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An Alternative Methylation Pathway in Lignin Biosynthesis in *Zinnia*

Zheng-Hua Ye,[a] Richard E. Kneusel,[b] Ulrich Matern,[b] and Joseph E. Varner [a,1]

[a] Department of Biology, Washington University, St. Louis, Missouri 63130

[b] Institut für Biologie II, Lehrstuhl für Biochemie der Pflanzen, Universität Freiburg, Schänzlestr. 1, 79104 Freiburg, Germany

S-Adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT) is implicated in disease resistant response, but whether it is involved in lignin biosynthesis is not known. We isolated a cDNA clone for CCoAOMT in differentiating tracheary elements (TEs) induced from *Zinnia*-isolated mesophyll cells. RNA gel blot analysis showed that the expression of the CCoAOMT gene was markedly induced during TE differentiation from the isolated mesophyll cells. Tissue print hybridization showed that the expression of the CCoAOMT gene is temporally and spatially regulated and that it is associated with lignification in xylem and in phloem fibers in *Zinnia* organs. Both CCoAOMT and caffeic acid *O*-methyltransferase (COMT) activities increased when the isolated *Zinnia* mesophyll cells were cultured, whereas only CCoAOMT activity was markedly enhanced during lignification in the in vitro–differentiating TEs. The induction pattern of the OMT activity using 5-hydroxyferuloyl CoA as substrate during lignification was the same as that using caffeoyl CoA. Taken together, the results indicate that CCoAOMT is associated with lignification during xylogenesis both in vitro and in the plant, whereas COMT is only involved in a stress response in vitro. We propose that CCoAOMT is involved in an alternative methylation pathway in lignin biosynthesis. In *Zinnia* in vitro–differentiating TEs, the CCoAOMT mediated methylation pathway is dominant.

INTRODUCTION

Lignin is a complex phenylpropanoid polymer found in vascular plants. It is mainly deposited in the walls of those cells that make up systems for conduction and support, such as xylem and phloem fibers. Thus, the evolution of the ability to synthesize lignin is thought to be an important step in the evolution of land plants (Raven et al., 1992). In addition, lignin deposition upon wounding or microbial invasion may play a defense role (Vance et al., 1980; Davin and Lewis, 1992). Lignin contributes about 20 to 30% of the dry weight of most woods and represents the second most abundant natural product (Brown, 1969; Gross, 1979). Although it is important for plant growth and development, lignin is undesirable in some aspects. For example, lignin is extracted during pulp and paper production by the use of chemicals, which have an adverse impact on the environment. Further, lignin decreases digestibility in animal forage (Cherney et al., 1990). Therefore, reduction and/or alteration of lignin composition could almost certainly reduce the pollution from pulping and improve the digestibility of animal forage. For these reasons, the structure, biosynthesis, and function of lignin have been intensively investigated since its discovery by A. Payen in 1838 (see Gross, 1979).

Lignin is formed through dehydrogenative polymerization of monomeric lignin precursors including *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The biosynthetic pathway of lignin precursors has been well defined using tracer and enzyme studies (Grisebach, 1981; Lewis and Yamamoto, 1990; Davin and Lewis, 1992). The chemical structures of monomeric lignin precursors differ only by methoxyl groups. Thus, the methylation of 3- and/or 5-hydroxyl groups of hydroxycinnamic acids is an important step influencing lignin composition. The *O*-methyltransferases (OMTs) involved in lignin formation have been characterized in a number of species. They either use both caffeic acid and 5-hydroxyferulic acid (in angiosperm dicots) or mainly use caffeic acid (in gymnosperms) as substrates. Thus, only free hydroxycinnamic acids are considered to be the in vivo substrates of the OMTs, and caffeic acid *O*-methyltransferase (COMT) is the only one known to be involved in the methylation reaction of lignin biosynthesis (Grisebach et al., 1981; Bugos et al., 1991; Gowri et al., 1991; Collazo et al., 1992; Davin and Lewis, 1992).

However, it was found that in wheat and barley derivatives of cinnamic, *p*-coumaric, caffeic, and ferulic acids were present. It was proposed that these derivatives instead of free acids were the intermediates in lignin biosynthesis (El-Basyouni et al., 1964; Brown, 1966, 1969; El-Basyouni and Neish, 1966). This is consistent with the evidence that derivatives of hydroxycinnamic acids are widely distributed in vascular plants (El-Basyouni and Neish, 1966). In addition, the free acids would

[1] To whom correspondence should be addressed.

tend to be insoluble at the acid pH of most plant saps (Brown, 1969). Therefore, Neish (1968) proposed that the carboxyl group is first activated either on cinnamic acid or on *p*-coumaric acid, and that subsequent hydroxylation and methylation are performed on these ester forms instead of on the free acids. However, this proposed pathway has been neglected since then, probably because the hydroxylases and OMTs in lignin biosynthesis characterized so far use free acid forms. It is not established whether different types of hydroxylases and OMTs specifically using derivatives as substrates are involved in lignin biosynthesis.

In the phenylpropanoid biosynthetic pathway, another OMT, *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT) was found in parsley and carrot cell suspension cultures (Matern et al., 1988; Kühnl et al., 1989; Pakusch et al., 1989). The enzyme activity rapidly increases in response to fungal elicitor treatment in both parsley and carrot cell suspension cultures (Matern et al., 1988; Kühnl et al., 1989; Pakusch et al., 1989). Because no lignin synthesis–specific enzyme activity, such as that of feruloyl-CoA:NADP oxidoreductase, and no lignin were detected in elicitor-treated parsley cells, CCoAOMT was purported to play a role in rapid defense response to form cell wall bound–ferulic polymers (Matern et al., 1988; Pakusch et al., 1989). Although Kühnl et al. (1989) questioned the sole role of OMT acting on free hydroxycinnamic acids in phenylpropanoid metabolism and proposed the possible involvement of CCoAOMT in the production of phenylpropanoid-derived compounds, including lignin, no evidence has been presented about the involvement of CCoAOMT in lignin biosynthesis.

The *Zinnia*-isolated mesophyll cell culture system has long been used to study the process of tracheary element (TE) formation (Fukuda and Komamine, 1985; Fukuda, 1992; Church, 1993). The *Zinnia* system provides an ideal system to search for the genes involved in lignin biosynthesis. In this system, up to 60% of isolated mesophyll cells semisynchronously differentiate into TEs in response to auxin and cytokinin treatment within 72 hr. Secondary wall thickening occurs at ~48 hr after culture, whereas lignin deposition proceeds after ~60 hr. Lignification and autolysis are the two dominant features that occur after 60 hr of culture. In the *Zinnia* system, lignin deposition is easily detected by phloroglucinol staining, and it is mainly associated with secondary wall thickening. A number of enzymes in the lignin biosynthetic pathway, such as phenylalanine ammonia-lyase (Fukuda and Komamine, 1982; Lin and Northcote, 1990), 4-coumarate:CoA ligase (Church and Galston, 1988), and peroxidase (Church and Galston, 1988; Sato et al., 1993), have been shown to be up-regulated. Thus, analysis of up-regulated genes during active lignification in the *Zinnia* system should help elucidate possible alternative pathways in lignin biosynthesis.

During the isolation of up-regulated genes involved in TE differentiation induced from *Zinnia*-isolated mesophyll cells, we identified a cDNA clone for CCoAOMT. To determine the possible role of the CCoAOMT in TE formation, we examined the patterns of CCoAOMT mRNA accumulation in both in vitro–differentiating TEs and *Zinnia* organs and analyzed spatial distribution of CCoAOMT mRNA in *Zinnia* and parsley organs. In addition, we assayed the time courses of both CCoAOMT and COMT activity changes during TE differentiation. The results indicated that CCoAOMT is involved in an alternative methylation pathway of lignin biosynthesis and that it is the dominant pathway in *Zinnia*. Thus, the results support the hypothesis that derivatives of hydroxycinnamic acids are intermediates in lignin biosynthesis.

RESULTS

Isolation of *Zinnia* CCoAOMT cDNA by Subtractive Hybridization

A number of cDNAs whose mRNAs were present at elevated levels were isolated from the subtractive library, and their expression patterns during TE differentiation were confirmed by RNA gel blot analysis. By DNA sequence analysis, we determined that one clone of these cDNAs encodes a CCoAOMT. The cDNA clone was used as a probe to isolate a full-length cDNA clone from a *Zinnia* cDNA library for further analysis. The CCoAOMT cDNA was cloned in an *Escherichia coli* expression vector. No activity was detected in the *E. coli* JM109 containing the pKK388-1 vector, whereas when pKK388-1 CCoAOMT was transformed into JM109, OMT enzyme activity using caffeoyl CoA and methyl-^{14}C-*S*-adenosyl-L-methionine (SAM) as substrates was detected with a specific activity of 276 pmol/min/mg of protein.

Analysis of the CCoAOMT cDNA Sequence

The CCoAOMT cDNA is 918 bp long (Figure 1), which is approximately the length of the 1.2-kb mRNA estimated from the RNA gel blot. The longest open reading frame encodes a polypeptide of 245 amino acids with a predicted molecular mass of 27,629 D. By comparing this cDNA sequence with the nucleotide and peptide sequences in sequence data bases, we found 77% nucleotide identity in the open reading frame with parsley CCoAOMT cDNA and 93% amino acid similarity (85% identity) with parsley CCoAOMT cDNA (Figure 2; Schmitt et al., 1991). In addition, the *Zinnia* CCoAOMT shows significant amino acid similarity with several other OMTs (Figure 2): 71% similarity (51% identity) with *Stellaria* CCoAOMT (GenPept peptide sequence data base accession number L22203); 55% similarity (39% identity) with OMT from *Streptomyces mycarofaciens* (Hara and Hutchinson, 1992); 45% similarity (21% identity) with rat catechol OMT at the N-terminal residues 13 to 126 (Salminen et al., 1990); and 48% similarity (21% identity) with human catechol OMT at the N-terminal residues 90 to 176 (sequence not shown; Bertocci et al., 1991).

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                TAACACCAACCATTTTACAAACACATAACCAAAAATCATAGCATAA    46

ATG GCG ACA CCC ACC GGT GAA ACT CAA CCT GCT AAA CAC CAA GAA    91
Met Ala Thr Pro Thr Gly Glu Thr Gln Pro Ala Lys His Gln Glu    15

GTT GGT CAC AAA AGC CTC CTT CAA AGT GAT GCT CTT TAC CAA TAC   136
Val Gly His Lys Ser Leu Leu Gln Ser Asp Ala Leu Tyr Gln Tyr    30

ATT CTT GAA ACC AGC GTC TAC CCG AGA GAA CCA CAA CCC ATG AAA   181
Ile Leu Glu Thr Ser Val Tyr Pro Arg Glu Pro Gln Pro Met Lys    45

GAG CTA CGC AGG ATC ACC GCC AAA CAC CCC TGG AAT CTT ATG ACC   226
Glu Leu Arg Arg Ile Thr Ala Lys His Pro Trp Asn Leu Met Thr    60

ACG TCT GCT GAT GAA GGA CAG TTT CTG AAC TTA CTT CTT AAG CTT   271
Thr Ser Ala Asp Glu Gly Gln Phe Leu Asn Leu Leu Leu Lys Leu    75

ATA AAT GCA AAG AAC ACG ATG GAG ATC GGT GTC TAC ACC GGT TAT   316
Ile Asn Ala Lys Asn Thr Met Glu Ile Gly Val Tyr Thr Gly Tyr    90

TCT CTT CTT TCT ACC GCC CTT GCT CTC CCT GAA GAC GGA AAG ATA   361
Ser Leu Leu Ser Thr Ala Leu Ala Leu Pro Glu Asp Gly Lys Ile   105

TTG GCT TTG GAT ATA AAC CGC GAG AAT TAT GAA ATC GGT CTT CCT   406
Leu Ala Leu Asp Ile Asn Arg Glu Asn Tyr Glu Ile Gly Leu Pro   120

ATT ATT CAG AAA GCC GGT GTT GCT CAC AAG ATT GAC TTT AGA GAA   451
Ile Ile Gln Lys Ala Gly Val Ala His Lys Ile Asp Phe Arg Glu   135

GGT CCT GCC CTC CCA CTT CTT GAC CAA ATG CTC CAA GAT GAA AAA   496
Gly Pro Ala Leu Pro Leu Leu Asp Gln Met Leu Gln Asp Glu Lys   150

TGT CAT GGT TCA TTT GAC TTT ATC TTT GTG GAT GCG GAT AAA GAC   541
Cys His Gly Ser Phe Asp Phe Ile Phe Val Asp Ala Asp Lys Asp   165

AAC TAT CTT AAC TAC CAT AAA AGG TTA ATC GAT CTG GTG AAA TTC   586
Asn Tyr Leu Asn Tyr His Lys Arg Leu Ile Asp Leu Val Lys Phe   180

GGG GGA GTG ATT GGC TAC GAT AAC ACC CTA TGG AAT GGG TCA TTG   631
Gly Gly Val Ile Gly Tyr Asp Asn Thr Leu Trp Asn Gly Ser Leu   195

GTG GCA CCA GCA GAC GCG CCA CTA AGA AAG TAT GTA AGG TAT TAC   676
Val Ala Pro Ala Asp Ala Pro Leu Arg Lys Tyr Val Arg Tyr Tyr   210

AGG GAT TTC GTG TTA GAG CTT AAC AAA GCT CTG GCT GTT GAC CCG   721
Arg Asp Phe Val Leu Glu Leu Asn Lys Ala Leu Ala Val Asp Pro   225

AGA GTG GAG ATC TGT CAG CTT CCG GTC GGT GAT GGA ATC ACT TTG   766
Arg Val Glu Ile Cys Gln Leu Pro Val Gly Asp Gly Ile Thr Leu   240

TGT CGC CGC ATA AGC TAA TCACAATTGACAAACCAACGAAAGCGTATGCACTT   819
Cys Arg Arg Ile Ser *                                        245

TTATGATTCAGTGTGACAAAACTATGTATTCTTTCTAATGTTTGTTTGATATGTTTAAG   878
AATTGTAAAATGAAAAGATATGGGCAGTTTACTTTTCCTTAAAAAAAAAAAAAAAA      934
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Figure 1. The cDNA and Deduced Amino Acid Sequence of *Zinnia* CCoAOMT.

The translation termination codon is marked with an asterisk. The nucleotide sequence was submitted to GenBank with the accession number U13151.

Genomic Organization of *Zinnia* CCoAOMT

A DNA gel blot containing genomic DNA digested with EcoRI, XbaI, BamHI (enzymes that do not cut the CCoAOMT cDNA), and HindIII (enzyme that cuts once in the CCoAOMT cDNA) was hybridized with the ^{32}P-labeled *Zinnia* CCoAOMT cDNA (Figure 3). It shows that all of these digests have at least five (EcoRI, XbaI, and BamHI) or 10 (HindIII) hybridizing fragments, indicating that a CCoAOMT gene family exists in *Zinnia*.

Accumulation of CCoAOMT Transcript in *Zinnia*-Cultured Cells and Organs

To determine whether the expression of the CCoAOMT gene in *Zinnia*-cultured cells is associated with TE formation or resulted from the mechanical isolation and subsequent culture of cells, we analyzed the accumulation of CCoAOMT transcript in *Zinnia*-isolated mesophyll cells cultured under different conditions (Figure 4). No CCoAOMT mRNA was detected in freshly isolated mesophyll cells (Figure 4A). A low level of mRNA accumulated between 12 and 36 hr after culture in the induction medium containing naphthaleneacetic acid (NAA) and benzyladenine (BA). During this period, cells undergo dedifferentiation and prepare for differentiation, but no differentiation features were visible. However, mRNA accumulated to a high level by 48 hr and remained at that level up to 60 hr of culture in the induction medium. During this period, secondary wall thickening initiated at ~48 hr, and lignin

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Zi CCoAOMT  MATPTGETQPAKHQEVGHKSLLQSDALYQYILETSVYPRE 40
Pa CCoAOMT       MASNGKS::S:::::::::::::::::::::::::: 36
St CCoAOMT     MLTK:MGNFFT::KDTG::::EQ:H::::D::F::: 37
   Sm OMT                    MADQTTLSPALLDYARSVALRED 23
  Rat OMT                      MGDTKEQRILRYVQQNAK:GD 21

Zi CCoAOMT  PQPMKELRRITAKHPWNLMTTSADEGQFLNLLLKLINAKN 80
Pa CCoAOMT  :EA:::::EV:::::::::::::::::::M:::::::::: 76
St CCoAOMT  SEHL::::KA:ES::MSF:G::PLA::L:SFM::TVKP:K 77
   Sm OMT   GLLRELHDMTAQLPGGRA:QIMPE:A:::G::IR:VG:RR 63
  Rat OMT   ::SVL:AIDTYCTQKEWA:NVGDAK::IMDAVIREYSPSL 61
                              * *
Zi CCoAOMT  TMEIGVYTGYSLLSTALALPEDGKILALDINRENYEIGLP 120
Pa CCoAOMT  :::::::::::::A::::::D::::::M:::::::::::: 116
St CCoAOMT  :I:V::F::::::A:::SI:D::::T:V::D::A:NV::A 117
   Sm OMT   VL:::TF::::T:CM:R:::AG:R:VTC::SDKWPG::A: 103
  Rat OMT   VL:L:A:C:::AVRM:RL:QPGARL:TMEM:PDYAA:TQQ 101
              *     *            **
Zi CCoAOMT  IIQKAGVAHKIDFREGPALPLLDQMLQDEKCHGSFDFIFV 160
Pa CCoAOMT  ::E::::G:::::::::::::V::H::E:G:Y::T:::V:: 156
St CCoAOMT  L:K::::ES::S:IVSD:MT:::DL:A:GRYQ::Y::A:: 157
   Sm OMT   FW:R:::DGL::L:I:D:ART:AE-:RERDGD:A::LV:: 142
  Rat OMT   MLNF::LQD:VTILN:ASQD:IP:-:KKKYDVDTL:MV:L 140
                            *
Zi CCoAOMT  DADKDNYLNYHKRLIDLVKFGGVIGYDNTLWNGSLVAP-A 199
Pa CCoAOMT  :::::::I:::::::::::I::L:::::::::::VAQ:-: 195
St CCoAOMT  ::::T::V:::E:::E:::V::I:A::::::G:TVAL:-- 195
   Sm OMT   ::::AG::H:YEQALA::RP::LVAI::::FF:RVAD:A: 182
  Rat OMT   :HW::R::PDTLL:EKCGLLRKGTVLLADNVIVPGTPDFL 180
             **
Zi CCoAOMT  DAPLRKYVRYYRDFVLELNKALAVDPRVEICQLPVGDGIT 239
Pa CCoAOMT  :::M:::::::::::I:::::::A:::I:::M:::::V: 235
St CCoAOMT  ESEVPDFMKNNWVC:TK::EI:GS:A:ID:AH:::::::: 235
   Sm OMT   :D:DTVA::T-------::DL:RD:E::D:AL:T:A:::: 215
  Rat OMT   AYVRGSSSFECTHYSSY:EYMKV::GLEKAIYQGPSSPDK 220

Zi CCoAOMT  LCRRIS                                   245
Pa CCoAOMT  ::::::                                   241
St CCoAOMT  F:::VY                                   241
   Sm OMT   :A::RE                                   221
  Rat OMT   S                                        221
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Figure 2. Deduced Amino Acid Sequence Comparison.

Shown is a deduced amino acid sequence comparison of *Zinnia* CCoAOMT (Zi CCoAOMT) with that of parsley CCoAOMT (Pa CCoAOMT), *Stellaria* CCoAOMT (St CCoAOMT), *S. mycarofaciens* OMT (Sm OMT), and rat catechol OMT (Rat OMT). Identical amino acid residues are indicated by colons. Dashed lines are gaps introduced to maximize identity. The consensus region involved in the binding of SAM in rat catechol OMT is underlined. The residues that are involved in the binding of SAM in rat catechol OMT are marked with asterisks underneath according to Vidgren et al. (1994).

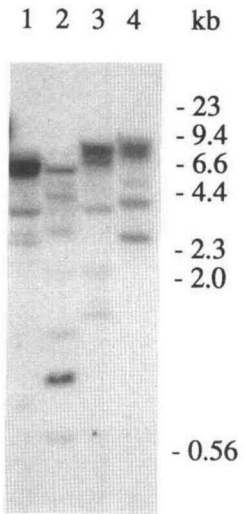

Figure 3. Genomic DNA Gel Blot Analysis of CCoAOMT.

Zinnia genomic DNA was digested with the restriction endonucleases EcoRI (lane 1), HindIII (lane 2), XbaI (lane 3), or BamHI (lane 4), separated on a 0.8% agarose gel, and transferred to a nitrocellulose membrane. The membrane was probed with ^{32}P-labeled CCoAOMT cDNA. HindIII-digested λ DNA fragments were used as length markers and are indicated at right in kilobases.

deposition started at ~60 hr. Thus, the marked accumulation of the mRNA correlates with the TE differentiation, and it occurs ~12 hr before visible lignin deposition. In contrast, only a low level of the mRNA accumulated in the cells cultured in the basal medium without any hormone or with either NAA or BA alone (Figures 4A and 4B). This indicates that the marked accumulation of CCoAOMT mRNA is specific to the process of TE formation.

We examined the expression of the CCoAOMT gene in *Zinnia* organs (Figure 4A). The results show that the mRNA was barely detectable in leaves but accumulated to a higher level in stems, roots, and flower buds. This indicates that the CCoAOMT gene is not only induced in the cultured cells but also expressed in normal developing tissues.

Cinnamic acid 4-hydroxylase (CA4H) converts cinnamic acid to *p*-coumaric acid in the biosynthesis of monolignols. We used a CA4H gene isolated from *Zinnia* (Z.-H. Ye and J.E. Varner, unpublished data) to examine the expression pattern in the *Zinnia*-cultured cells and organs for comparison (Figure 4A). The results show that the expression pattern of the CCoAOMT gene was almost the same as that of the CA4H gene in the cultured cells as well as in *Zinnia* organs. These results indicate that CCoAOMT is associated with lignification during TE formation.

Localization of CCoAOMT mRNA in *Zinnia* and Parsley Organs

To know whether the expression of the CCoAOMT gene is associated with lignification in the plant, we determined the spatial distribution of CCoAOMT mRNA in different internodes of *Zinnia* stem using tissue print hybridization (Figure 5). Vascular systems at different internodes have different degrees of lignification. The location of the signal on the tissue prints was determined by superimposition of the signals with the corresponding anatomies. At the first internode (the uppermost extended internode), mRNA was predominantly present in the differentiating xylem cells, which showed little lignin staining, but not in the developing phloem fibers (little lignin staining) and mature xylem (intensive lignin staining) (Figures 5A and 5B). At the second internode, mRNA was present in both differentiating xylem cells and phloem fibers (light lignin staining) (Figures 5C and 5D). At the third internode, both mature xylem and phloem fibers showed intensive lignin staining (Figure 5E). Concomitantly, the mRNA signal was much less in the phloem fibers in the third internode (Figure 5F) than in the second one (Figure 5D). Intensive mRNA signal was still present in some xylem bundles but not in others (Figure 5F), indicating that the activity of lignification is not the same in these xylem bundles. Figures 5G and 5H show that mRNA

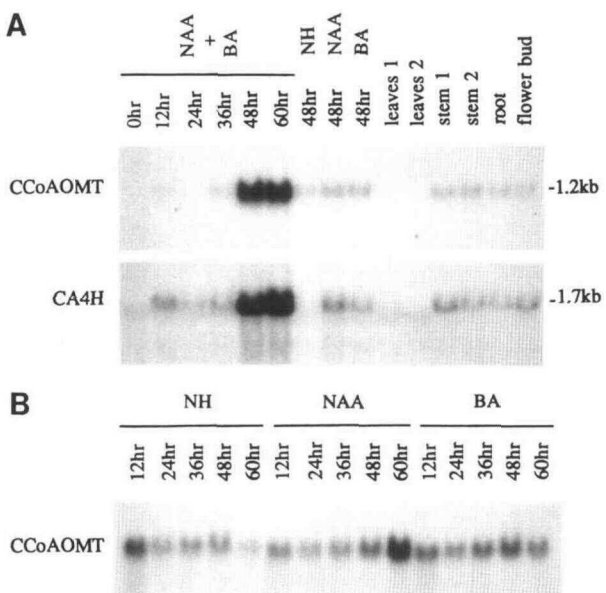

Figure 4. RNA Gel Blot Analysis of CCoAOMT and CA4H Gene Expression in the Isolated *Zinnia* Leaf Mesophyll Cells and *Zinnia* Organs.

Cells were cultured in basal medium without hormone (NH), or with 0.5 μM 1-naphthaleneacetic acid alone (NAA), or with 0.5 μM benzyladenine alone (BA), or with the combination of 0.5 μM 1-naphthaleneacetic acid and 0.5 μM benzyladenine (NAA+BA) and collected for total RNA isolation after different culture times as indicated above each lane. Leaves 1, stem 1, and root are from 4-week-old plants. Leaves 2, stem 2, and flower bud are from 6-week-old plants. The probes used for hybridization are identified at left. The lengths of the RNAs are indicated at right.

(A) Comparison of the expression patterns of CCoAOMT and CA4H genes.

(B) Accumulation of CCoAOMT mRNA in cells cultured in non-TE induction medium.

was present in differentiating xylem cells in both leaf and nodal xylem bundles and in developing phloem fibers in node vascular bundles. When the tissue prints were hybridized with the CCoAOMT sense RNA probe, no positive signals were shown. Moreover, a different localization pattern was observed when the tissue prints were hybridized with p48h-10 cDNA probe (Ye and Varner, 1994). Taken together, the results indicate that the expression of the CCoAOMT gene is temporally and spatially regulated and that it is coincident with lignification in xylem and in phloem fibers.

In parsley, CCoAOMT mRNA was present in the organs under normal physiological conditions (Schmitt et al., 1991). Tissue print hybridization shows that the CCoAOMT mRNA was predominantly present in the differentiating xylem cells in the parsley petiole (Figure 6). These results indicate that the expression of the CCoAOMT gene in xylogenesis is a general pattern in different plants.

CCoAOMT and COMT Activities during in Vitro TE Formation

The results described previously in this article demonstrate that CCoAOMT mRNA accumulation is closely associated with lignification in cultured cells and in the plant. Therefore, we examined whether the increase of CCoAOMT activity is associated with the timing of lignin deposition during in vitro TE formation. We first showed that crude protein extract from the cells cultured in the induction medium for 96 hr possesses the ability to transfer the methyl-^{14}C group from methyl-^{14}C-SAM to caffeoyl CoA, and the reaction product is feruloyl CoA as shown in Figure 7. Next, we prepared crude protein extracts from the cultured cells for CCoAOMT activity assay using caffeoyl CoA and methyl-^{14}C-SAM as substrates. Figure 8A shows the time course of TE formation from *Zinnia*-isolated mesophyll cells cultured in the induction medium. TE formation began between 48 and 60 hr, and increased thereafter. The percentage of TEs reached 62% by 60 hr and then decreased after 60 hr of culture. This is a result of the increase of undifferentiated cells by cell division. Heavy lignin deposition occurred between 60 and 72 hr, as was determined by phloroglucinol staining. CCoAOMT activity in the cells cultured in the induction medium increased in a similar pattern as the time course of TE formation (Figure 8B). Activity was detectable after 12 hr of culture and stayed low until 36 hr of culture. By 48 hr, activity was increasing and reached the highest level by 72 and 96 hr of culture. The increase of CCoAOMT activity between 72 and 96 hr was a result of the increase of newly differentiating TEs as shown in Figure 8A (please note that activity is expressed per milligram of protein, not per cell). A high level of CCoAOMT activity at 96 hr also indicates that the enzyme is not significantly degraded when TEs are mature. In contrast, activity was low in the cells cultured in the basal medium, which was almost the same as that in the cells cultured in the induction medium before 36 hr. CCoAOMT activity in the cells after 96 hr of culture was approximately six times higher in the induction medium (754 pmol/min/mg) than in the basal medium (125 pmol/min/mg). The results indicate that the increase in CCoAOMT activity is closely associated with lignification. The time course of the induction of CCoAOMT activity is similar to its mRNA accumulation, but the marked increase of activity was ~12 hr later than its mRNA accumulation.

COMT is known to participate in the methylation steps in plant lignin biosynthesis; therefore, we decided to determine whether the induction of COMT activity shows the same pattern as that of CCoAOMT. The crude protein extracts from the cultured cells were used for COMT activity assay using caffeic acid and methyl-^{14}C-SAM as substrates. Figure 8C shows that COMT activity increased similarly throughout the culture in both the basal medium and the induction medium. The specific activity of COMT was at a level similar to that of CCoAOMT in the cells cultured in the basal medium. Thus, the increase of COMT does not correlate with the timing of lignification. The results indicate that COMT is most likely not involved in the lignification during in vitro TE formation from isolated mesophyll cells.

To determine whether OMTs from crude protein extract can add methyl groups at both 3′ and 5′ hydroxyl groups, we assayed OMT activity using 5-hydroxyferuloyl CoA and methyl-^{14}C-SAM as substrates and compared it with the activity using caffeoyl CoA. The results in Figure 9 show that 5-hydroxylferuloyl CoA is methylated efficiently and that the time course of induction of activity using 5-hydroxyferuloyl CoA is the same as that using caffeoyl CoA. The specific activity using 5-hydroxyferuloyl CoA is ~0.55 times that using caffeoyl CoA.

CCoAOMT and COMT Activities in Organs from Different Plant Species

The above-mentioned results show that CCoAOMT activity increased markedly when lignin deposition started during TE formation, whereas COMT activity did not. The specific activity of CCoAOMT was approximately six times higher than that of COMT in the cultured cells (Table 1). COMT is known to be widely distributed in different species. We assayed CCoAOMT activity in several different species and compared this activity with COMT activity. The results in Table 1 show that CCoAOMT activity was detectable in all species examined, and the specific activity of CCoAOMT is approximately two to nine times higher than that of COMT in *Zinnia*, tobacco, tomato, parsley, and alfalfa. In maize and Arabidopsis, the specific activity of CCoAOMT was slightly lower than or the same as that of COMT, respectively.

DISCUSSION

CCoAOMT Shares Consensus SAM Binding Elements with Catechol OMT but Not with COMT

The CCoAOMT cDNA was isolated from in vitro–differentiating TEs induced from cultured *Zinnia* mesophyll cells. Its

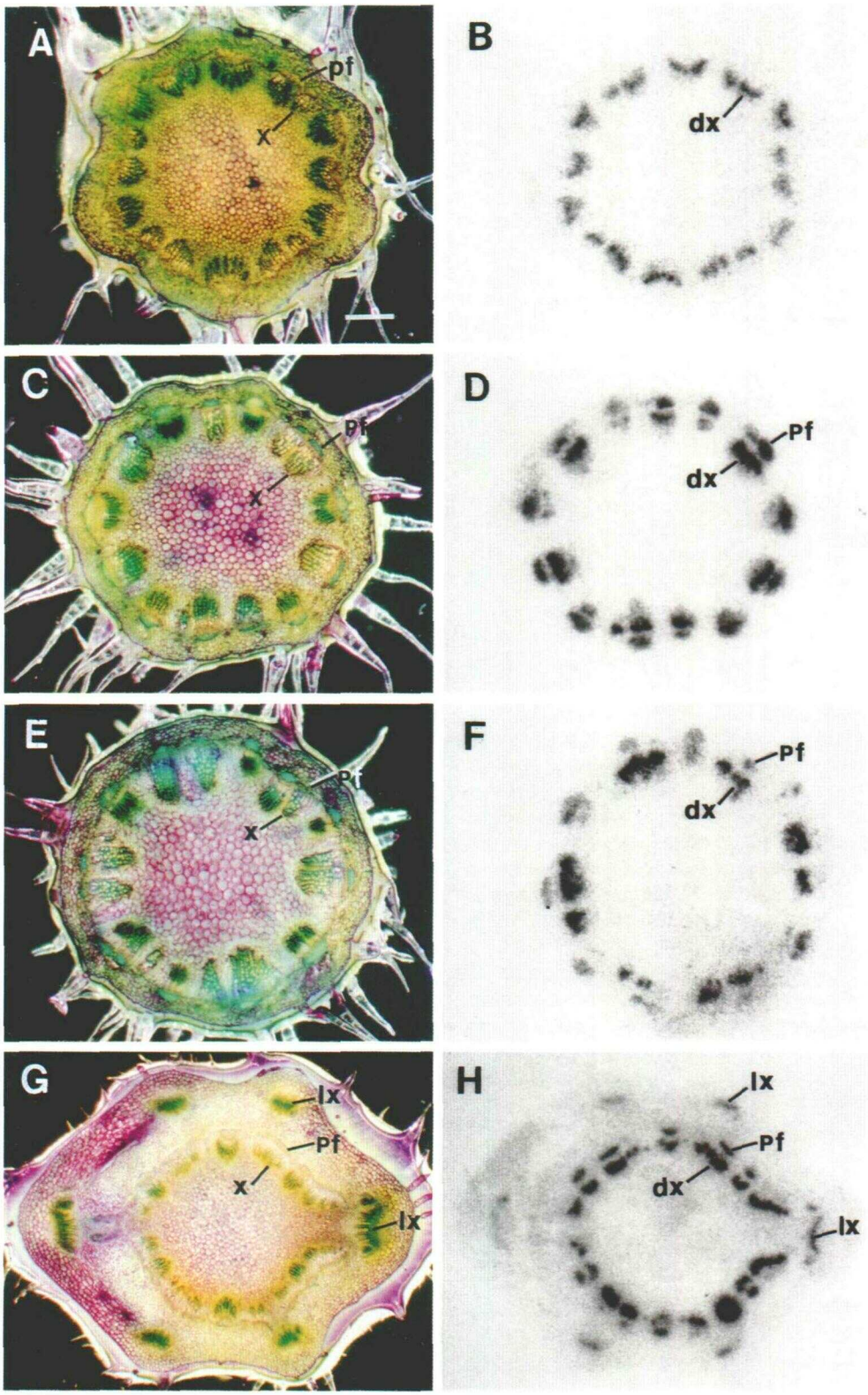

Figure 5. Tissue Print Hybridization of CCoAOMT mRNA in the *Zinnia* Stem.

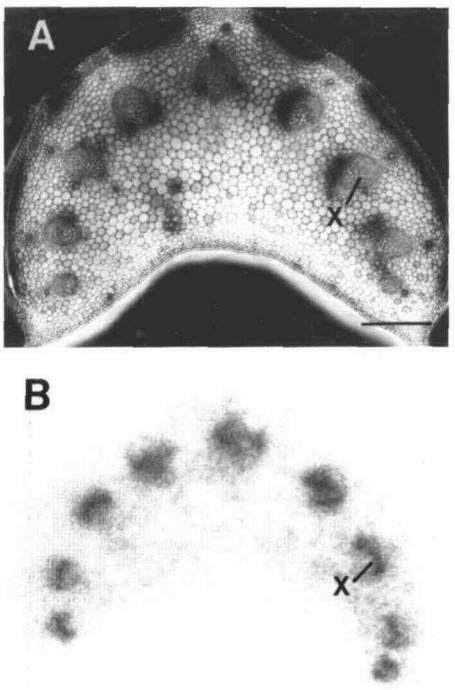

Figure 6. Tissue Print Hybridization of CCoAOMT mRNA in a Parsley Petiole.

(A) Anatomy of a cross-section of the parsley petiole stained with toluidine blue.
(B) Localization of CCoAOMT mRNA in xylem cells in the petiole.
x, xylem. Bar in **(A)** = 0.55 mm.

identity was determined according to both nucleotide and deduced amino acid sequence comparison and the activity assay in *Escherichia coli*. Recently, the rat catechol OMT crystal structure was solved, and the amino acid residues involved in the binding of SAM were determined (Vidgren et al., 1994). Comparison of the amino acid sequence of *Zinnia* CCoAOMT with that of rat catechol OMT shows that several important residues involved in the binding of SAM are conserved. Specifically, the amino acid residues 82 to 89 of *Zinnia* CCoAOMT show significant similarity with the consensus region involved in the binding of SAM in rat catechol OMT (underlined in Figure 2). This region is considered to be the location for consensus residues in nucleotide binding proteins. In addition, the conserved acidic residue that binds to the ribose hydroxyls of SAM (Glu-90 in rat catechol OMT) is conserved in *Zinnia* CCoAOMT (Asp-109). These comparisons suggest that CCoAOMT shares structural similarities and consensus elements with some other SAM-dependent OMTs.

However, *Zinnia* CCoAOMT does not exhibit any significant similarity with COMT and some other OMTs from plants. COMT cDNAs or genes have been characterized from alfalfa (Gowri et al., 1991), aspen (Bugos et al., 1991), and maize (Collazo et al., 1992). Several other OMT cDNAs have also been isolated, including a putative OMT possibly involved in suberin biosynthesis from maize (Held et al., 1993), an isoliquiritigenin OMT from alfalfa (Maxwell et al., 1993), an orthodiphenol OMT from tobacco (Pellegrini et al., 1993), and a myoinositol

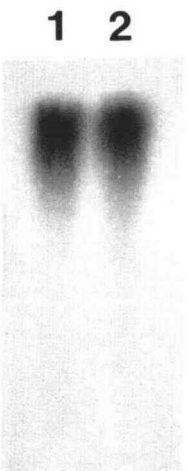

Figure 7. *Paper Chromatography* Analysis of CCoAOMT Activity in the Cultured Cells.

Lane 1 contains the reaction product catalyzed by CCoAOMT from cells cultured for 96 hr in the induction medium. The protein extract was used for CCoAOMT assay with caffeoyl CoA and methyl-^{14}C-SAM as substrates. After the reaction, the CoA ester was removed by alkaline hydrolysis. The resulting ferulic acid was extracted into ethyl acetate, dried, redissolved in ethanol, and then applied to 3MM Whatman paper for paper chromatographic separation. Lane 2 contains the standard ^{14}C-ferulic acid. The retardation factor values of ferulic acid and caffeic acid are 0.90 and 0.77, respectively.

Figure 5. (continued).

Hand-cut sections of stems were printed on a nylon membrane for RNA transfer. ^{35}S-labeled CCoAOMT antisense RNA was used to probe the membrane. At left are toluidine blue-stained sections; at right are the corresponding CCoAOMT mRNA localizations. Lignified walls stain blue with toluidine blue.
(A) and **(B)** Anatomy in **(A)** and the mRNA localization in **(B)** of a section from the first internode.
(C) and **(D)** Anatomy in **(C)** and the mRNA localization in **(D)** of a section from the second internode.
(E) and **(F)** Anatomy in **(E)** and the mRNA localization in **(F)** of a section from the third internode.
(G) and **(H)** Anatomy in **(G)** and the mRNA localization in **(H)** of a section from the second node.
dx, differentiating xylem; lx, leaf xylem; pf, phloem fibers; x, xylem. Bar in **(A)** = 0.5 mm for all panels.

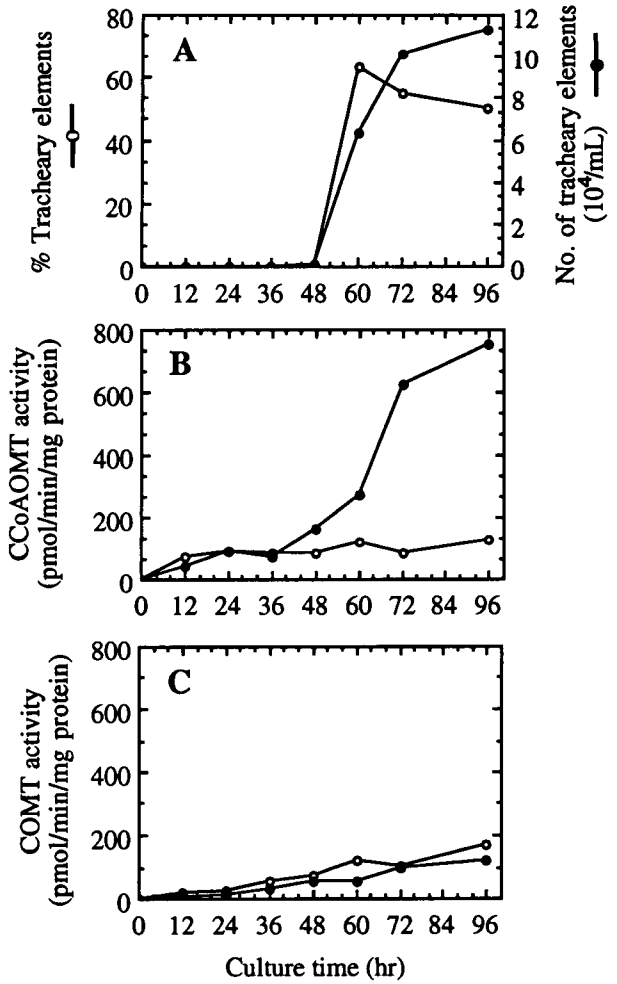

Figure 8. Time Course of TE Formation and CCoAOMT and COMT Activities in Isolated *Zinnia* Mesophyll Cells.

Mesophyll cells were isolated from 11-day-old *Zinnia* leaves and cultured in basal medium and TE induction medium. Crude protein extracts were prepared from cells cultured for various times and used for the enzyme activity assay. CCoAOMT- and COMT-specific activities are expressed as picomoles of methyl-^{14}C-residue from methyl-^{14}C-SAM transferred to caffeoyl CoA and caffeic acid per minute per milligram of protein, respectively. Each data point is the mean of two separate assays.

(A) TE formation in cells cultured in TE induction medium as the function of culture time.

(B) Time course of the induction of CCoAOMT activity in cells cultured in basal medium (○) and in TE induction medium (●).

(C) Time course of the induction of COMT activity in cells cultured in basal medium (○) and in TE induction medium (●).

OMT from the ice plant (Vernon and Bohnert, 1992). All of these OMTs, but not CCoAOMT, show significant amino acid similarity, especially in several conserved regions identified for OMTs (Bugos et al., 1991). Some of these conserved regions are suggested to be involved in the SAM binding. However, these conserved regions do not share any significant similarity with the consensus SAM binding region identified in rat catechol OMT. Thus, CCoAOMT may have an evolution different from COMT for the utilization of SAM.

CCoAOMT Is Involved in an Alternative Methylation Pathway in Lignin Biosynthesis

Our results show that the marked induction of CCoAOMT mRNA and enzyme activity is specifically associated with the process of lignification during TE formation from *Zinnia*-cultured cells. Significantly, the patterns of CCoAOMT mRNA accumulation in both cultured cells and organs are almost the same as those of CA4H, which is known to be involved in lignin biosynthesis. The low level accumulation of CCoAOMT mRNA and enzyme activity in non-TE-differentiating cells is probably a result of stress induction during cell maceration and subsequent culture. The expression of the CCoAOMT gene in differentiating xylem and in phloem fiber cells in organs further confirms its involvement in lignification in the plant. Taken together, these results lead us to propose that CCoAOMT is involved in an alternative methylation pathway in lignification (Figure 10).

The OMTs in the known methylation pathway use hydroxycinnamic acids as substrates (Figure 10A; Davin and Lewis, 1992). The reaction in this pathway starts from *p*-coumaric acid. After hydroxylation and methylation, the resulting caffeic acid and 5-hydroxyferulic acid together with *p*-coumaric acid undergo CoA ligation individually. In contrast, the OMTs in the alternative methylation pathway utilize CoA esters of hydroxycinnamic acids as substrates (Figure 10B). The reaction in this pathway starts from *p*-coumaroyl CoA. Thus, the CoA ligation

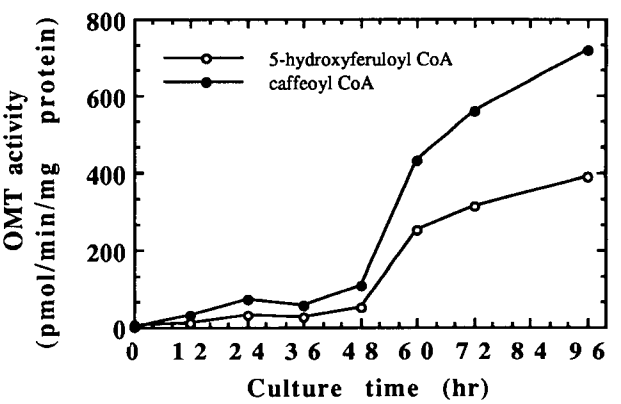

Figure 9. Comparison of OMT Activity Using 5-Hydroxyferuloyl CoA and Caffeoyl CoA as Substrates.

Crude protein extracts were isolated from cells cultured in TE induction medium and used for the enzyme activity assay with methyl-^{14}C-SAM as the methyl group donor. The specific activities are expressed as picomoles of methyl-^{14}C-residue from methyl-^{14}C-SAM transferred to 5-hydroxyferuloyl CoA (○) or caffeoyl CoA (●) per minute per milligram of protein.

Table 1. CCoAOMT and COMT Activities from Different Plant Species

Source	pmol/min/mg Protein[a]	
	CCoAOMT Activity	COMT Activity
Zinnia l96h[b]	754	124
Zinnia leaf	28	10
Zinnia stem	27	5
Zinnia root	270	126
Tobacco stem	78	18
Tomato stem	58	6
Parsley petiole	110	39
Maize root	220	302
Alfalfa stem	30	9
Arabidopsis seedling	19	18

[a] CCoAOMT and COMT activities are expressed as picomoles of methyl-^{14}C-residue from methyl-^{14}C-SAM transferred to caffeoyl-CoA and caffeic acid per minute per milligram of protein extract, respectively.

[b] Isolated mesophyll cells cultured for 96 hr in the induction medium.

step only uses *p*-coumaric acid. In contrast to the known methylation pathway in which lignin biosynthesis branches from *p*-coumaric acid while flavanoid branches from *p*-coumaroyl CoA, the alternative methylation pathway centers *p*-coumaroyl CoA as the branch point for the biosynthesis of these two important phenylpropanoids.

In *Zinnia*-cultured cells induced to TE differentiation, both caffeoyl CoA and 5-hydroxyferuloyl CoA are efficiently methylated (Figure 9). The ratio of sinapoyl CoA to feruloyl CoA being formed by OMTs in vitro is 0.55. This is different from the ratio of sinapic acid to ferulic acid (1.2 to 6.4) being formed by dicot COMT (Davin and Lewis, 1992). Dicot COMT is bispecific; it methylates both caffeic acid and 5-hydroxyferulic acid (Bugos et al., 1991). It is not known whether CCoAOMT can methylate both caffeoyl CoA and 5-hydroxyferuloyl CoA or if another OMT is involved.

As indicated in Figure 10, caffeoyl CoA is proposed to be derived from the 3-hydroxylation of *p*-coumaroyl CoA. *p*-Coumaroyl CoA 3-hydroxylase activities were detected in *Silene dioica* (Kamsteeg et al., 1981) and parsley (Kneusel et al., 1989). It was found that the enzyme was elicitor-induced in parsley cultured cells. The rapid induction of *p*-coumaroyl CoA 3-hydroxylase, together with CCoAOMT, by elicitor treatment was proposed to play a defense role (Matern et al., 1988; Kneusel et al., 1989). Using the assay method described by Kneusel et al. (1989), we detected *p*-coumaroyl CoA 3-hydroxylase activity in both in vitro–differentiating TEs from isolated *Zinnia* mesophyll cells and *Zinnia* roots (data not shown). Thus, it seems likely that *p*-coumaroyl CoA 3-hydroxylase is involved in the production of caffeoyl CoA in *Zinnia*. Nevertheless, there

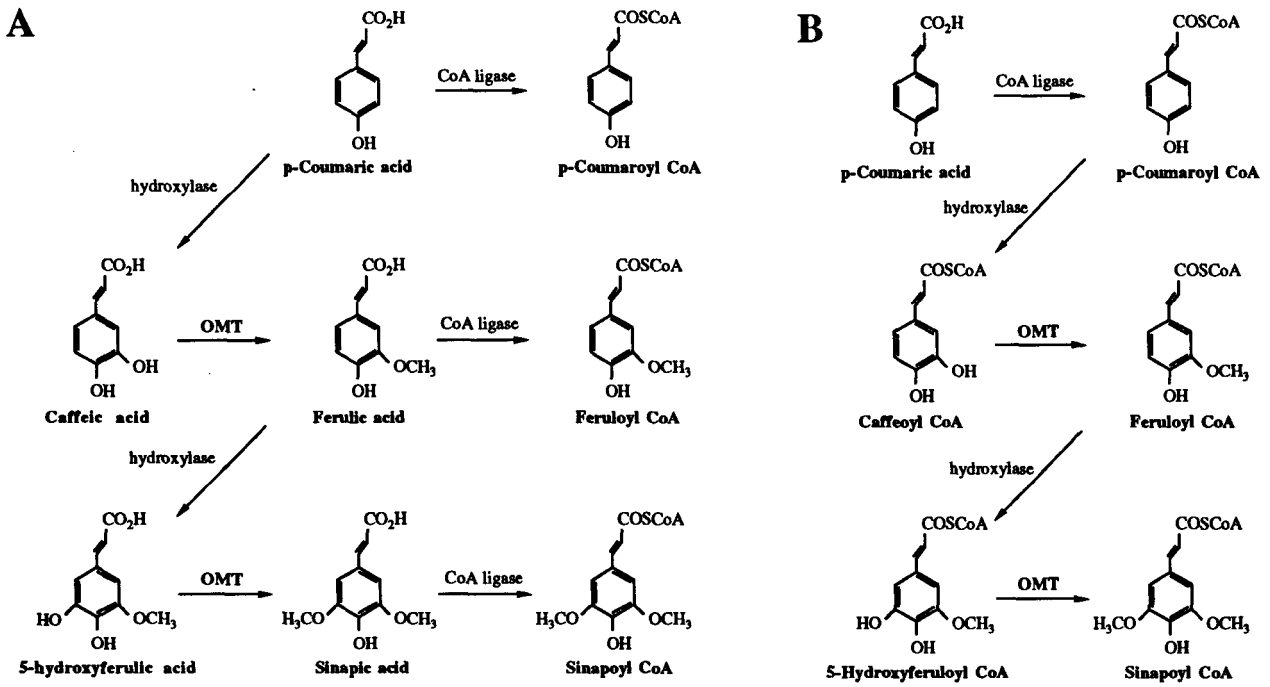

Figure 10. Two Methylation Pathways in Lignin Biosynthesis.

(A) A known methylation pathway (Davin and Lewis, 1992). OMT acts on free hydroxycinnamic acids. Each methylated hydroxycinnamic acid then proceeds to CoA ligation.

(B) An alternative methylation pathway. OMT acts on CoA esters of hydroxycinnamic acids but not on free acids. Thus, CoA ligation precedes methylation.

are some other possibilities in the production of caffeoyl CoA as proposed by Kühnl et al. (1989). One is that caffeoyl CoA is derived from CoA ligation of caffeic acid. It is known that CoA ligase has wide substrate specificities, including using caffeic acid as substrate (Davin and Lewis, 1992). The other is that caffeoyl CoA is derived from 5-*O*-caffeoylshikimate by the hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (Kühnl et al., 1989).

5-Hydroxyferuloyl CoA is proposed to be derived from the 5-hydroxylation of feruloyl CoA. We were unable to detect the feruloyl CoA 5-hydroxylase activity in crude protein extracts from in vitro–differentiating TEs, probably because this is a membrane-bound cytochrome P-450–dependent monooxygenase as is ferulic acid 5-hydroxylase (Grand, 1984). In addition, Chapple et al. (1992) were also unable to detect the hydroxylase activity using both ferulate and feruloyl CoA as substrates in wild-type Arabidopsis plants.

The CCoAOMT-Mediated Methylation Pathway in Lignification Is Dominant

Our results showed that CCoAOMT activity increased markedly when *Zinnia*-isolated mesophyll cells were undergoing lignification, whereas COMT activity did not. Fukuda and Komamine (1982) also found that COMT activity is higher in the cells cultured in the control medium than in the cells cultured in the TE induction medium. These results indicate that the CCoAOMT-mediated methylation pathway in lignin biosynthesis is dominant in *Zinnia*-differentiating TEs. Whether this is true in *Zinnia* organs is not known. It seems likely that this pathway is also prevalent in the plant because the specific activity of CCoAOMT in organs from *Zinnia* and several other species (Table 1) is higher than that of COMT. However, several researchers have documented that the expression of COMT is associated with lignification. For example, Bugos et al. (1991) found that COMT is localized in differentiating xylem in aspen. It was reported that lignin deposition is reduced in transgenic tobaccos plants that express an antisense alfalfa COMT gene (Chasan, 1994). Thus, it is possible that CCoAOMT and COMT have differential expressions in different cell types in the plant. CCoAOMT is involved in the lignification in both xylem TEs and fibers and phloem fibers, whereas COMT may be mainly involved in the lignification in xylem fibers and phloem fibers but not in xylem TEs. It was found that different tissue and cell types have different lignin monomer compositions (Monties, 1985). Differential expression of CCoAOMT and COMT could be one of the steps affecting the lignin compositions because these OMTs have different methylation rates on their corresponding substrates. The existence of two pathways for methylation reactions is intriguing. It raises the question of whether some of the other steps in lignification also involve parallel reactions. At least in the polymerization of monolignols, both peroxidase and laccase are proposed to be involved in this reaction (Sterjiades et al., 1993).

CCoAOMT Is Widely Distributed in Plants

Schmitt et al. (1991) demonstrated that CCoAOMT mRNA and enzyme activity were detected in taxonomically widely diverse plants such as carnation, safflower, parsley, and carrot. We show here that CCoAOMT activity is present in a number of other species, and the accumulation of CCoAOMT mRNA is associated with xylogenesis in both *Zinnia* and parsley organs. Therefore, it seems likely that CCoAOMT is widely distributed, and the methylation pathway it catalyzes is a general one in lignin biosynthesis in plants.

It was reported that unknown derivatives of hydroxycinnamic acids from wheat and barley shoots were detected in acetone- and ethanol-insoluble fractions (El-Basyouni et al., 1964; El-Basyouni and Neish, 1966). Consequently, a lignin biosynthetic pathway employing derivatives of hydroxycinnamic acids as natural intermediates was proposed by Neish (1968). Now it seems likely that these derivatives are CoA esters because hydroxycinnamate CoA ligases are expressed in xylem cells (Douglas et al., 1991). The demonstration of the involvement of CCoAOMT in lignin biosynthesis further supports the existence of this pathway. However, it is still possible that derivatives other than CoA esters are natural intermediates, as some evidence indicates that some of these derivatives are acylated enzymes or glucose esters (Brown, 1966, 1969; El-Basyouni and Neish, 1966; Bland and Logan, 1967).

The identification of the alternative methylation pathway may be helpful for the genetic engineering of lignin. Fibers from softwoods (gymnosperms) are preferred to those from hardwoods (angiosperms). However, delignification of softwoods by kraft pulping is more difficult probably because of the absence of syringyl groups in softwoods, thus resulting in more condensed lignin structure (see Bugos et al., 1991, for discussion). It was proposed that introduction of angiosperm bispecific COMT and ferulic acid 5-hydroxylase into softwoods could change the composition of lignin, thus increasing the efficiency of kraft pulping (Bugos et al., 1991; Whetten and Sederoff, 1991; Chapple et al., 1992). If CoA esters of hydroxycinnamic acids are also natural intermediates in softwoods, it seems that introduction of only bispecific COMT and ferulic acid 5-hydroxylase into softwoods would not be efficient in the production of guaiacyl-syringyl lignin. It is possible that simultaneous introduction of bispecific CCoAOMT and feruloyl CoA 5-hydroxylase into softwoods could improve efficiency in the production of guaiacyl-syringyl lignin.

METHODS

Materials

Zinnia (*Zinnia elegans* var Peter Pan), tobacco (*Nicotiana tabacum* cv Xanthi), tomato (*Lycopersicum esculentem* cv UC82B), alfalfa (*Medicago sativa* cv CUF 101), parsley (*Petroselinum crispum* cv Plain or Single), maize (*Zea mays* cv Silver Queen), and Arabidopsis (*Arabidopsis*

thaliana cv Columbia) plants were grown in the greenhouse. Plants of ~1 month old were used in all experiments unless otherwise indicated. The internodes were arbitrarily numbered according to the order from young (top) to older (bottom). 5-Hydroxyvanillin was synthesized from 5-iodovanillin according to Banerjee et al. (1962). 5-Hydroxyferulic acid was prepared by condensation with malonic acid (Pearl and Beyer, 1951). *trans*-2-^{14}C-Ferulic acid and *trans*-4-2-^{14}C-coumaric acid were synthesized by condensation reaction of vanillin and 4-hydroxybenzaldehyde, respectively, with 2-^{14}C-malonic acid. All the hydroxycinnamoyl-coenzyme A (CoA) esters were prepared according to Stöckigt and Zenk (1975). Methyl-^{14}C-*S*-adenosyl-L-methionine (SAM) (57.6 mCi/mmol) was purchased from Du Pont-New England Nuclear Research Products.

Zinnia Mesophyll Cell Isolation and Culture

Mesophyll cells were isolated from the first true leaves of 11-day-old *Zinnia* seedlings as described previously (Fukuda and Komamine, 1980; Ye and Varner, 1993). The cells were cultured in basal medium without any hormone as described by Fukuda and Komamine (1980), or in induction medium containing the basal medium with the addition of 0.5 μM 1-naphthaleneacetic acid (NAA) and 0.5 μM benzyladenine (BA). Cells cultured in the basal medium did not differentiate into tracheary elements (TEs), while those in the induction medium did. The cell culture conditions were the same as described by Ye and Varner (1993). After culture, the cells were collected, quickly frozen in liquid N_2, and stored at −80°C until used.

cDNA Library Construction and Subtractive Hybridization

The cells after 60 hr of culture in the induction medium were used in poly(A)$^+$ RNA isolation. Double-strand cDNAs were synthesized from poly(A)$^+$ RNA using oligo(dT) as the primer (Sambrook et al., 1989). One half of the products was used for an in vitro cDNA library construction as described previously (Duguid and Dinauer, 1990). The other half of the products was used for construction of a cDNA library in λgt10 (Sambrook et al., 1989). The in vitro cDNA library constructed from cells cultured for 60 hr in the induction medium was subtracted with that from freshly isolated mesophyll cells to generate a subtractive library (Duguid and Dinauer, 1990; Ye and Varner, 1993). The cDNAs whose mRNAs were up-regulated during TE formation were isolated from the subtractive library and confirmed by cross-hybridization, RNA gel blot analysis, and partial DNA sequence analysis (Ye and Varner, 1993).

RNA Isolation and Gel Blot Analysis

Total RNA was isolated from *Zinnia*-cultured cells and organs as described previously (Ye and Varner, 1993). Poly(A)$^+$ RNA used for cDNA synthesis was isolated using Promega's poly-A-Tract mRNA isolation system following the manufacturer's protocol. RNA gel blot analysis was performed as described previously (Ye and Varner, 1993).

DNA Sequence Analysis

S-Adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT) cDNA insert was cloned into the EcoRI site in pBluescript SK+ plasmid vector. The cDNA was sequenced from both strands with T7 or T3 primer and synthetic oligonucleotide primers. United States Biochemical's Sequenase (version 2.0) DNA sequencing kit was used for sequencing, and manufacturer's protocol was followed. DNA and protein sequence comparisons were performed using the BLAST network service from the National Center for Biotechnology Information (Bethesda, MD).

Expression of CCoAOMT in *Escherichia coli*

The coding region of *Zinnia* CCoAOMT cDNA was cloned into pKK388-1 expression vector (Clontech, Palo Alto, CA). The cell extracts used for activity assay were prepared as described previously (Gowri et al., 1991).

Genomic DNA Isolation and Gel Blot Analysis

Young leaves from 3-week-old *Zinnia* plants were used for genomic DNA isolation. The DNA was digested with EcoRI, HindIII, XbaI, and BamHI, separated on a 0.8% agarose gel, and transferred onto a nitrocellulose membrane as described by Sambrook et al. (1989). Prehybridization, hybridization, and washing conditions were the same as described for RNA gel blot analysis in Ye and Varner (1993).

Preparation of Crude Extracts and Assay of Enzyme Activity

Cells and tissues were homogenized in the extraction buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL leupeptin, 10 μg/mL aprotinin) with a mortar and pestle as described previously (Pakusch et al., 1989). After homogenization, the extracts were centrifuged at 12,000*g* for 15 min. The supernatants were passed through Sephadex G25 column to remove small molecules with molecular weight under 5000. The eluents were saved as crude extracts for assay of enzyme activity. Freshly prepared crude extracts were used for enzyme assay directly or stored at −80°C until used. Freezing and thawing once did not result in any loss of activities.

CCoAOMT activity was determined essentially as described (Pakusch et al., 1989). Fifty microliters of reaction mixture (50 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 0.2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 nmol caffeoyl CoA, 2 nmol methyl-^{14}C-SAM, 20 μg crude protein extract) was incubated at 30°C for 15 min. The reaction mixture omitting either caffeoyl CoA or crude extract was used as blank. The reaction was stopped by the addition of 5.5 μL of 5 N NaOH, and CoA ester was hydrolyzed by the incubation of the reaction at 40°C for 15 min. After hydrolysis, the reaction was acidified by the addition of 6.2 μL of 6 N HCl. The hydrolyzed product (ferulic acid) was separated from methyl-^{14}C-SAM by extraction with 200 μL of ethyl acetate. The extracted products in ethyl acetate were taken for radioactivity counting in a Beckman liquid scintillation counter.

Caffeic acid *O*-methyltransferase (COMT) activity was assayed in 50 μL of reaction mixture (50 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 0.2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 nmol caffeic acid, 2 nmol methyl-^{14}C-SAM, 20 μg crude protein extract). The reaction mix was incubated at 30°C for 15 min and then stopped by the addition of 1 μL of 6 N HCl. The acidified reaction mixture was extracted with 200 μL of ethyl acetate to separate the reaction product (ferulic acid) from methyl-^{14}C-SAM. The

extracted ferulic acid in ethyl acetate was taken for radioactivity counting in a Beckman liquid scintillation counter.

Protein content was determined according to Bradford's method with Bio-Rad's protein assay dye.

Paper Chromatography Analysis

Ethyl acetate extracts from CCoAOMT reaction products were dried in a speed-vacuum centrifuge and then dissolved in 5 μL of 95% ethanol. The reaction products were separated on 3MM Whatman chromatography paper in 1-butanol–acetic acid–H_2O (5:2:3 [v/v]) (Pakusch et al., 1989). ^{14}C-Ferulic acid was run beside as a standard. After chromatography, the paper was dried and exposed to Kodak x-ray film.

Tissue Print Hybridization

Zinnia stems and parsley petioles were hand-sectioned and printed onto a Zeta probe nylon membrane (Bio-Rad, Richmond, CA) as described previously (Ye and Varner, 1991). The membrane was UV-illuminated using a UV cross-linker (Bio-Rad). The same sections were saved for recording anatomy after printing. The sections were stained with toluidine blue O, and the photographs were taken under the Nikon stereomicroscope SMZ-U with dark-field illumination. The hybridization conditions were essentially the same as described previously (McClure and Guilfoyle, 1989; Ye and Varner, 1991). The pBluescript SK+ vector containing the CCoAOMT cDNA insert was used for the synthesis of ^{35}S-labeled sense or antisense RNA probe (Sambrook et al., 1989). After washing, the membrane was completely dried and exposed to Kodak Tmax 400 film at room temperature.

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