ethanol for 2 h, 70% (v/v) ethanol overnight, 95% (v/v) ethanol for 2 h, and 95% (v/v) ethanol overnight. The samples were embedded by incubating them successively in a 1:1 (v/v) ratio of 95% (v/v) ethanol:LR White (London Resin Co., Basingstoke, UK) at 4°C overnight and then for three changes, at least 8 h each, in pure resin at 4°C. The samples were placed in gelatin capsules containing fresh nitrogen-fluxed resin. Polymerization was performed by UV illumination at 4°C for 24 h followed by 24 h at 65°C to ensure complete polymerization of the resin. These LR White-embedded samples were ready to be sectioned for immunocytochemical localization of proteins using light or electron microscopy.

Light Microscopy

LR White-embedded samples were cut in semithin sections (1–3 μm) using a 2050 microtome (Reichert-Jung). The sections were collected on Vectabond-coated glass slides. Rabbit polyclonal anti-CCoAOMT antibodies (Kersey et al., 1999) were used as primary antibody. The AuroProbe One and IntenSE reagents were used for immunogold silver staining according to the manufacturer's protocol (Amersham). Sections were examined with a light microscope Jenalumar (Zeiss, Jena, Germany).

Electron Microscopy

Ultrathin sections of gold interference color (60–90 nm) were made from the LR White-embedded samples using an ultracut E (Reichert-Jung), and were collected on collodion-coated Cu grids. These sections were floated with the tissue-containing side downward for 5 min on a droplet of blocking solution (0.1% [w/v] bovine serum albumin and 0.05% [w/v] NaN_3 in phosphate-buffered saline) to avoid non-specific adsorption of the antibodies. Subsequently, the sections were incubated for 1 h with primary antiserum raised against alfalfa CCoAOMT (Kersey et al., 1999), diluted 1:100 in blocking solution, followed by a 30-min incubation with a protein A-gold conjugate (15 nm; Amersham) diluted 1:50 in gold buffer (1% [w/v] bovine serum albumin and 0.05% [w/v] NaN_3 in phosphate-buffered saline). Sections were washed three times with gold buffer, three times with distilled water, and then allowed to air-dry. The grids were post-stained for 12 min with 2% (w/v) uranyl acetate, washed five times with distilled water, and then left to air dry. Sections were examined using transmission electron microscopy (Elmiskop 101, Siemens, Karlsruhe, Germany).

ACKNOWLEDGMENTS

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