Plant Physiol. (1997) 115: 1341–1350

Association of Caffeoyl Coenzyme A 3-*O*-Methyltransferase Expression with Lignifying Tissues in Several Dicot Plants[1]

Zheng-Hua Ye*

Department of Botany, University of Georgia, Athens, Georgia 30602

Caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) was previously shown to be associated with lignification in both in vitro tracheary elements (TEs) and organs of zinnia (*Zinnia elegans*). However, it is not known whether this is a general pattern in dicot plants. To address this question, polyclonal antibodies against zinnia recombinant CCoAOMT fusion protein were raised and used for immunolocalization in several dicot plants. The antibodies predominantly recognized a protein band with a molecular mass of 28 kD on western analysis of tissue extracts from zinnia, forsythia (*Forsythia suspensa*), tobacco (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), and soybean (*Glycine max*). Western analyses showed that the accumulation of CCoAOMT protein was closely correlated with lignification in in vitro TEs of zinnia. Immunolocalization results showed that CCoAOMT was localized in developing TEs of young zinnia stems and in TEs, xylem fibers, and phloem fibers of old stems. CCoAOMT was also found to be specifically associated with all lignifying tissues, including TEs, xylem fibers, and phloem fibers in stems of forsythia, tobacco, alfalfa, soybean, and tomato (*Lycopersicon esculentum*). The presence of CCoAOMT was evident in xylem ray parenchyma cells of forsythia, tobacco, and tomato. In forsythia and alfalfa, pith parenchyma cells next to the vascular cylinder were lignified. Accordingly, marked accumulation of CCoAOMT in these cells was observed. Taken together, these results showed a close association of CCoAOMT expression with lignification in dicot plants. This supports the hypothesis that the CCoAOMT-mediated methylation branch is a general one in lignin biosynthesis during normal growth and development in dicot plants.

CCoAOMT (EC 2.1.1.104) converts caffeoyl CoA into feruloyl CoA in the phenylpropanoid biosynthetic pathway. CCoAOMT activity was first identified in cultured cells of parsley and carrot (Kühnl et al., 1989; Pakusch et al., 1989). Since its activity was induced by the challenge of fungal elicitors, CCoAOMT was proposed to play a role in defense by catalyzing the formation of cell wall-bound ferulic polymers (Matern et al., 1988; Pakusch et al., 1989; Schmitt et al., 1991). Recently, the association of CCoAOMT expression with lignification was demonstrated. It was found that the CCoAOMT gene was specifically expressed in all lignifying tissues of zinnia (*Zinnia elegans*; Ye et al., 1994; Ye and Varner, 1995). Therefore, CCoAOMT may play roles in both defense and lignification.

CAOMT (EC 2.1.1.68) has long been proposed to mediate methylation reactions in monolignol biosynthesis (Neish, 1968). Recent antisense experiments indicated that CAOMT might not be the sole OMT involved in methylation (Dwivedi et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995). It has been shown that, although antisense expression of the CAOMT gene resulted in a dramatic decrease of syringyl lignin in tobacco (*Nicotiana tabacum*), it did not change the total lignin content. Therefore, it is possible that other OMTs such as CCoAOMT compensate for the decrease of CAOMT (Atanassova et al., 1995). However, it is not known whether the expression of CCoAOMT is associated with lignification in tobacco stems. Although CCoAOMT activities are detected in a number of species, the tissue distribution of CCoAOMT has not been examined except in zinnia and parsley. Thus, it is important to investigate whether the association of CCoAOMT with lignification is a general pattern in different plant species.

In this paper immunolocalization of CCoAOMT in several dicot species is presented. CCoAOMT was localized preferentially, using antibodies prepared against zinnia recombinant CCoAOMT, in lignifying tissues such as TEs, xylem fibers, and phloem fibers of zinnia, tobacco, tomato (*Lycopersicon esculentum*), soybean (*Glycine max*), alfalfa (*Medicago sativa*), and forsythia (*Forsythia suspensa*). CCoAOMT also accumulated markedly in xylem ray parenchyma and sclerified pith parenchyma. These results suggest that CCoAOMT expression is associated with lignification in diverse dicot plants.

MATERIALS AND METHODS

Zinnia (*Zinnia elegans* var Peter Pan), tobacco (*Nicotiana tabacum* cv Xanthi), tomato (*Lycopersicon esculentum* cv UC 82B), alfalfa (*Medicago sativa* cv CUF101), and soybean (*Glycine max* cv William) were grown in the greenhouse. Forsythia (*Forsythia suspensa* cv Fortunei) was collected on the campus during the early spring season.

Fusion Protein Preparation and Antibody Production

The coding region of CCoAOMT cDNA from zinnia was amplified by PCR using two primers (primer 1, 5′-CTTG-

[1] This work was supported in part by the U.S. Department of Energy and by a research start-up fund from the University of Georgia.

* E-mail ye@dogwood.botany.uga.edu; fax 1–706–542–1805.

Abbreviations: CAOMT, caffeic acid 3-*O*-methyltransferase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; OMT, *O*-methyltransferase; TE, tracheary element.

AATTCATGGCGACACCCACCGGTG-AAACT-3′; primer 2, 5′-CTTTCTAGAGCTTATGCGGCGACACAAAGTGAT-3′). The *Eco*RI and *Xba*I recognition sites were designed in primers 1 and 2, respectively. The PCR-amplified DNA was purified using a PCR purification kit (Qiagen, Chatsworth, CA). After digestion with *Eco*RI and *Xba*I restriction endonucleases, the DNA fragment was ligated into *Eco*RI and *Xba*I sites in the expression vector pMAL-C2 (New England Biolabs), creating the fusion protein construct pMAL-CCoAOMT. The construct was sequenced to confirm that CCoAOMT was fused in frame with the malE gene. The pMAL-CCoAOMT fusion protein was expressed in the *Escherichia coli* DH5αF′ strain. A fusion protein with a molecular mass of about 70 kD (42 kD malE and 28 kD CCoAOMT) was detected on SDS-PAGE. A large amount of fusion protein was purified on an amylose resin affinity column and the purified protein was used to raise polyclonal antibodies in rabbits. The antibodies were purified by passing through a minicolumn of Sepharose conjugated with protein extracts of *E. coli* DH5αF′ (Sambrook et al., 1989) and used for all subsequent experiments.

Cell Culture and Preparation of Tissue Extracts

Mesophyll cells of zinnia were isolated and cultured as described previously (Fukuda and Komamine, 1980; Ye and Varner, 1993). For extract preparation, cells and tissues were homogenized in extraction buffer (50 mM Tris-Cl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, and 0.2 mM PMSF) using a mortar and pestle. After homogenization, the extracts were cleared by centrifugation at 12,000*g* for 15 min. The supernatants were saved for western analysis. Membrane fractions of zinnia extracts were prepared by further centrifugation at 100,000*g* for 60 min.

CCoAOMT Activity Assay

Protein concentration was determined using the Bradford method (Bradford, 1976) with BSA as the standard protein. CCoAOMT activity in cell extracts was assayed as described previously (Pakusch et al., 1989; Ye et al., 1994). CCoAOMT-specific activity was expressed as picomoles of methyl-^{14}C residue from [methyl-^{14}C]*S*-adenosyl-L-Met transferred to caffeoyl CoA per minute per milligram of protein. Each data point was the mean of two separate assays. For antibody inhibition experiments, 10 μL of antibodies was mixed with tissue extracts containing 10 μg of proteins at 4°C for 4 h before the assay of CCoAOMT activity.

Western Analysis

Proteins were separated on 6 to 20% gradient SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Sambrook et al., 1989). The membranes were incubated in the blocking buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, and 5% nonfat milk) for 4 h and then incubated with the polyclonal antibodies against CCoAOMT fusion protein (1:5,000 dilution) in the blocking buffer overnight. In the control the antibodies were replaced with preimmune serum. After the membranes were washed, they were incubated with peroxidase-conjugated goat anti-rabbit polyclonal antibodies (1:10,000 dilution in the blocking buffer) for 1 h. The signals were detected with chemiluminescent reaction reagents (ECL, Amersham) according to the manufacturer's protocol. To reprobe membranes, original primary and secondary antibodies on membranes were completely removed by incubation of membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-Cl, pH 6.7) at 50°C for 30 min.

Immunolocalization by Light Microscopy

Stem and root segments were fixed in the fixation solution (4% freshly depolymerized paraformaldehyde in 0.1 M sodium phosphate, pH 7.0) for 12 h at 4°C after vaccum infiltration for 10 min. After dehydration through a gradient series of ethanol, the tissue segments were embedded in paraffin. Thin sections (15 μm) were cut from paraffin-embedded tissues with a microtome. Sections were mounted on glass slides coated with polylysine (Jackson, 1991). After rehydration, sections were blocked in 5% BSA in PBS solution (10 mM sodium phosphate, pH 7.2, 138 mM NaCl, and 3 mM KCl) for 1 h and then incubated with the polyclonal antibodies against CCoAOMT fusion protein (1:500 dilution in PBS containing 0.1% BSA) for 4 h. After the sections were washed, they were incubated with gold-conjugated goat anti-rabbit polyclonal antibody (Auro-Probe LM GAR, Amersham; 1:40 dilution in PBS solution containing 0.1% BSA) for 1 h. Bound gold particles were revealed by a silver enhancement kit (IntenSe, Amersham). After the sections were counterstained with 0.5% safranine O for 5 min, they were dehydrated through a gradient series of ethanol and embedded in mounting medium (Fisher Scientific). The signals were observed under either bright-field or epipolarized illumination. The presence of lignin was revealed as red color by phloroglucinol-HCl staining or epipolarized illumination.

Immunolocalization by Electron Microscopy

Zinnia stem slices were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at room temperature for 4 h and then postfixed in 2% uranyl acetate. After dehydration through a gradient series of acetone, the tissue slices were infiltrated with K4M embedding medium and polymerized overnight under UV light at −20°C. Ultrathin sections (80 nm thick) were prepared for immunogold electron microscopy (Holwerda et al., 1990). After incubation in the blocking buffer (PBS solution containing 1% BSA and 0.1% gelatin) at room temperature for 30 min, sections were incubated with the polyclonal antibodies against CCoAOMT fusion protein (1:500 dilution in the blocking buffer) overnight at 4°C. Sections were then probed with 10-nm colloidal gold-conjugated goat anti-rabbit polyclonal antibody (Auro-Probe EM GAR, Amersham; 1:25 dilution in the blocking buffer). The signals were visualized under a transmission electron microscope.

RESULTS

Accumulation of CCoAOMT Closely Correlated with Lignification during in Vitro Xylogenesis

To investigate the time course of CCoAOMT accumulation in the process of lignification, isolated mesophyll cells of zinnia cultured in either TE induction medium or basal medium were collected for detection of CCoAOMT by western analysis (Fig. 1). Polyclonal antibodies against zinnia CCoAOMT detected a major band of 28 kD and two minor bands of 26 and 31 kD in the extracts of developing TEs. Freshly isolated mesophyll cells did not show any cross-reactivity with the antibodies. Only faint bands were observed in cells cultured in TE induction medium for 60 h or less (Fig. 1A). At this stage, the cells did not show lignin deposition, as indicated by phloroglucinol-HCl staining (data not shown). At 72 h of induction the cells started to show visible differentiation. By 96 h about 30% of cells showed secondary wall thickening. Concomitantly, CCoAOMT accumulated dramatically between 60 and 84 h. A significant level of CCoAOMT was still detected in cells cultured for 96 h (Fig. 1A).

In contrast, only a low level of CCoAOMT was detected in cells cultured in basal medium (Fig. 1B). This is consistent with the fact that the cells cultured in basal medium did not differentiate into TEs and did not show positive lignin staining by phloroglucinol-HCl (data not shown). When the same membrane was reprobed with the preimmune serum after removal of original first and secondary antibodies, no signals were observed (data not shown).

CCoAOMT Antibodies Showed Cross-Reactivity with Extracts from Several Dicot Plants

To explore the possibility of using the zinnia CCoAOMT antibodies for immunolocalization, extracts from stems or roots of several species, including zinnia, forsythia, tobacco, tomato, alfalfa, and soybean, were used in western analysis. The results in Figure 2 show that the major band of 28 kD was present in all extracts. As in in vitro TEs, zinnia stems and roots had an additional band of 30 kD. An additional 35-kD band was observed in forsythia stems, and soybean stems had an additional minor band of 26 kD, similar to the 26-kD band in developing TEs of zinnia. When the same membrane was reprobed with the preimmune serum after removal of the original first and secondary antibodies, no signal was detected.

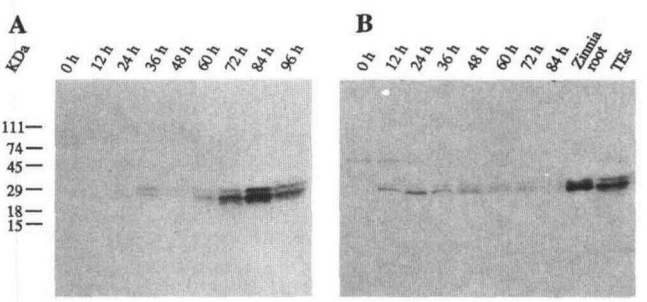

Figure 1. Time course of CCoAOMT accumulation in isolated zinnia mesophyll cells. Mesophyll cells were isolated from young leaves and cultured in either the TE induction medium (A) or the basal medium (B). Cells were collected for total protein extraction at various times, as indicated above each lane. Proteins separated on SDS-polyacrylamide gels were blotted onto nitrocellulose membranes. The membranes were probed with the polyclonal antibodies against zinnia recombinant CCoAOMT. The molecular mass of protein standards is shown on the left. A, CCoAOMT accumulation during in vitro TE differentiation. Lignin deposition in TEs was detected after 72 h of culture in the induction medium. B, CCoAOMT accumulation in cells cultured in the basal medium. Extracts from zinnia roots and in in vitro TEs at the 72-h time were included as positive controls.

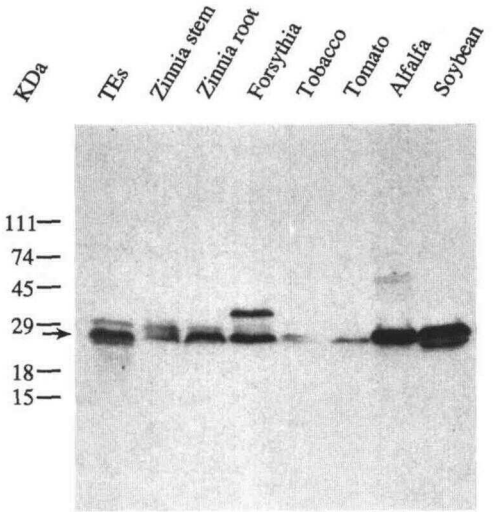

Figure 2. Immunodetection of CCoAOMTs in dicot plants. Crude extracts were prepared from in vitro TEs, stems, and roots of zinnia and from stems of forsythia, tobacco, tomato, alfalfa, and soybean. Proteins were separated on an SDS-polyacrylamide gel. After proteins were blotted onto a nitrocellulose membrane, the CCoAOMT protein was detected with the polyclonal antibodies against zinnia recombinant CCoAOMT.

To confirm the specificity of the antibodies, tissue extracts from different species were mixed with the antibodies before the activity assay. This resulted in a more than 90% decrease in CCoAOMT activities in the extracts. In contrast, the preimmune serum had no significant effect on the enzyme activity (data not shown). These results indicate that the antibodies against zinnia CCoAOMT specifically reacted with CCoAOMTs from zinnia, forsythia, tobacco, tomato, alfalfa, and soybean. They also indicate that the antibodies were useful for immunolocalization of CCoAOMTs in these species. However, the antibodies did not show obvious cross-reactivity on western analysis with extracts from maize roots (data not shown), although CCoAOMT activity was detected in maize roots (Ye et al., 1994).

CCoAOMT Expression Was Spatially Associated with Lignified Tissues

To investigate the tissue distribution of CCoAOMT, immunocytochemical localization was performed in zinnia stems and roots. In the top internode, only TEs were lignified, as indicated by phloroglucinol-HCl staining (data not

shown). The CCoAOMT staining was obvious only in developing TEs (Fig. 3A). No staining was observed in mature TEs and xylem parenchyma cells on the same section (Fig. 3A). In the second internode, both phloem fibers and TEs were lignified. Concomitantly, the marked staining was seen in phloem fibers and TEs (Fig. 3B). Starting from the third internode, xylem fibers were differentiated and lignified, as were phloem fibers and TEs. Coincidentally, the CCoAOMT staining appeared in both xylem fibers and developing TEs (Fig. 3C).

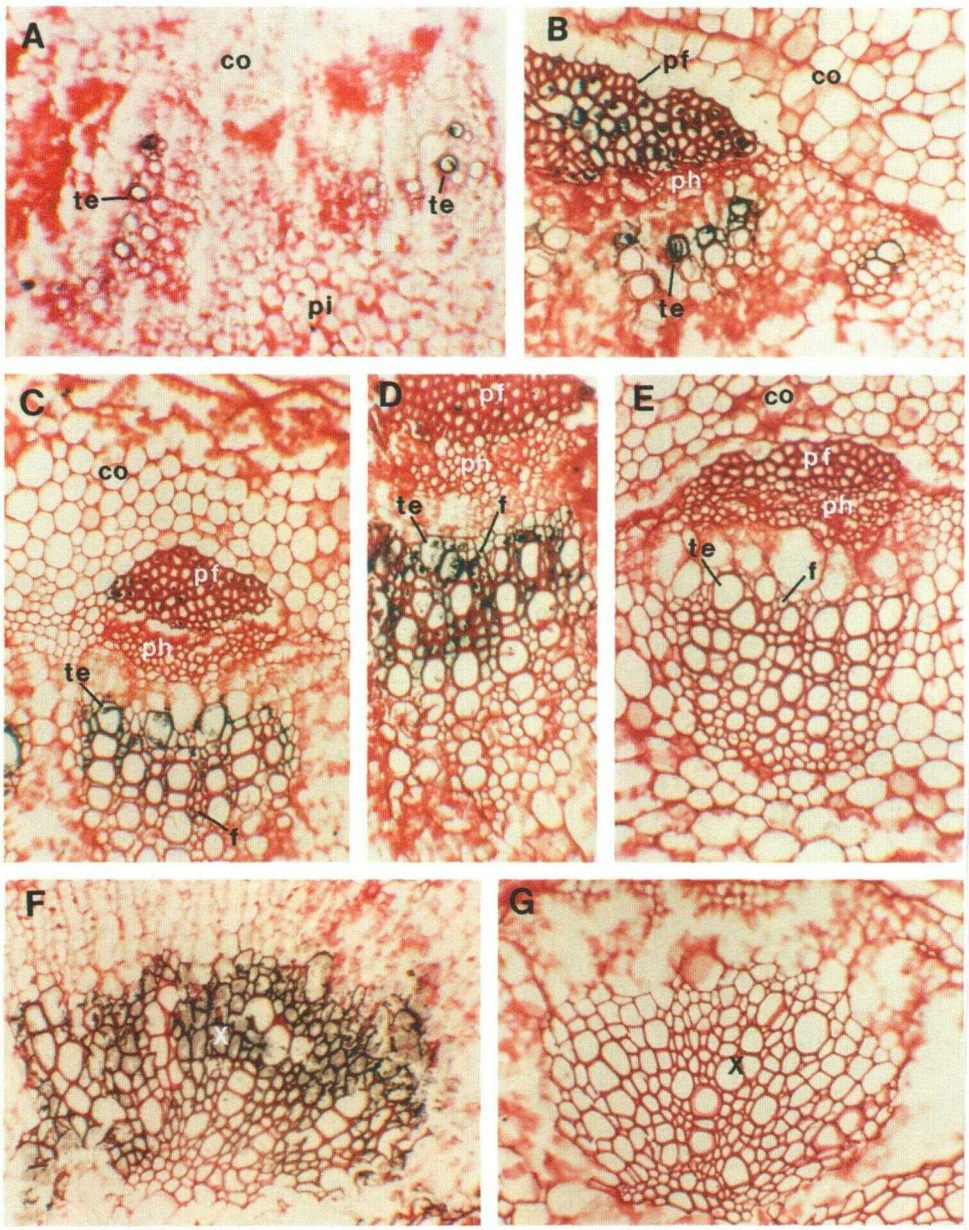

Figure 3. Immunolocalization of CCoAOMT in zinnia stems and roots. Stems and roots from a 6-week-old zinnia plant were used. Sections were probed with the polyclonal antibodies against zinnia recombinant CCoAOMT or the preimmune serum and then detected with gold-conjugated secondary antibody. The signal was amplified with silver enhancement and seen as black. Sections were counterstained with safranine O to reveal the anatomy (red staining). The pictures were taken under a compound microscope with bright-field illumination. A, Section from the first internode stained with the CCoAOMT antibodies. The signal was seen in TEs. B, Section from the second internode stained with the CCoAOMT antibodies. The signal was seen in both TEs and phloem fibers. C, Section from the third internode stained with the CCoAOMT antibodies. The signal was seen in TEs, xylem fibers, and phloem fibers. D, Section from the fourth internode stained with the CCoAOMT antibodies. The signal was seen mainly in TEs and xylem fibers. E, Control section from the third internode stained with the preimmune serum. F, Section from the root stained with the CCoAOMT antibodies. The signal was seen in xylem. G, Section from the root stained with the preimmune serum. co, Cortex; f, xylem fiber; pf, phloem fiber; ph, phloem; pi, pith; te, tracheary element; and x, xylem. Magnification, ×450.

In the fourth internode, which was still undergoing strong secondary xylem differentiation, an intense signal was found in developing TEs and xylem fibers (Fig. 3D), with little staining in phloem fibers (Fig. 3D), presumably because fibers were mature at this stage. The tissue distribution of CCoAOMT was also examined in zinnia roots. As shown in Figure 3F, marked staining was present in both TEs and xylem fibers. The control section stained with the preimmune serum did not show any signal in vascular tissues of either stem (Fig. 3E) or root (Fig. 3G) sections. The results indicate that the presence of CCoAOMT is temporally and spatially associated with lignification in zinnia.

To investigate whether the association of CCoAOMT with lignification is a general pattern in dicot plants, stems from forsythia, tobacco, tomato, alfalfa, and soybean were used for immunolocalization with the antibodies against zinnia CCoAOMT. In forsythia stems marked staining was observed in TEs and fibers, as well as in ray parenchyma cells (Fig. 4, A and C). Intense CCoAOMT staining was found in sclerified pith parenchyma cells next to the vascular cylinder (Fig. 4, A and C). Both phloroglucinol-HCl staining and epifluorescence showed positive lignin staining in walls of these sclerified cells (Fig. 4D), indicating a close correlation between CCoAOMT localization and lignification. The control sections stained with the preimmune serum did not show positive signals (Fig. 4, B and D).

In young tobacco stems CCoAOMT staining was evident only in TEs (Fig. 5A). In older stems xylem fibers and phloem fibers were differentiated and lignified. Accordingly, CCoAOMT staining was seen in both xylem fibers and phloem fibers in addition to TEs (Fig. 5C). A close-up of a portion of xylem in Figure 5C showed a clearer image for the distribution of CCoAOMT in lignified cells (Fig. 5D). Noticeably, marked staining was evident in ray parenchyma cells. The control sections incubated with the preimmune serum did not show positive staining (Fig. 5, B and E).

The distribution of CCoAOMT was also investigated in alfalfa, soybean, and tomato (Fig. 6). In alfalfa stems CCoAOMT staining was obvious in both TEs and xylem fibers (Fig. 6A). In addition, significant staining was seen in a few layers of sclerified pith parenchyma cells adjacent to xylem bundles. Both phloroglucinol-HCl staining and epifluorescence indicated the presence of lignin in walls of these cells (Fig. 6, A and B). In soybean stems intense signals were seen in TEs and xylem fibers (Fig. 6C). Phloem fibers did not show any staining, presumably because these fibers were mature at this stage. Significant staining was found in phloem fibers of younger stems (data not shown). In tomato stems CCoAOMT staining was seen in all lignifying tissues, including TEs and xylem and phloem fibers, as well as in ray parenchyma cells (Fig. 6E). The control sections of alfalfa, soybean, and tomato stems incubated with the preimmune serum did not show any signal (Fig. 6, B, D, and F).

Subcellular Localization of CCoAOMT in Zinnia Stems

To investigate the subcellular localization of CCoAOMT in lignifying cells, young zinnia stems were sectioned for electron microscopic immunolocalization. In developing TEs (next to procambial region), electron-dense gold particles were seen only in the cytoplasm but not in the cell wall (Fig. 7A). It was not clear whether the signal was associated with any organelle, because the subcellular structures were not well revealed in the sections. In one maturing TE, in which cytoplasmic structures seemed to be partially degraded, the CCoAOMT signal was still seen in the cytoplasm (Fig. 7B). The control section incubated with preimmune serum did not show any gold particles in the cytoplasm (Fig. 7C). Some nonspecific gold particles were seen in lignified walls in both antibody- and preimmune-treated samples (Fig. 7, B and C). These results indicate that the distribution of CCoAOMT is somewhat different from that of Phe ammonia-lyase and cinnamyl alcohol dehydrogenase. Both of these enzymes were shown to be present in both the cytoplasm and the secondary cell walls of TEs of zinnia (Nakashima et al., 1997).

To investigate whether CCoAOMT was associated with any membrane structure, soluble and membrane fractions were prepared from in vitro differentiating TEs of zinnia. Enzyme activity assay showed no significant difference of total CCoAOMT activity between the crude extract (480 ± 60 pmol min^{-1} mg^{-1} after 12,000*g* centrifugation) and the soluble fraction (460 ± 60 pmol min^{-1} mg^{-1} after 100,000*g* centrifugation). The membrane fraction had a low level of CCoAOMT activity (16 ± 7 pmol min^{-1} mg^{-1}), indicating that CCoAOMT is not associated with membranes.

DISCUSSION

The polyclonal antibodies against zinnia recombinant CCoAOMT recognized both denatured and native CCoAOMT from zinnia. This is different from the polyclonal antibodies against parsley CCoAOMT, which reacted only with the native CCoAOMT but not with the denatured form (Pakusch et al., 1989). The molecular mass (28 kD) of zinnia CCoAOMT detected on western analysis corresponds to the molecular weight (27,629) predicted from its cDNA-encoded sequence (Fig. 1). Two minor bands of 26 and 31 kD may represent isoforms of CCoAOMTs. This possibility was supported by the fact that several CCoAOMT cDNAs with different lengths of coding regions were found in a zinnia cDNA library (Z.-H. Ye, unpublished data).

The antibodies also recognized CCoAOMTs from forsythia, tobacco, tomato, alfalfa, and soybean (Fig. 2). This is consistent with the high similarity of CCoAOMT protein sequences among dicot plants. For example, zinnia CCoAOMT shares 93% amino acid similarity (85% identity) with parsley CCoAOMT (Ye et al., 1994). All of these dicot plants have a major band of CCoAOMT of 28 kD on western analysis, indicating that CCoAOMT from different species have a similar molecular mass. The only exception is forsythia, in which an additional major band of 35 kD was also observed. It is not known whether this 35-kD protein is an isoform of CCoAOMT. It is unlikely that the antibodies react with CAOMT, because CCoAOMT and CAOMT do not share protein sequence similarity. In addition, the CCoAOMT antibodies did not recognize zinnia

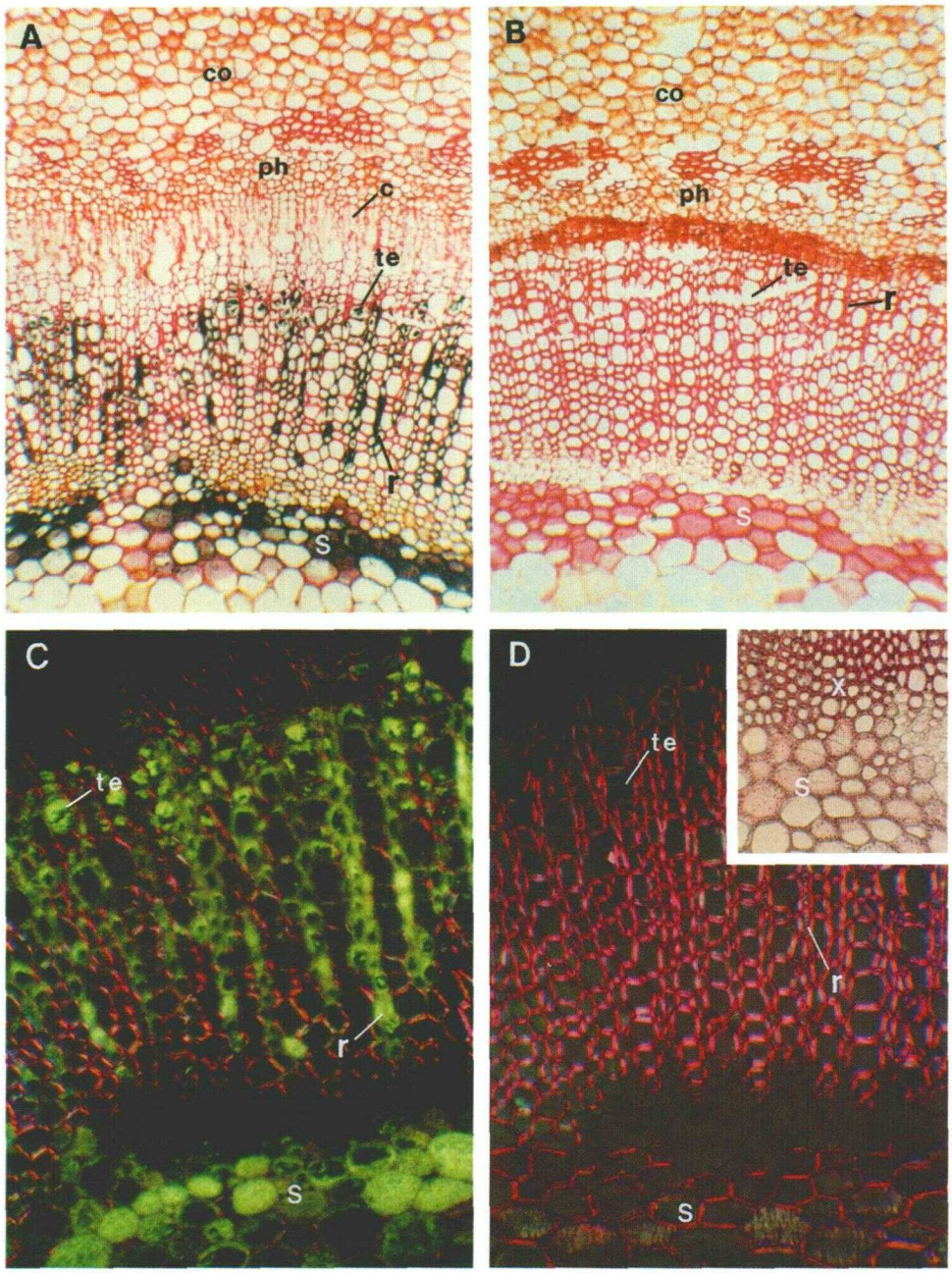

Figure 4. Immunolocalization of CCoAOMT in forsythia stems. A, Section stained with zinnia CCoAOMT antibodies. The signal was seen as black color in xylem and sclerified pith parenchyma cells under bright-field illumination. B, A section incubated with the preimmune serum. No signal was seen under bright-field illumination. C, Close-up of a portion of xylem in A. The signal was seen as yellow-green under UV epifluorescence. Lignin showed red autofluorescence under UV light. D, Close-up of a portion of xylem in B. No signal was seen with the preimmune serum. Lignin autofluorescence was seen under UV epifluorescence. Inset in D shows phloroglucinol-HCl staining of lignin in xylem cells and adjacent sclerified pith cells. c, Cambium; co, cortex; ph, phloem; r, ray parenchyma cells; s, sclerified pith parenchyma cells; te, tracheary element; and x, xylem. Magnification, ×450.

CAOMT, which has a molecular mass of 38.6 kD (data not shown).

The marked increase of CCoAOMT detected on western analysis was closely correlated with the process of lignification in in vitro TEs of zinnia (Fig. 1). The temporal and spatial distribution patterns of CCoAOMT protein in zinnia stems detected by immunolocalization was similar to the localization of CCoAOMT mRNA (Ye et al., 1994). Immunolocalization gave more detailed information at the cellular level. For example, in young stems in which only TEs showed lignin deposition, CCoAOMT was seen only in these TEs but not in xylem parenchyma cells (Fig. 3). In older stems in which both TEs and xylem fibers showed lignin deposition, CCoAOMT was seen in both TEs and

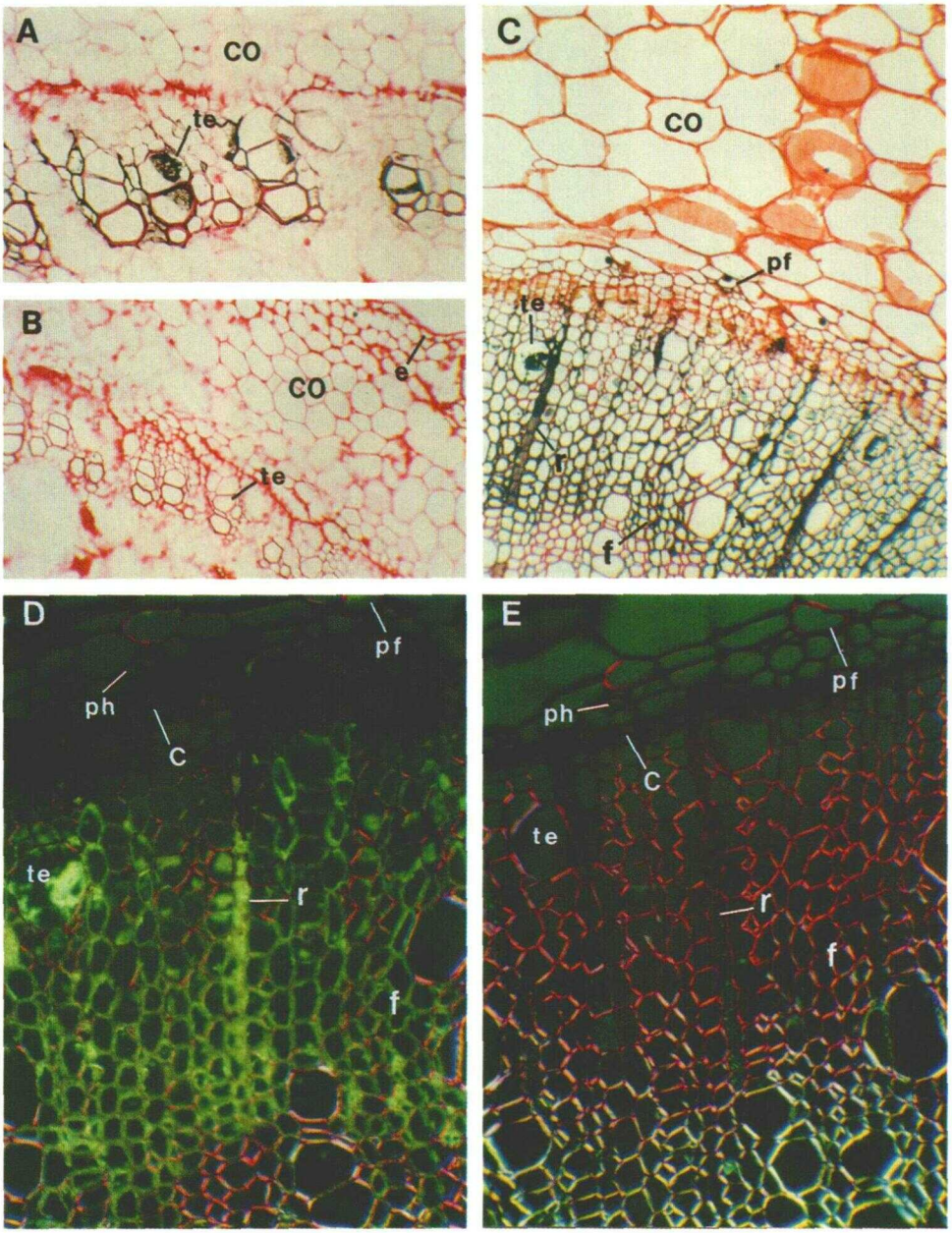

Figure 5. Immunolocalization of CCoAOMT in tobacco stems. A, Section from the young stem stained with the CCoAOMT antibodies. The signal was seen as black color in TEs under bright-field illumination. B, Section stained with the preimmune serum. No signal was observed under bright-field illumination. C, Section from the old stem stained with the CCoAOMT antibodies. The signal was seen as black color in TEs, xylem fibers, phloem fibers, and ray parenchyma cells under bright-field illumination. D, Close-up of a portion of xylem in C. The signal was seen as yellow-green under UV epifluorescence. Lignin showed red autofluorescence under UV light. E, Close-up of a portion of xylem in the old stem stained with the preimmune serum. No CCoAOMT signal was observed under UV epifluorescence except lignin autofluorescence seen as red color. c, Cambium; CO, cortex; e, epidermis; f, xylem fiber; pf, phloem fiber; ph, phloem; r, ray parenchyma cells; and te, tracheary element. Magnification, ×450.

xylem fibers. Since CCoAOMT was present only in TEs of young stems, this suggests that lignin precursors are synthesized in TEs rather than transported from adjacent parenchyma cells. Other lignin pathway enzymes, such as cinnamate 4-hydroxylase, phenylalanine ammonia-lyase, and cinnamyl alcohol dehydrogenase, were also localized in TEs (Smith et al., 1994; Nakashima et al., 1997).

Circumstantial evidence has suggested that xylem parenchyma cells may contribute monolignols to TEs for the synthesis of lignin (Boudet et al., 1995). For example, Pickett-Heaps (1968) showed that mature TEs were still able to incorporate labeled lignin precursors into lignin in walls of TEs. Furthermore, it has been shown that promoters of lignin pathway genes directed GUS gene expression

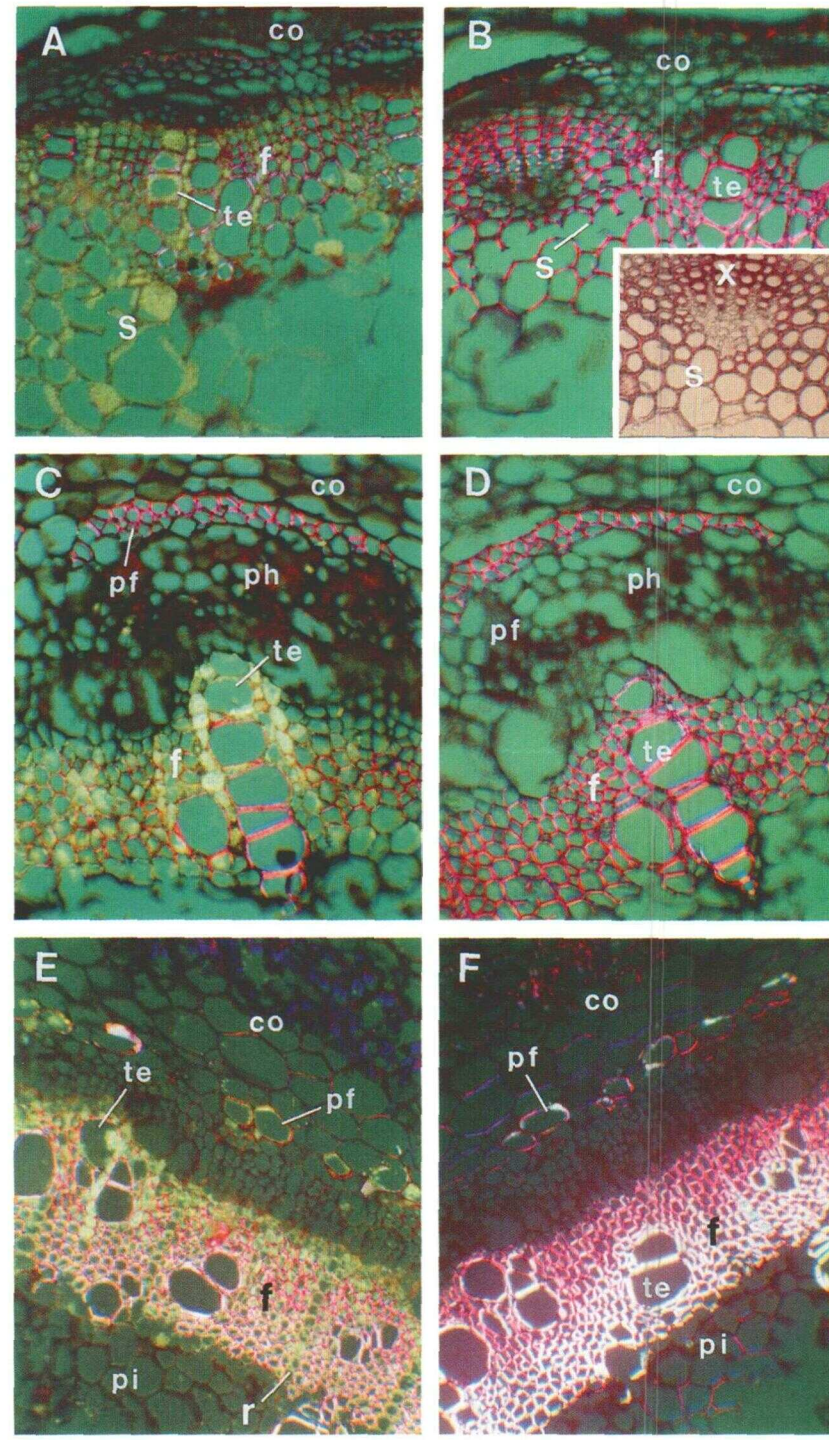

Figure 6. Immunolocalization of CCoAOMT in stems of alfalfa, soybean, and tomato. The sections were probed with zinnia CCoAOMT polyclonal antibodies or the preimmune serum and then incubated with gold-conjugated secondary antibody. After amplification with silver enhancement, the signal was visualized as yellow-green under UV epifluorescence illumination. Lignin showed red autofluorescence under UV light. A, Section of alfalfa stem stained with the CCoAOMT antibodies. The signal was seen in TEs, xylem fibers, and sclerified pith parenchyma cells. B, Section of alfalfa stem incubated with the preimmune serum. Inset in B showed phloroglucinol-HCl staining of lignin in xylem and adjacent pith parenchyma cells. C, Section of soybean stem stained with the CCoAOMT antibodies. The signal was seen in TEs and xylem fibers. D, Section of soybean stem incubated with the preimmune serum. E, Section of tomato stem stained with the CCoAOMT antibodies. The signal was seen in TEs, xylem fibers, phloem fibers, and ray parenchyma cells. F, Section of tomato stem incubated with the preimmune serum. co, Cortex; f, xylem fiber; pf, phloem fiber; ph, phloem; pi, pith; r, ray parenchyma cells; s, sclerified pith parenchyma cells; te, tracheary element; and x, xylem. Magnification, ×450.

in ray parenchyma cells in transgenic plants (Bevan et al., 1989; Feuillet et al., 1995). The immunolocalization results in this study (Figs. 4–6) also showed the presence of CCoAOMT in ray parenchyma cells. This indicates that in secondary xylem ray parenchyma cells may synthesize monolignols for use by adjacent TEs and fibers.

Immunolocalization of CCoAOMTs in other dicot species such as forsythia, tobacco, alfalfa, soybean, and tomato showed the same CCoAOMT distribution pattern as in zinnia (Figs. 4–6). CCoAOMTs were expressed in TEs, phloem fibers, and xylem fibers, which are lignified tissues in stems. These results support the early hypothesis that the CCoAOMT-mediated methylation branch is a general one in lignin biosynthesis during normal growth and development (Ye et al., 1994). It is likely that in dicot plants both CCoAOMT- and CAOMT-mediated methylation branches are involved in lignification, although this may not hold true for loblolly pine (Li et al., 1997). This was further suggested

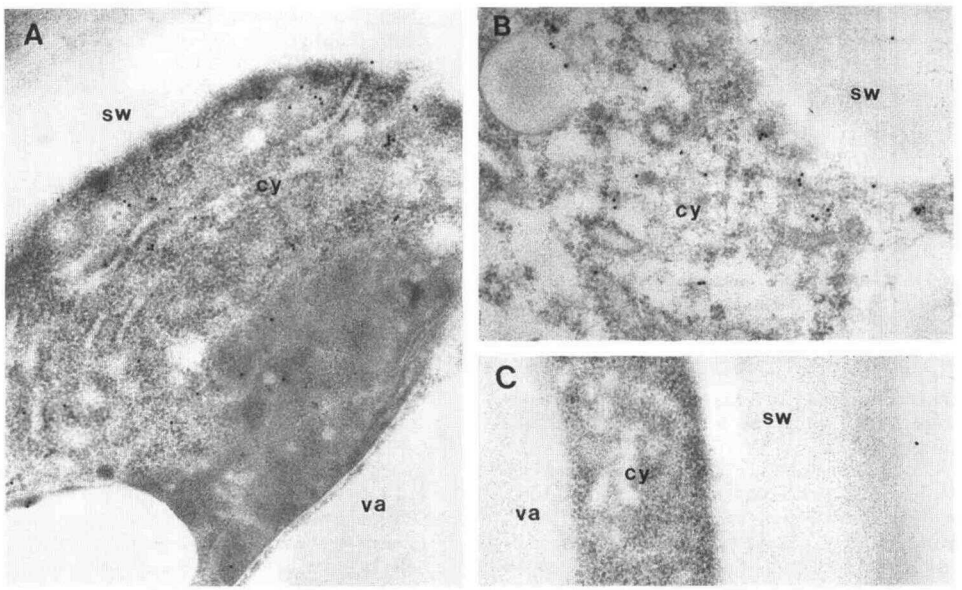

Figure 7. Electron microscopic immunolocalization of CCoAOMT in TEs. Sections from zinnia stems were probed with the CCoAOMT antibodies and then incubated with gold-labeled secondary antibody. The signal was seen as black dots. A, Section of a developing TE stained with the CCoAOMT antibodies. B, Section of a maturing TE stained with the CCoAOMT antibodies. C, Section of a developing TE stained with the preimmune serum. cy, Cytoplasm; sw, secondary wall; and va, vacuole. Magnification, ×45,000.

by the CAOMT antisense experiments (Dwivedi et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995).

In transgenic tobacco plants expressing the antisense CAOMT gene, a dramatic decrease of CAOMT activity did not change the lignin content (Atanassova et al., 1995; Doorsselaere et al., 1995), indicating that other OMTs may compensate for the loss of CAOMT. However, a decrease of CAOMT activity did alter the lignin composition, i.e. syringyl lignin was significantly reduced, suggesting that syringyl lignin synthesis is controlled mainly by CAOMT. This seems contradictory to what was proposed for the role of CCoAOMT in lignification (Ye and Varner, 1995). However, as demonstrated for some lignin pathway genes such as CAOMTs (Davin and Lewis, 1992; Whetten and Sederoff, 1995), CCoAOMTs in different species may have different substrate specificities and differential expression pattern in different developmental stages. Therefore, it is possible that CCoAOMT in tobacco may compensate for the loss of CAOMT in the synthesis of guaiacyl lignin, but it may not use 5-hydroxylferuloyl CoA as a substrate efficiently or the substrate 5-hydroxylferuloyl CoA may not be available. Further experiments on the decrease of both CCoAOMT and CAOMT in transgenic tobacco will unequivocally answer these questions.

ACKNOWLEDGMENTS

I am grateful to Dr. Candace H. Haigler for critical reading of the manuscript and to two anonymous reviewers for their suggestions.

Received July 18, 1997; accepted September 11, 1997.
Copyright Clearance Center: 0032–0889/97/115/1341/10.

LITERATURE CITED

Atanassova R, Favet N, Martz F, Chabbert B, Tollier M-T, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequence in sense and antisense orientation. Plant J **8:** 465–477

Bevan M, Shufflebottom D, Edwards K, Jefferson R, Schuch W (1989) Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J **8:** 1899–1906

Boudet AM, Lapierre C, Grima-Pettenati J (1995) Biochemistry and molecular biology of lignification. New Phytol **129:** 203–236

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Davin LB, Lewis NG (1992) Phenylpropanoid metabolism: biosynthesis of monolignols, lignans and neolignans, lignins and suberins. *In* HA Stafford, RK Ibrahim, eds, Phenolic Metabolism in Plants. Plenum Press, New York, pp 325–375

Doorsselaere JV, Baucher M, Chognot E, Chabbert B, Tollier M-T, Petit-Conil M, Leplé J-C, Pilate G, Cornu D, Monties B, and others (1995) A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid *O*-methyltransferase activity. Plant J **8:** 855–864

Dwivedi UN, Campbell WH, Yu J, Datla RSS, Bugos RC, Chiang VL, Podila GK (1994) Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. Plant Mol Biol **26:** 61–71

Feuillet C, Lauvergeat V, Deswarte C, Pilate G, Boudet A, Grima-Pettenati J (1995) Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants. Plant Mol Biol **27:** 651–667

Fukuda H, Komamine A (1980) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of zinnia *elegans*. Plant Physiol **65:** 57–60

Holwerda BC, Galvin NJ, Baranski TJ, Rogers JC (1990) In vitro processing of aleurain, a barley vacuolar thiol protease. Plant Cell **2:** 1091–1106

Jackson D (1991) In situ hybridization in plants. *In* DJ Bowles, SJ Gurr, MJ McPherson, eds, Molecular Plant Pathology: A Practical Approach. Oxford University Press, London, pp 163–174

Kühnl T, Koch U, Heller W, Wellmann E (1989) Elicitor induced *S*-adenosyl-L-methionine:caffeoyl-CoA 3-*O*-methyltransferase from carrot cell suspension cultures. Plant Sci **60:** 21–25

Li L, Popko JL, Zhang X-H, Osakabe K, Tsai C-J, Joshi CP, Chiang VL (1997) A novel multifunctional *O*-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. Proc Natl Acad Sci USA **94:** 5461–5466

Matern M, Wendorff H, Hamerski D, Pakusch AE, Kneusel RE (1988) Elicitor-induced phenylpropanoid synthesis in *Apiaceae* cell cultures. Bull Liaison Group Polyphenols **14:** 173–184

Nakashima J, Awano T, Takabe K, Fujita M, Saiki H (1997) Immunocytochemical localization of phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase in differentiating tracheary elements derived from zinnia mesophyll cells. Plant Cell Physiol **38:** 113–123

Neish AC (1968) Monomeric intermediates in the biosynthesis of lignin. *In* K Freudenberg, AC Neish, eds, Constitution and Biosynthesis of Lignin. Springer-Verlag, New York, pp 1–43

Ni W, Paiva NL, Dixon RA (1994) Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. Transgenic Res **3:** 120–126

Pakusch A-E, Kneusel RE, Matern U (1989) *S*-Adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase from elicitor-treated parsley cell suspension cultures. Arch Biochem Biophys **271:** 488–494

Pickett-Heaps JD (1968) Xylem wall deposition. Radioautographic investigations using lignin precursors. Protoplasma **65:** 181–205

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmitt D, Pakusch A-E, Matern U (1991) Molecular cloning, induction, and taxonomic distribution of caffeoyl-CoA 3-*O*-methyltransferase, an enzyme involved in disease resistance. J Biol Chem **266:** 17416–17423

Smith CG, Rodgers MW, Zimmerlin A, Ferdinando D, Bolwell GP (1994) Tissue and subcellular immunolocalisation of enzymes of lignin synthesis in differentiating and wounded hypocotyl tissue of French bean (*Phaseolus vulgaris* L.). Planta **192:** 155–164

Whetten R, Sederoff R (1995) Lignin biosynthesis. Plant Cell **7:** 1001–1013

Ye Z-H, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in zinnia. Plant Cell **6:** 1427–1439

Ye Z-H, Varner JE (1993) Gene expression patterns associated with *in vitro* tracheary element formation in isolated single mesophyll cells of *Zinnia elegans*. Plant Physiol **103:** 805–813

Ye Z-H, Varner JE (1995) Differential expression of two *O*-methyltransferases in lignin biosynthesis in *Zinnia elegans*. Plant Physiol **108:** 459–467