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Stress Responses in Alfalfa (*Medicago sativa* L.)

X. Molecular Cloning and Expression of *S*-Adenosyl-L-Methionine:Caffeic Acid 3-*O*-Methyltransferase, a Key Enzyme of Lignin Biosynthesis

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ABSTRACT

S-Adenosyl-L-methionine:caffeic acid 3-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the conversion of caffeic acid to ferulic acid, a key step in the biosynthesis of lignin monomers. We have isolated a functionally active cDNA clone (pCOMT1) encoding alfalfa (*Medicago sativa* L.) COMT by immunoscreening a λZAPII cDNA expression library with anti-(aspen COMT) antibodies. The derived amino acid sequence of pCOMT1 is 86% identical to that of COMT from aspen. Southern blot analysis indicates that COMT in alfalfa is encoded by at least two genes. Addition of an elicitor preparation from bakers' yeast to alfalfa cell suspension cultures resulted in a rapid accumulation of COMT transcripts, which reached a maximum level around 19 hours postelicitation. Northern blot analysis of total RNA from different organs of alfalfa plants at various developmental stages showed that COMT transcripts are most abundant in roots and stems. Transcripts encoding ATP: L-methionine-S-adenosyl transferase (AdoMet synthetase, EC 2.5.1.6), the enzyme responsible for the synthesis of the methyl donor for the COMT reaction, were co-induced with COMT transcripts in elicitor-treated cells and exhibited a similar pattern of expression to that of COMT in different organs of alfalfa plants at various stages of development.

Lignin is a three-dimensional phenolic structure resulting from the free-radical polymerization of 4-coumaryl, coniferyl, and sinapyl alcohols within the plant cell wall (11). The monomeric alcohols are elaborated from phenylalanine via the central phenylpropanoid pathway that is common to the synthesis of a wide range of phenylpropanoid products in plants, including, in addition to lignin, flavonoid pigments, isoflavonoid phytoalexins, and hydroxycinnamic acid esters (14). In both angiosperms and gymnosperms, production of lignin monomers involves methylation of caffeic acid, derived from the central phenylpropanoid pathway intermediate 4-coumaric acid, to ferulic acid by the enzyme COMT[1] (EC 2.1.1.6) (11). OMT specificities have been shown to account for the difference between angiosperm lignin, which contains guiacyl- and syringyl-type units, and gymnosperm lignin, which contains predominantly guiacyl-type units (23).

Lignification or simple esterification of hydroxycinnamic acid derivatives in cell walls is a common response to infection or wounding in plants (21, 29). Deposition of lignin has been hypothesized to interfere with the enzymatic hydrolysis and mechanical penetration of plant tissue by fungal pathogens and may also impair the movement of water and diffusible molecules between the plant and fungus (24). Alfalfa cell suspension cultures accumulate the isoflavonoid phytoalexin medicarpin and wall-bound phenolic material following exposure to elicitor molecules from fungal pathogens and yeast (3). This is preceded by increases in the extractable activities of isoflavonoid and lignin-specific enzymes, including COMT (3). Recent studies in our laboratory (5, C. Maxwell, R. Edwards, R. A. Dixon, unpublished results) indicate that alfalfa plants and cell cultures contain at least three distinct OMT activities that catalyze transfer of the methyl group of SAM to either the 3-hydroxyl group of caffeic acid (COMT), the 7-hydroxyl group of isoflavones (IOMT), or the 2′-hydroxyl group of chalcones, respectively. COMT has been purified to homogeneity from several plant sources including aspen (2), tobacco (15), and alfalfa (5).

In contrast to the large body of information available on the molecular characterization of the enzymes of the central phenylpropanoid pathway (14), there is little information at the molecular genetic level on the later enzymes involved in specific branch pathways such as isoflavonoid or lignin biosynthesis. The molecular cloning of enzymes specific for these branch pathways will provide a basis for fundamental studies on the mechanisms of developmental and environmental regulation of plant genes and, in the case of lignin-specific enzymes, for applied studies aimed at altering lignin composition by gene transfer. We report here the isolation and characterization of a cDNA encoding COMT from alfalfa, and the expression patterns of COMT transcripts in response to elicitor treatment in alfalfa cell suspension cultures and during development of alfalfa plants from seed to maturity. We also describe the induction pattern of AdoMet synthetase, the enzyme responsible for production of the methyl group donor in the COMT reaction.

[1] Abbreviations: COMT, caffeic acid 3-*O*-methyltransferase (EC 2.1.1.6); SAM, *S*-adenosyl-L-methionine; IOMT, isoflavone *O*-methyltransferase; IPTG, isopropyl β-D-thiogalactoside; PAL, L-phenylalanine ammonia-lyase (EC 4.3.1.5); PCR, polymerase chain reaction; OMT, *O*-methyl transferase; bp, base pairs; kb, kilobase pairs.

MATERIALS AND METHODS

Cell Cultures and Elicitor Treatment

Alfalfa (*Medicago sativa* L. cv Apollo) cell cultures were initiated and maintained in suspension as described previously (3). Cultures were treated 4 d after subculture with elicitor prepared from yeast (27) at a final concentration of 70 μg glucose equivalents mL^{-1} plant cell culture medium. Elicitor was added in aqueous solution and an equivalent amount of water was added to control cultures. At various times after the addition of elicitor, cells were harvested by vacuum filtration on nylon mesh, frozen in liquid N_2, and stored at −70°C.

Plant Materials

Alfalfa seeds were inoculated with *Rhizobium meliloti* at the time of planting. Plants were grown at 25/20°C at 16 h day/8 h night cycles in trays with a 50:50 peat/sand mix. Plants were harvested at weekly intervals up to 6 weeks after planting, and the final harvest occurred when the plants were 9 weeks old. Tissue samples were separated into roots, root nodules, stems, leaves, growing points, petioles, flower buds, and flowers. Roots and stems were collected from the first week after planting. Root nodules, leaves, growing points, and petioles were collected starting from the third week. Flower buds were collected at the sixth and ninth weeks and flowers at the ninth week.

Screening of cDNA Expression Library

A λZAPII cDNA expression library, constructed from poly(A)$^+$ RNA from pooled alfalfa cell suspension cultures that had been exposed to elicitor from cell walls of *Colletotrichum lindemuthianum* for 2, 3, and 4 h, was used. *Escherichia coli* XL-1 Blue (Stratagene) cells were infected with the library, induced with IPTG, and positive clones selected by direct immunoscreening with monospecific polyclonal antibodies for aspen (*Populus tremuloides*) COMT (5, R.C. Bugos, V.L.C. Chiang, W.H. Campbell, unpublished results) utilizing alkaline phosphatase detection with 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium (32). Putative clones were purified by three rounds of screening and processed to homogeneity. The helper phage R408 (Stratagene) was used to rescue plasmids (pBluescript SK-) from phages giving a positive signal. Rescreening of the same library was carried out by plaque hybridization using DNA probes that were ^{32}P-labeled by random oligonucleotide-primed synthesis (9). Washing of membranes was as described below for Northern and Southern blot analysis, except that the high temperature wash was performed at 50°C.

Isolation and Analysis of RNA and DNA

Total RNA from cell suspension cultures and plant material was prepared according to Logemann *et al.* (18) and contaminating polysaccharides were removed by successive lithium chloride precipitations. For Northern hybridizations, 10 μg of total RNA was denatured in formamide and formaldehyde and fractionated on 1% agarose gels containing formaldehyde (20). For Southern hybridizations, alfalfa leaf genomic DNA (10 μg) was prepared, cleaved with various restriction enzymes, and electrophoresed on 0.8% agarose gels. RNA and DNA gel blot analyses were performed on nitrocellulose membranes (Schleicher & Schull) according to Maniatis *et al.* (20). Unless otherwise stated, a 1.3 kb *Eco*RI/*Eco*RI cDNA insert from pCOMT1 was used as hybridization probe. Prehybridizations were performed at 42°C in 50% (v/v) formamide, 6 × SSC, 7.5 × Denhardt's solution, 0.2% (w/v) SDS, 0.2 M sodium phosphate buffer (pH 6.5), and 100 μg/mL denatured salmon sperm DNA. Hybridizations were carried out at 42°C in the same solution for 24 to 36 h. The blots were washed three times for 10 min at room temperature with 2 × SSC, 0.1% (w/v) SDS, twice with 0.2 × SSC, 0.1% (w/v) SDS at 60 to 65°C, and finally rinsed with 0.2 × SSC at room temperature. Northern blots were probed with a soybean actin gene sequence under the same conditions to check for loading and transfer efficiencies. Relative RNA transcript levels were estimated by direct scanning of Northern blots with an Ambis radioisotope scanner.

Nucleotide Sequencing

Double-stranded template DNAs were prepared and sequenced by the dideoxy chain-termination method (26) with T7 DNA polymerase (T7 Sequencing Kit, Pharmacia) according to the manufacturer's recommendations. Both strands of the cDNA insert were sequenced from their ends using M13 universal primers. In addition, oligonucleotides (21-mers) were synthesized according to sequence information obtained and used directly as primers for further sequencing.

Polymerase Chain Reaction

Primary plaques obtained after rescreening the cDNA library were analyzed by PCR. Aliquots of phage lysates (5 μL) were heated for 5 min at 70°C and used as templates for PCR. The amplification mixture consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each oligonucleotide primer, and 1 unit of Taq polymerase in a total volume of 100 μL. The amplification program consisted of 30 cycles of denaturation (94°C, 1.5 min), annealing (55°C, 2 min), and primer extension (72°C, 2 min). The 3′ noncoding region of the cDNA insert in pCOMT1 was amplified with Taq polymerase as described above with 0.2 μg of purified plasmid DNA as template. Products of the reactions were separated on agarose gels. The PCR-generated 3′ end-specific fragments were separated and excised from 1.5% low gelling temperature agarose gels (SeaPlaque, FMC) and subsequently labeled with ^{32}P by random oligonucleotide-primed synthesis (9).

Expression of pCOMT1 in *E. coli*

E. coli XL-1 Blue cells harboring pCOMT1 were grown to an optical density of 0.6, and expression was induced by addition of IPTG to a final concentration of 10 mM. Cells were collected by centrifugation 2 and 6 h after addition of IPTG. The cells were resuspended in a buffer containing 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA,

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aat ctc aca aaa acc tca tca atc aca acc ATG GGT TCA ACA GGT GAA ACT CAA    54
                                       M   G   S   T   G   E   T   Q
ATA ACA CCA ACC CAC ATA TCA GAT GAA GAA GCA AAC CTC TTC GCC ATG CAA CTA   108
 I   T   P   T   H   I   S   D   E   E   A   N   L   F   A   M   Q   L
GCA AGT GCT TCA GTT CTT CCC ATG ATT TTG AAA TCA GCT CTT GAA CTT GAT CTC   162
 A   S   A   S   V   L   P   M   I   L   K   S   A   L   E   L   D   L
TTA GAA ATC ATT GCT AAA GCT GGA CCT GGT GCT CAA ATT TCA CCT ATT GAA ATT   216
 L   E   I   I   A   K   A   G   P   G   A   Q   I   S   P   I   E   I
GCT TCT CAG CTA CCA ACA ACT AAC CCT GAT GCA CCA GTT ATG TTG GAC CGA ATG   270
 A   S   Q   L   P   T   T   N   P   D   A   P   V   M   L   D   R   M
TTG CGT CTC TTG GCT TGT TAC ATA ATC CTC ACA TGT TCA GTT CGT ACT CAA CAA   324
 L   R   L   L   A   C   Y   I   I   L   T   C   S   V   R   T   Q   Q
GAT GGA AAG GTT CAG AGA CTT TAT GGT TTG GCT ACT GTT GCT AAG TAT TTG GTT   378
 D   G   K   V   Q   R   L   Y   G   L   A   T   V   A   K   Y   L   V
AAG AAT GAA GAT GGT GTA TCC ATT TCT GCT CTT AAT CTC ATG AAT CAG GAT AAA   432
 K   N   E   D   G   V   S   I   S   A   L   N   L   M   N   Q   D   K
GTG CTC ATG GAA AGC TGG TAC CAC CTA AAA GAT GCA GTC CTT GAT GGG GGC ATT   486
 V   L   M   E   S   W   Y   H   L   K   D   A   V   L   D   G   G   I
CCA TTC AAC AAG GCT TAT GGA ATG ACA GCC TTT GAA TAC CAT GGA ACA GAT CCA   540
 P   F   N   K   A   Y   G   M   T   A   F   E   Y   H   G   T   D   P
AGG TTT AAC AAG GTT TTC AAC AAG GGG ATG TCT GAT CAC TCT ACC ATC ACA ATG   594
 R   F   N   K   V   F   N   K   G   M   S   D   H   S   T   I   T   M
AAG AAA ATT CTT GAG ACC TAC ACA GGT TTT GAA GGC CTT AAA TCT CTT GTT GAT   648
 K   K   I   L   E   T   Y   T   G   F   E   G   L   K   S   L   V   D
GTA GGT GGT GGT ACT GGA GCT GTA ATT AAC ACG ATT GTC TCA AAA TAT CCC ACT   702
 V   G   G   G   T   G   A   V   I   N   T   I   V   S   K   Y   P   T
ATA AAG GGT ATA AAT TTT GAT TTA CCC CAT GTC ATT GAA GAT GCT CCA TCT TAT   756
 I   K   G   I   N   F   D   L   P   H   V   I   E   D   A   P   S   Y
CCA GGA GTT GAG CAT GTT GGT GGA GAC ATG TTT GTC AGT ATT CCA AAG GCT GAT   810
 P   G   V   E   H   V   G   G   D   M   F   V   S   I   P   K   A   D
GCT GTT TTT ATG AAG TGG ATT TGT CAT GAC TGG AGT GAT GAG CAC TGC TTG AAA   864
 A   V   F   M   K   W   I   C   H   D   W   S   D   E   H   C   L   K
TTT TTG AAG AAC TGC TAT GAG GCA CTG CCA GAC AAT GGA AAA GTG ATT GTG GCA   918
 F   L   K   N   C   Y   E   A   L   P   D   N   G   K   V   I   V   A
GAA TGC ATA CTT CCA GTG GCT CCA GAT TCA AGC CTG GCC ACA AAA GGT GTG GTT   972
 E   C   I   L   P   V   A   P   D   S   S   L   A   T   K   G   V   V
CAC ATT GAT GTG ATC ATG TTG GCT CAT AAT CCT GGT GGG AAA GAG AGA ACA CAA  1026
 H   I   D   V   I   M   L   A   H   N   P   G   G   K   E   R   T   Q
AAA GAG TTT GAG GAT CTT GCC AAA GGT GCT GGA TTC CAA GGT TTC AAA GTC CAT  1080
 K   E   F   E   D   L   A   K   G   A   G   F   Q   G   F   K   V   H
TGT AAT GCT TTC AAC ACA TAC ATC ATG GAG TTT CTT AAG AAG GTT TAA ttt ctt  1134
 C   N   A   F   N   T   Y   I   M   E   F   L   K   K   V  ***
tgg tgt gtt gca tct gag ttt tga tat tga gat tgt ggt tgt gct tct act tac  1188
cta agc ttt ccc cat aaa aat atg tga ttt cca ctt cta ttc ggt agg aaa ata  1242
ata atg aga aag ttc att gta ata ttg cct ata taa atg aac att gtt tca tat  1296
tgt gga tta taa aaa aaa aaa                                             1317
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Figure 1. Nucleotide and deduced amino acid sequences of the alfalfa COMT cDNA, pCOMT1. Nucleotides are numbered from the first base of the cDNA insert. The deduced amino acid sequence for alfalfa COMT is indicated below the nucleotide sequence in single-letter code. The first methionine of the open reading frame is designated as the first amino acid of the putative polypeptide. The termination codon is indicated by asterisks. Noncoding 5′ and 3′ regions are represented by lower case letters. Potential polyadenylation signals are underlined.

and flash-frozen in liquid N_2 or kept at −70°C. Immediately before the enzyme assay, the cells were thawed quickly, lysozyme was added at a concentration of 1 mg/mL, and the mixture was incubated on ice for 15 min. The cells were centrifuged once again, and the supernatant was used for assay of COMT activity. XL-1 Blue and XL-1 Blue cells containing pBluescript plasmid grown in the presence or absence of IPTG were treated as above and used as controls.

Assay of COMT Activity

COMT activity in bacterial extracts and alfalfa cell suspension samples was assayed as described previously (3) except a lower concentration of [^{14}C-methyl]SAM was used (16 μM) at a higher specific activity (603 kBq μmol^{-1}). The reaction was stopped with 0.4 N HCl and partitioned against ethyl acetate:hexane (1:1, v/v). After centrifugation, the organic phase was analyzed by scintillation counting. Protein content was determined with a commercial dye binding reagent (Bio-Rad) with BSA as standard.

RESULTS

Isolation and Characterization of COMT cDNA Clones

A λZAPII cDNA expression library constructed from poly(A)$^+$ RNA from elicitor-treated alfalfa cell suspension cultures was screened with anti-(aspen COMT) antiserum. This antiserum immunoprecipitates alfalfa COMT activity but not IOMT (5). Five antibody-positive plaques were identified on screening approximately 2×10^5 plaque-forming units from the amplified library. After *in vivo* excision of the pBluescript plasmid followed by digestion with *Eco*RI, all of the five clones were found to have similar sized cDNA inserts of about 1.3 kb and cross-hybridized strongly on Southern blots (data not shown). These five clones also had identical restriction maps. The complete nucleotide sequence and deduced amino acid sequence of one of the alfalfa COMT clones (pCOMT1) are shown in Figure 1. The sequence contains a 1095 bp open reading frame coding for 365 amino acids, a 30 bp putative 5′ noncoding region, and a 192 bp 3′ noncoding region including a poly(A) tail. The cDNA has two potential polyadenylation sites (AATAAT and AATGAA). The relative mol wt of COMT was calculated to be 39,964 based on the deduced amino acid sequence of the cDNA clone, which corresponds well with the reported subunit molecular mass of 41 kD (determined by SDS-PAGE) for alfalfa COMT (5). We cannot, however, rule out the possibility that translation starts at a methionine codon upstream of the 5′ end of the pCOMT1 clone, although the sequence around the ATG codon at position 31 matches reasonably with the consensus for plant initiation sequences (19). Comparison (Fig. 2) of the deduced amino acid sequence of pCOMT1 to that of a previously isolated aspen COMT cDNA (R.C. Bugos, V.L.C. Chiang, W.H. Campbell, unpublished results) revealed a high degree of similarity (86%), suggesting that the cDNA clone indeed codes for COMT from alfalfa. At the nucleotide level, the similarity between the alfalfa and aspen sequences was 77% in the open reading frame and 52% in the 3′-untranslated region. Computer searches of protein sequence data banks (National Biomedical Research Foundation and European Molecular Biological Laboratory Swissprot Library) with the pCOMT1 sequence revealed little sequence similarity with a bovine hydroxyindole *O*-methyltransferase. A recently published sequence of a 221 amino acid rat liver catechol *O*-methyltransferase (25) also showed little similarity to the predicted amino acid sequence of pCOMT1.

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Alfalfa   MGSTGETQITPTHISDEEANLFAMQLASASVLPMILKSALELDLLEIIAKAGPGA    55
Aspen              M   QV     H                  T I       M

Alfalfa   QISPIEIASQLPTTNPDAPVMLDRMLRLLACYIILTCSVRTQQDGKVQRLYGLAT   110
Aspen     FL TS    H  K            I    S S     LKDLP    E     P

Alfalfa   VAKYLVKNEDGVSISALNLMNQDKVLMESWYHLKDAVLDGGIPFNKAYGMTAFEY   165
Aspen      C F T       V P C             Y    I

Alfalfa   HGTDPRFNKVFNKGMSDHSTITMKKILETYTGFEGLKSLVDVGGGTGAVINTIVS   220
Aspen                                    K    T          V

Alfalfa   KYPTIKGINFDLPHVIEDAPSYPGVEHVGGDMFVSIPKADAVFMKWICHDWSDEH   275
Aspen       S                              V                A

Alfalfa   CLKFLKNCYEALPDNGKVIVAECILPVAPDSSLATKGVVHIDVIMLAHNPGGKER   330
Aspen               D         LV                   V

Alfalfa   TQKEFEDLAKGAGFQGFKVHCNAFNTYIMEFLKKV                       365
Aspen      E   G          E M C    HVI  R  A
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Figure 2. Comparison of the deduced amino acid sequences of alfalfa and aspen COMTs. The amino acid sequences are in single-letter code. Only amino acid residues different from the alfalfa sequence are shown in the aspen sequence.

To provide unequivocal data for the identity of the alfalfa COMT clones, the pCOMT1 sequence was expressed in *E. coli*. *E. coli* extracts were tested for activity against caffeic acid, 5-hydroxy ferulic acid (also an intermediate in lignin biosynthesis), and the isoflavone daidzein, a presumed intermediate in the biosynthesis of the phytoalexin medicarpin in alfalfa. *E. coli* lysates prepared 2 and 6 h after addition of IPTG were analyzed for *O*-methyltransferase activity. As shown in Table I no activity was found in the control host (*E. coli*, XL-1 Blue) or in XL-1 Blue containing control pBluescript plasmid. Two hours after the addition of IPTG, a 2.5-fold induction of COMT activity was observed in XL-1 Blue cells containing pCOMT1 compared with the uninduced control. At 6 h, the activity remained the same as at 2 h in the IPTG-treated cultures, whereas a 2.8-fold increase was seen in the uninduced controls. Because pCOMT1 is out of frame with the lacZ open reading frame in pBluescript, it may be expressed from its own ribosome binding site. The expressed COMT did not exhibit any activity against the isoflavone daidzein as a substrate but was active against 5-hydroxy ferulate (ratio of activity 5-hydroxy-ferulate:caffeate was 2.1:1). The aspen COMT clone, likewise, appears to encode a bifunctional OMT in view of its similar activity ratio following expression in *E. coli* (R.C. Bugos, V.L.C. Chiang, W.H. Campbell, unpublished results).

Rescreening of the alfalfa cDNA library with the insert from pCOMT1 yielded a further 60 primary putative COMT clones. PCR amplification of each of these 60 clones, using two internal primers spanning about 1 kb of the pCOMT1 coding region, yielded a single amplified species of approximately 1 kb in each case, suggesting that these clones were all probably COMT (data not shown). This conclusion was strengthened by similar results of PCR amplification using two primers from the 3′ noncoding region of pCOMT1.

Genomic Organization of Alfalfa COMT

Genomic DNA from alfalfa leaves was digested with a variety of restriction endonucleases and analyzed by Southern blot hybridization (Fig. 3). The *Eco*RV, *Pst*I, and *Hin*dIII digests each showed one strongly hybridizing band, two somewhat weaker bands, and up to three minor bands when the entire 1.3 kb insert of pCOMT1 was used as probe (Fig. 3A). When the 3′ noncoding region of pCOMT1 amplified by PCR was used as probe (Fig. 3B), we could observe some very faint bands in the *Eco*RV digest (lane 1), one strong band and two moderately strong bands in the *Pst*I digest (lane 2), and one strong band in the *Hin*dIII digest (lane 3). It should be noted that the signal intensities in these two blots are not directly comparable, because A and B were exposed for different times. We conclude that COMT in alfalfa is either encoded by at least two genes or exists as two or three allelic variants, a reasonable possibility in a tetraploid species.

Table I. *Expression of COMT Activity in E. coli XL-1 Blue Cells Harboring pCOMT1 and Control Plasmids*

Source of *E. coli* Extract	COMT Specific Activity
	pkat·kg protein^{-1}
XL-1 Blue minus plasmid	0.0
XL-1 Blue/pBluescript	0.0
pCOMT1 uninduced (2 h)	1.7
pCOMT1 + IPTG (2 h)	4.2
pCOMT1 uninduced (6 h)	4.7
pCOMT1 + IPTG (6 h)	5.1

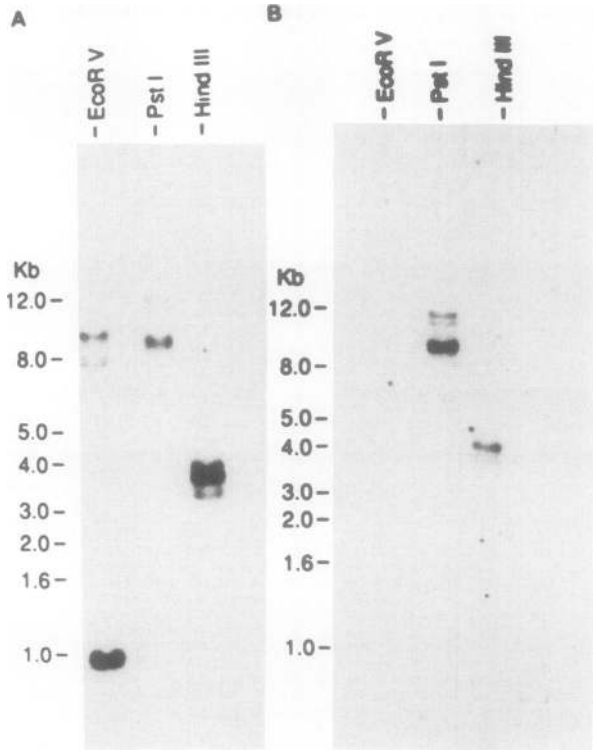

Figure 3. Southern blot analysis of alfalfa genomic DNA. Alfalfa genomic DNA (10 μg) was digested with the restriction endonucleases *Eco*RV, *Pst*I, or *Hin*dIII, electrophoresed on a 0.8% agarose gel, and blotted onto a nitrocellulose membrane. A, Membrane probed with ^{32}P-labeled full-length (1.3 kb) pCOMT1 insert. B, Membrane probed with PCR-generated, ^{32}P-labeled, 3′ noncoding region of pCOMT1.

Induction of COMT and AdoMet Synthetase Transcripts in Alfalfa Cell Suspension Cultures in Response to Elicitor Treatment

To study the kinetics of COMT transcript appearance upon elicitor treatment of alfalfa cell suspension cultures, a time course experiment was set up with elicitor-treated and water-treated (control) cultures. As seen in Figure 4A, there was a rapid accumulation of COMT message by 2 to 4 h following addition of elicitor (lanes 3 and 4). The induced transcript level remained nearly constant up to 12 h, was further elevated by 19 h (lane 8), and then decreased slowly thereafter up to 48 h (lane 12) postelicitation. The COMT transcript size of approximately 1.6 kb suggests that the pCOMT1 cDNA lacks about 300 bp of 5′-untranslated region. The rapid initial induction of COMT transcripts is similar to that reported for the penultimate enzyme in lignin synthesis, cinnamyl-alcohol dehydrogenase, in bean cell cultures exposed to fungal cell wall elicitor preparations (10).

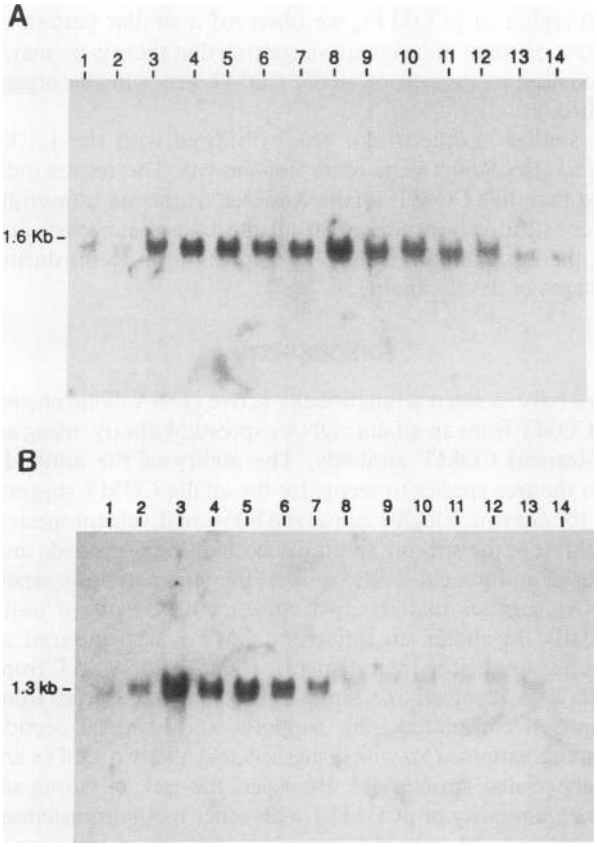

Figure 4. Time course for the induction of COMT and AdoMet synthetase steady-state transcript levels by elicitor treatment of alfalfa cell suspension cultures. A, Total RNA (10 μg) was denatured and fractionated on a denaturing agarose gel containing formaldehyde and transferred to a nitrocellulose membrane. Lanes 1 through 12: RNA from cells treated with elicitor for 0, 1, 2, 4, 6, 8, 12, 19, 24, 30, 38, and 48 h, respectively. Lanes 13 and 14: RNA from 6 and 48 h water-treated (control) cell samples, respectively. The blot was probed with ^{32}P-labeled 1.3 kb insert of pCOMT1. B, As in A. The blot was probed with a ^{32}P-labeled 1.3 kb *PstI/PstI* fragment of the *Arabidopsis* Sam-1 gene. Hybridization of Northern blots with a 3 kb soybean actin gene was included to confirm that the RNA preparations used in Figures 4 and 6 were intact and to preclude effects due to unequal gel loadings (data not shown).

The large increase in COMT transcript levels was accompanied by a relatively modest increase in COMT extractable activity over that observed in unelicited cultures (Fig. 5). This is in contrast to the situation observed for PAL in the same cultures, in which its extractable activity increases from approximately 10 to 550 μkat/kg protein over the first 12 h of elicitation (G. Gowri, R. A. Dixon, unpublished results). Furthermore, PAL enzyme activity declines to basal levels in parallel with decreased transcript levels, whereas COMT activity remains elevated. It would appear that both posttranslational and transcriptional regulation underlie the changes in phenylpropanoid pathway enzyme activities in elicited cells. The less than twofold increase in COMT activity in the elicited alfalfa cultures correlates with a 1.5- to twofold increase in wall bound phenolic material over 24 h (data not shown).

AdoMet synthetase is the enzyme that catalyzes the biosynthesis of SAM in a wide range of organisms (28). SAM serves as methyl group donor in numerous transmethylation reactions, including the reaction catalyzed by COMT. To determine whether the levels of transcripts encoding AdoMet synthetase changed coordinately with COMT transcripts in response to elicitor, we probed Northern blots of the elicitation time course with an *Arabidopsis Sam*-1 gene probe (22) (Fig. 4B). AdoMet synthetase transcript levels also increased rapidly within 2 h of elicitation from a low basal level (Fig. 4B, lanes 1–3) but declined more rapidly than COMT transcript levels after 12 h postelicitation.

Levels of COMT and AdoMet Synthetase Transcripts during Alfalfa Development

Expression of COMT from several plant sources has thus far been studied only at the enzyme activity and protein levels and mostly in cultured cells (3, 12, 13, 15). To study the developmental expression of COMT transcripts in alfalfa plants grown under controlled environmental conditions, we subjected total RNA isolated from roots, stems, root nodules, leaves, growing points (shoot tips), petioles, flower buds, and flowers of alfalfa plants grown at various stages of development to Northern blot analysis. As shown in Figure 6, we observed a high level of COMT transcripts in roots, stems, petioles, flower buds, and flowers compared with root nodules, leaves, and growing points. Due to differences in exposure times, signal strengths in panels A and B are not directly comparable. The major difference in the profiles between the two panels is that there is approximately 20 to 40 times as much mRNA hybridizing to the pCOMT1 cDNA from roots and stems (estimated by directly scanning the Northern blots for radioactivity, not shown) as compared with root nodules, leaves, growing points, and petioles. Thus there is a strong preference for expression of COMT transcripts in roots and stems, the highest transcript level being observed in stems. In roots, there is a gradual increase in the transcript level from

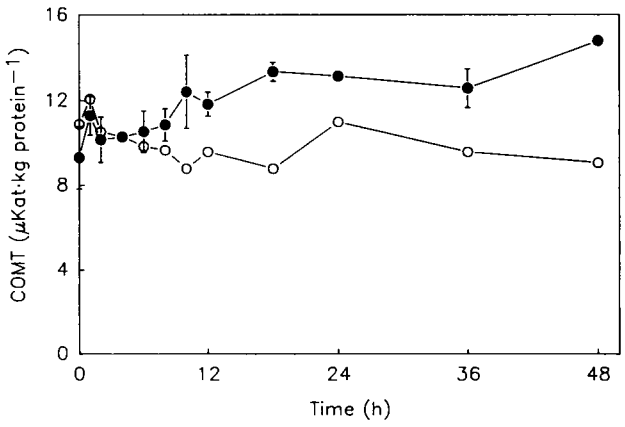

Figure 5. Induction of COMT enzyme activity in elicitor-treated alfalfa cell suspension cultures. Cultures were exposed to yeast elicitor (●) or an equal volume of water (○) and harvested at the times shown. Error bars represent the spread of values of two independent determinations.

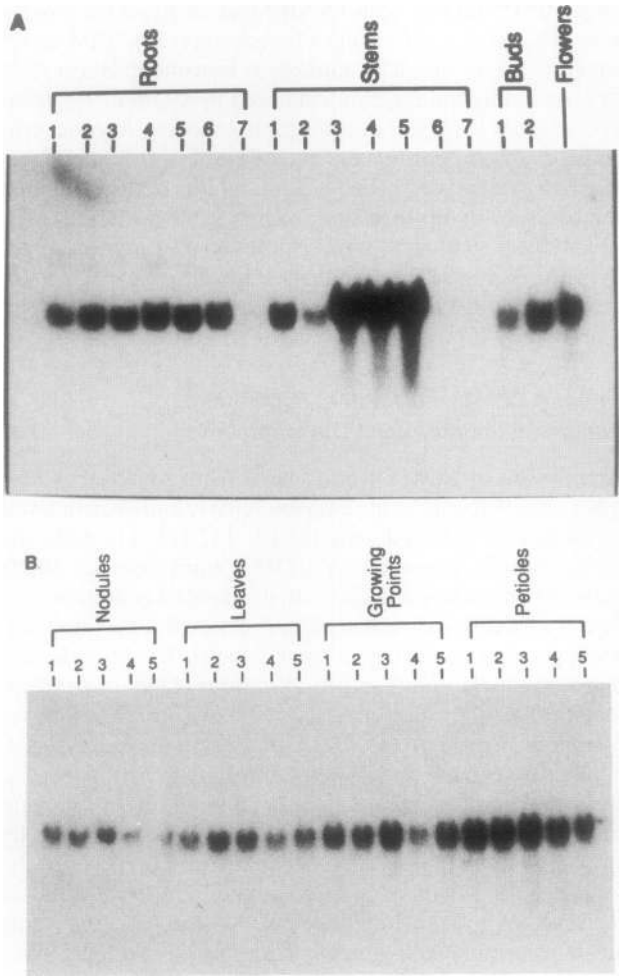

Figure 6. Northern blot analysis of COMT mRNA levels in various organs of alfalfa plants during development. Total RNA (10 μg) from the indicated organs was separated and blotted as described in the legend to Figure 4, and hybridized with ^{32}P-labeled 1.3 kb insert of pCOMT1. A, Roots and stems: lanes 1 through 6 and 7 show RNA from 1- to 6-week and 9-week-old plants, respectively. Flower buds: lanes 1 and 2 show RNA from 6- and 9-week-old plants, respectively. Flowers were from 9-week-old plants. B, Nodules, leaves, growing points, and petioles: lanes 1 through 4 and 5 show RNA from 3- to 6-week and 9-week-old plants, respectively.

the first to the sixth week postgermination. The lack of signal in the 7-week root sample and the 6- and 7-week stem samples was subsequently shown to be due in part to a gel loading error. In stems, it appears that a constant steady-state level of the transcript is maintained at least between the third and fifth weeks postgermination. It should be noted that, during the first and second weeks postgermination, there are no well defined stems. Therefore, we collected mostly shoots, which included all plant parts except roots. The sudden apparent drop in the RNA level in the second week (Fig. 6, panel A, stems, lane 2) may therefore be due to nonhomogeneity of the sampled material. In root nodules, leaves, growing points, and petioles, the kinetics of appearance of COMT transcripts follow nearly the same pattern observed in roots and stems. When the Northern blots were probed with the 3′-untranslated region of pCOMT1, we observed a similar pattern of expression (data not shown), suggesting that there is no major differential expression of alfalfa COMT genes in the organs studied.

A similar Northern blot was hybridized with the 1.3 kb *Arabidopsis Sam*-1 gene (data not shown). The results indicated that, like COMT, alfalfa AdoMet synthetase transcripts are constitutively expressed in all the tissues analyzed, and that there is some preference for expression in stems during all stages of development.

DISCUSSION

We have isolated a functionally active cDNA clone encoding COMT from an alfalfa cDNA expression library, using an anti-(aspen) COMT antibody. The ability of the antibody from the tree species to recognize the alfalfa COMT suggests that the enzyme is highly conserved between divergent species. In addition, the striking similarity both at the nucleotide and deduced amino acid levels between the alfalfa and the aspen cDNAs suggests that the two enzymes have evolved quite similarly. In alfalfa, an isoflavone OMT is also induced in response to elicitor treatment (3). IOMT and COMT from alfalfa have identical M_rs, similar elution characteristics from a range of chromatographic supports, and identical peptide mapping patterns (5), which suggests that the two OMTs are closely related structurally. However, the lack of strong sequence similarity of pCOMT1 with other methyltransferases that utilize SAM as substrate suggests that the recognition of SAM by each of these enzymes may depend on tertiary rather than primary structure.

The genomic blot data obtained for the COMT 3′-untranslated region probe along with the full-length pCOMT1 insert reveals the presence of either up to three genes, or possible allelic variants of one or two genes, for COMT in alfalfa. Previous work has shown the presence of two isoforms of the enzyme in alfalfa cell suspension cultures, based on their isoelectric points and relative affinities for SAM and *S*-adenosyl-L-homocysteine (5). It also appears that one of these isoforms preferentially accumulates in elicited cells. Our isolation of a large number of apparently identical COMT clones from the elicited library suggests, however, that only one gene may be induced in alfalfa cell cultures.

The rapid increase in COMT transcript levels by 2 h after exposure of cultured cells to elicitor is similar to the rapid and dramatic elicitor-induced appearance of PAL transcripts in alfalfa cell suspension cultures (G. Gowri, R.A. Dixon, unpublished results). However, COMT transcript levels remained approximately constant up to 48 h postelicitation, whereas PAL transcripts measured in the same batch of cells returned almost to the basal level within this time period. COMT transcripts, therefore, may have a longer half-life than PAL transcripts in alfalfa cell suspensions.

In addition to PAL and COMT transcripts, elicitor has been shown to induce the appearance of 4-coumarate:CoA ligase, chalcone synthase, and isoflavone reductase transcripts in alfalfa cells (4, N. Paiva, R.A. Dixon, unpublished results). A parallel situation is observed in elicitor-treated bean cells, in which nuclear run-off analysis has revealed rapid transcriptional activation of a central phenylpropanoid pathway gene

(PAL), flavonoid pathway genes (chalcone synthase, chalcone isomerase), and a lignin pathway gene (presumed to be cinnamyl alcohol dehydrogenase, although this identification is now uncertain) (17, 30, 31). Therefore, it is likely that the increases in steady-state transcript levels determined in the present work predominantly reflect increased transcription.

Stimulation of COMT activity in alfalfa cell suspension cultures correlates with the accumulation of wall-bound phenolics that may include lignin or lignin-like material. Elicitor-induced lignin synthesis has been previously confirmed in soybean cell suspension cultures (8). In addition, compounds derived from lignin precursors, the dehydrodiconiferyl glucosides, have been reported to exhibit cytokinin-like activity in plant cells (1), and the rapid induction of COMT transcripts by elicitor may therefore play a role in the generation of secondary signal molecules. In this respect, studies on cultured bean cells have revealed a more rapid elicitation of cinnamyl alcohol dehydrogenase, the penultimate enzyme in the synthesis of lignin, than of PAL (10), suggesting a role for the pathway in signal production as well as lignification.

COMT activity may be regulated by the relative concentrations of the substrate SAM and the co-product *S*-adenosyl-L-homocysteine, which is a strong competitive inhibitor of binding of SAM to OMTs (16). Therefore, we were interested in analyzing the effect of elicitation on AdoMet synthetase expression in alfalfa cell suspension cultures. The rapid and coordinated induction of AdoMet synthetase mRNA along with COMT transcripts in elicitor-treated cell suspension cultures suggests that AdoMet synthetase is not simply a "housekeeping" gene, but is also regulated by biotic stress. This regulation may be linked to the requirement for increased SAM pools as a consequence of increased COMT and IOMT activities. Because SAM also serves as methyl group donor in pathways such as ethylene and polyamine biosynthesis and other transmethylation reactions, other factors may also necessitate the high expression of AdoMet synthetase transcripts in elicited cells.

The strong preference in the expression of COMT for roots, stems, and petioles presumably reflects the extent of lignification these tissues are undergoing. A similar preference for expression of AdoMet synthetase in roots and stems has recently been reported in *Arabidopsis* (22). The timing of expression probably reflects the proportion of differentiating lignifying tissues (xylem vessels and sclerenchyma) in these organs during development. We have observed a similar preference for expression of PAL transcripts in the same tissues (G. Gowri, R.A. Dixon, unpublished results). Therefore, it is likely that the production of lignin precursors during differentiation involves tightly regulated temporal and spatial coordination of gene expression. Such a correlation between COMT and PAL has been observed at the enzyme activity level in bean cell suspension cultures (13). At all stages of development, roots were found to have more COMT transcripts than nodules. This is probably due to the fact that roots, as supporting and conducting organs, have high levels of lignified tissues, whereas the only lignified tissues in root nodules are the vascular tracks. Clearly, nodulation by *Rhizobium* does not induce a defense response involving induction of COMT transcripts. The same conclusion was reached in earlier studies comparing COMT enzyme activities in effective and ineffective alfalfa root nodules (6). The continued presence of COMT transcripts up to 9 weeks in the petioles suggests that the petioles from the leaves of younger nodes (near the shoot tip) are still undergoing differentiation. Furthermore, it has been observed that the collenchyma, a juvenile supporting tissue, becomes sclerified through lignification in alfalfa petioles as the plant gets older (7).

The molecular cloning of alfalfa COMT will allow us to explore the possibility of perturbing lignin synthesis by overexpression or under-expression (using antisense RNA) of COMT transcripts in transgenic alfalfa and other species. Such studies may help address the role of lignin in plant defense, and the possible role of dehydrodiconiferyl glucosides as cell division factors. In addition, engineering reduced lignin in forage legumes may help improve digestibility of the cell wall material for grazing animals.

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LITERATURE CITED

1. **Binns AN, Chen RH, Wood HN, Lynn DG** (1987) Cell division promoting activity of naturally occurring dehydrodiconiferyl glucosides: do cell wall components control cell division? Proc Natl Acad Sci USA **84:** 980–984
2. **Bugos RC, Chiang VL, Campbell WH** (1989) Isolation of O-methyltranferase associated with lignin biosynthesis in aspen. Wood Pulp Chem TAAPI Proc 345–347
3. **Dalkin K, Edwards R, Edington B, Dixon RA** (1990) Defense responses in alfalfa (*Medicago sativa* L.) I. Elicitor induction of phenylpropanoid biosynthesis and hydrolytic enzymes in cell suspension cultures. Plant Physiol **92:** 440–446
4. **Dalkin K, Jorrin J, Dixon RA** (1990) Stress responses in alfalfa (*Medicago sativa* L.) VII. Induction of defense related mRNAs in elicitor-treated cell suspension cultures. Physiol Mol Plant Pathol **37:** 293–307
5. **Edwards R, Dixon RA** (1991) Purification and characterization of S-adenosyl-L-methionine: caffeic acid 3-O-methyltransferase from suspension cultures of alfalfa (*Medicago sativa* L.). Arch Biochem Biophys **287:** 372–379
6. **Egli MA, Griffith SM, Miller SS, Anderson MP, Vance, CP** (1989) Nitrogen assimilating enzyme activities and enzyme protein during development and senescence of effective and plant gene-controlled ineffective alfalfa nodules. Plant Physiol **91:** 898–904
7. **Fahn A** (1982) Plant Anatomy, Ed 3. Pergamon Press, Oxford, p 84
8. **Farmer E** (1985) Effects of fungal elicitor on lignin biosynthesis in cell suspension cultures of soybean. Plant Physiol **78:** 338–342
9. **Feinberg AP, Vogelstein B** (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **137:** 266–267
10. **Grand C, Sarni F, Lamb CJ** (1987) Rapid induction by fungal elicitor of the synthesis of cinnamyl alcohol dehydrogenase, a specific enzyme of lignin synthesis. Eur J Biochem **169:** 73–77
11. **Grisebach H** (1981) Lignins. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 7. Academic Press, New York, pp 457–478
12. **Gustine DL, Sherwood RT, Vance CP** (1978) Regulation of

phytoalexin synthesis in jackbean callus cultures. Stimulation of phenylalanine ammonia-lyase and *O*-methyltransferase. Plant Physiol **61:** 226–230

13. **Haddon LE, Northcote DH** (1976) Correlation of the induction of various enzymes concerned with phenylpropanoid and lignin synthesis during differentiation of bean callus (*Phaseolus vulgaris* L.). Planta **128:** 255–262
14. **Hahlbrock K, Scheel D** (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol **40:** 347–369
15. **Hermann C, Legrand M, Geoffroy P, Fritig B** (1987) Enzymatic synthesis of lignin: purification to homogeneity of the three O-methyltransferases of tobacco and production of specific antibodies. Arch Biochem Biophys **253:** 367–376
16. **Khouri HE, Tahara S, Ibrahim RK** (1988) Partial purification, characterization, and kinetic analysis of isoflavone 5-O-methyltransferase from yellow lupin roots. Arch Biochem Biophys **262:** 592–598
17. **Lawton MA, Lamb CJ** (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol **7:** 335–341
18. **Logemann J, Schell J, Willmitzer L** (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20
19. **Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA** (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J **6:** 43–48
20. **Maniatis T, Fritsch E, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. **Matern U, Kneusel RE** (1988) Phenolic compounds in plant disease resistance. Phytoparasitica **16:** 153–170
22. **Peleman J, Boerjan W, Engler G, Seurinck J, Botterman J, Alliotte T, Van Montagu M, Inzé D** (1989) Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding S-adenosylmethionine synthetase. Plant Cell **1:** 81–93
23. **Poulton JE** (1981) Transmethylation and demethylation reactions in the metabolism of secondary plant products. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 7. Academic Press, New York, pp 667–723
24. **Ride JP** (1978) The role of cell wall alterations in resistance to fungi. Ann Appl Biol **89:** 302–306
25. **Salminen M, Lundstrom K, Tilgmann C, Savolainer R, Kalkkinen N, Ulmanen I** (1990) Molecular cloning and characterization of rat liver catechol-O-methyltransferase. Gene **93:** 241–247
26. **Sanger F, Nicklen S, Coulson AR** (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **83:** 8073–8076
27. **Schumacher HM, Grundlach H, Fiedler F, Zenk MH** (1987) Elicitation of benzophenanthridine alkaloid synthesis in *Eschscholtzia* cell cultures. Plant Cell Rep **6:** 410–413
28. **Tabor CW, Tabor H** (1984) Methionine adenosyltransferase (S-adenosylmethionine synthetase) and S-adenosylmethionine decarboxylase. Adv Enzymol **56:** 251–282
29. **Vance CP, Kirk TK, Sherwood RT** (1980) Lignification as a mechanism of disease resistance. Annu Rev Phytopathol **18:** 259–288
30. **Walter MH, Grima-Pettenati J, Grand C, Boudet AM, Lamb CJ** (1988) Cinnamyl alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor. Proc Natl Acad Sci USA **85:** 5546–5550
31. **Walter MH, Grima-Pettenati J, Grand C, Boudet AM, Lamb CJ** (1990) Extensive sequence similarity of the bean CAD4 (cinnamyl alcohol dehydrogenase) to a maize malic enzyme. Plant Mol Biol **15:** 525–526
32. **Young D, Davis RW** (1983) Yeast RNA polymerase II genes: isolation with antibody probes. Science **222:** 778–782