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Structure and expression of the lignin *O*-methyltransferase gene from *Zea mays* L.

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Abstract

The isolation and characterization of cDNA and homologous genomic clones encoding the lignin *O*-methyltransferase (OMT) from maize is reported. The cDNA clone has been isolated by differential screening of maize root cDNA library. Southern analysis indicates that a single gene codes for this protein. The genomic sequence contains a single 916 bp intron. The deduced protein sequence from DNA shares significant homology with the recently reported lignin-bispecific caffeic acid/5-hydroxyferulic OMTs from alfalfa and aspen. It also shares homology with OMTs from bovine pineal glands and a purple non-sulfur photosynthetic bacterium. The mRNA of this gene is present at different levels in distinct organs of the plant with the highest accumulation detected in the elongation zone of roots. Bacterial extracts from clones containing the maize OMT cDNA show an activity in methylation of caffeic acid to ferulic acid comparable to that existing in the plant extracts. These results indicate that the described gene encodes the caffeic acid 3-*O*-methyltransferase (COMT) involved in the lignin biosynthesis of maize.

Introduction

Several plant *O*-methyltransferase (OMT) activities (EC 2.1.1.6) have been described and their function discussed in relation to the biosynthesis of lignins, flavonoids, furanocoumarins and meta- or para-*O*-methylation. In different tissues, in addition to lignin precursors, various methylated phenolic compounds often occur and multiple forms of OMTs have been reported [for reviews see 4, 21, 22, 27, 32, 36]. One of them is responsible for the methylation of cinnamic acids as precursors of cinnamyl alcohols in lignin biosynthesis. The sources for the purification of lignin OMTs were different tissues of apple tree [18], bamboo [45, 46], poplar [8, 46], pine [29, 44], suspension cultures of parsley [14] and soybean [37, 38], pampas grass [16, 17], tobacco [9, 47], or alfalfa [10, 48]. More detailed studies of this enzyme in lignin-related tissues have been re-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number M73235.

ported from mistletoe lignin [28], cultured tobacco cells [49], aspen secondary xylem [30], swede root disks [40] and tracheary elements from *Zinnia elegans* [19]. More recently, lignin-related OMT has been purified to homogeneity from aspen [5], tobacco [24], cabbage [11], poplar [13] and alfalfa [15].

In angiosperms, the biosynthesis of monolignols as precursors of lignins involves two methylation reactions. Caffeic acid is methylated to ferulic acid by caffeic acid 3-*O*-methyltransferase (COMT) [21], which is then hydroxylated at position 5 by ferulate-5-hydroxylase. Subsequent methylation by COMT at this new hydroxyl position yields sinapic acid. Then ferulic and sinapic acids, previously activated to the corresponding coenzyme-A esters, are converted into coniferyl and sinapyl alcohols (the two methylated lignin precursors), respectively, via a two-step reductive process. The other monolignol, *p*-coumaryl alcohol (very abundant in monocots), is directly synthesized from *p*-coumaric acid, without the methylation step. COMT appears to be a key enzyme in the lignin biosynthetic pathway. For example, the correlation between the mono- and bi-methylation activity ratio of purified enzyme and the amounts of the corresponding lignin precursors from plant tissues has been well established [27, 36].

Antibodies against COMT, have been used to isolate and characterize homologous cDNA clones from aspen [6] and alfalfa [20]. No information is available so far on COMT genomic sequences or its sequence and expression in monocotyledonous species. In this paper we report the characterization of the COMT gene from maize. The cloned gene appears interesting both in relation to its pattern of expression and to the possibility it offers to study the features of its enzymatic activity. Maize COMT mRNA is highly accumulated in the elongation zone of the radicular system, which is active in lignin formation. We have obtained methylation of caffeic acid as 3-methoxy-4-hydroxycinnamic acid (ferulic acid) from bacterial clones containing the maize COMT cDNA, as well as in plant extracts.

Materials and methods

Plants used in this work were *Zea mays* L. inbred line W64A, except when indicated otherwise, grown under greenhouse conditions in Barcelona, Spain. Plantlets were obtained by germinating dry seeds through imbibition in water at 25 °C in the dark for the indicated period of time.

Cloning procedures were carried out following standard published methods [2, 23, 42]. Enzymes were purchased from Boehringer (Mannheim) unless stated otherwise, labelled nucleotides were from Amersham. The construction of a cDNA library, screening procedures, northern and Southern blot analysis were carried out as described [33, 34]. DNA sequence analysis was done in both directions with overlapping clones and using a M13 or pUC dideoxy nucleotide sequencing method according to described protocols [2] with Klenow DNA polymerase (New England Biolabs). Sequence alignment was done using the Clustal V option from the software of BISANCE [12] and sequence analysis was done by using software from Micro-Genie (Beckman) [39].

For expression of the MC1 cDNA clone in *Escherichia coli* with the *lacZ* (β-galactosidase gene) promoter, the coding region was inserted in pBluescript (KS and SK) and pUC plasmids in the appropriate directions using the 5′ unique *Xho* I restriction site (see Fig. 5) and *E. coli* K12 DH5α strain. This construction leaves the MC1 protein sequence out of frame with respect to the *lacZ* polypeptide.

Enzymatic OMT activity was tested as follows: overnight bacterial cultures were centrifuged and resuspended in 200 mM Tris-HCl (pH 7.2) containing 14 mM 2-mercaptoethanol as previously reported [10], and the cells were sonicated for a few seconds. Then 2.5mM caffeic acid (Sigma) in dimethylsulfoxide (DMSO) was added with 0.1 μCi of *S*-adenosyl-*L*-(methyl-^{14}C)-methionine (50–60 mCi/mmol; Amersham). After 2 h incubation at 37 °C, 10 μg of ferulic acid (Aldrich) in DMSO was added as a carrier. Phenylpropanoids were extracted twice with one volume of ether by vortexing and the solvent was evaporated in a

new microfuge tube. The residual products were resuspended in 5 μl ethanol and applied to thin-layer chromatography (TLC) silica plates (Merck 60F-254, pre-coated plates). Finally, the plates were developed in C_6H_6-OHAc-H_2O (5:5:1, upper phase) and autoradiographed for 4–5 days. In order to assay the plant enzymatic activity, 7-day-old roots were frozen in liquid nitrogen, ground to a fine powder in a mortar in the presence of 200 mM Tris-HCl pH 7.2, 14 mM 2-mercaptoethanol and the assay carried out as described above.

Results

cDNA and genomic cloning

Searching for genes corresponding to mRNAs preferentially accumulated in developing maize roots, a number of clones were identified [34]. One of the cDNA clones (MR18) was isolated as a result of differential screening of a λgt10 cDNA library of two-month-old roots from *Zea mays* (pure inbred line W64A).

By northern blotting a single homologous mRNA band about 1600 nucleotides long is observed using the MR18 clone as a probe (Fig. 3). As expected from the result of the differential screening, a higher level of mRNA accumulation was observed in roots than in leaves. The insert of the MR18 clone was 637 bp long (Fig. 1), representing a partial fragment of the transcript detected by northern analysis. In order to obtain a longer cDNA clone, other libraries were screened. A new clone homologous to MR18 (clone MC1) was isolated from a pBR322 maize (W64A) coleoptile cDNA library. The insert of MC1 was 1360 bp long, and it had the same sequence as the MR18 clone except for some gaps in the 3′ non-coding region that may be the product of recombination either in the plant or during the cloning procedures. When compared with the genomic sequence (see below) it appears that the MC1 clone contains the complete coding sequence, and it is identical to the MR18 and the genomic ones. From the MC1 clone only one putative open reading frame including a methionine residue in its 5′ region could be deduced (Fig. 1).

By screening a maize (W64A) λCharon-35 genomic library [34], using the MR18 insert as a probe, two phages MG18/14 and MG18/32 containing homologous sequences were isolated. These clones have overlapping inserts of 13 and 14 kb, respectively, corresponding to 19 kb of the plant genome. The fragments hybridizing with the MR18 probe and their flanking regions were sequenced. Both clones were identical to the MR18 cDNA sequence. Figure 1 shows 2512 bp of this sequence, including the translation of the coding region. From the comparison of the genomic and cDNA sequences an intron of 916 bp was deduced. It is flanked by consensus intron splicing sequences and it contains a higher proportion of A + T sequences than the coding region, including some stretches of A or T nucleotide residues.

Nucleotide and protein sequences

Figure 1 shows the sequence of the gene homologous to the MR18 cDNA. From both the genomic and cDNA sequences the same open reading frame can be deduced. The nucleotide composition of the coding region sequence is very GC-rich with 68.3% of G + C and 31.7% of A + T, a feature already observed in many monocot genes [7]. By plotting the G + C distribution along the sequence of the genomic clone the exon and intron regions can be easily distinguished (not shown). The distribution of codons shows an extreme bias towards the use of G or C at the third position: among the 364 amino acids coded in the sequence only three of them use A or T in this position while C or G are evenly distributed. The same occurs by some other monocot genes as reviewed by Campbell *et al.* [7].

The two cDNAs have a poly(A) sequence beginning at two different sites indicated in the figure by triangles and the putative polyadenylation signal is underlined. The use of different polyadenylation sites in a single gene has already been described in a number of genes including an α-tubulin gene preferentially expressed in the

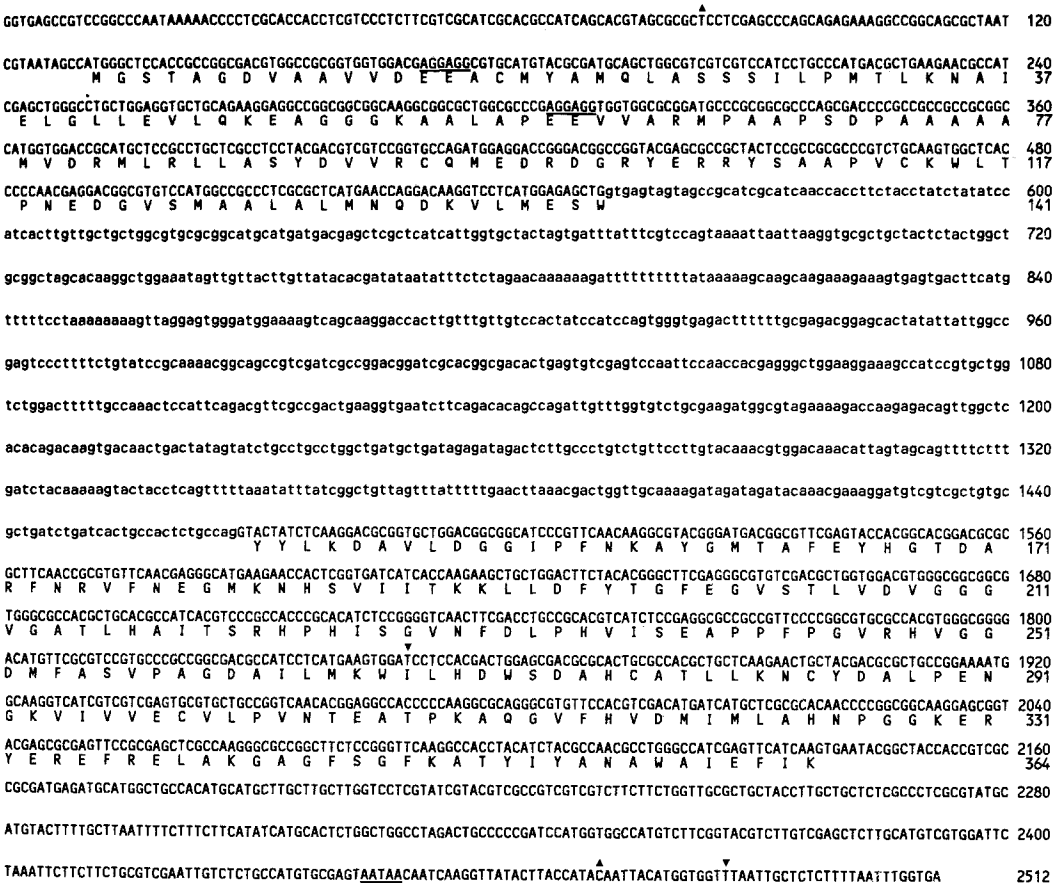

Fig. 1. Nucleotide sequence of the *O*-methyltransferase gene in *Zea mays*. The sequence (2512 bp long) obtained from the overlapping MG18/14 and /32 genomic clones corresponds to a *Hph* I-flanked fragment shown in Fig. 5 between asterisks. The MR18 cDNA sequence is indicated by two downward triangles from nucleotides 1849 to 2487. The homologous MC1 begins and ends in the upward triangles except for the differences mentioned in the text. The predicted amino acid sequence (single-letter code) is shown and numbered below the corresponding nucleotide triplets. The two putative *E. coli* ribosome-binding sequences at nucleotide positions 171 and 306 are underlined. The putative polyadenylation signal at position 2444 is also underlined. The intron sequence appears in lower-case letters.

radicular system of maize [35]. At 5′ of the sequence two motifs at positions 171 and 306 nt are also underlined. They are identical to the *E. coli* ribosome-binding site (Shine-Dalgarno sequence) and each one is located in the 5′-proximal region with respect to a methionine residue in the sequence of the protein.

The protein coded by the MC1 and MG18 clones is composed of 12.1% acidic residues (Asp + Glu), 9.1% of basic ones (Arg + Lys), 8.8% of aromatic (Phe + Trp + Tyr) and 25.5% of hydrophobic (Ile + Leu + Met + Val) amino acids giving an estimated pI of 6.03. The estimated molecular weight of the protein is 39572 Da. The hydropathy profile of the protein shows two main hydrophilic regions around positions 100 and 333 in the polypeptide sequence (not shown). Secondary structure predictions show a high probability of helix formation in different parts of the sequence, indicating that it may be a globular polypeptide in agreement with a protein having enzymatic activity.

By comparing the protein deduced from the cDNA and genomic clones with protein data

banks we could detect only two related sequences, both having an *O*-methyltransferase function: a hydroxyindole OMT from bovine pineal glands (23% identity) (OMTBO) [26] and a purple non-sulfur photosynthetic bacterium (*Rhodobacter capsulatus*) OMT [1] from the carotenoid biosynthesis gene cluster (OMTCA) (22% identity). Recently, the first two OMT sequences from plants have been published. Both OMTs, from alfalfa (OMTAL) [20] and aspen (OMTAS) [6], correspond to the caffeic acid *O*-methyltransferase (COMT) involved in lignin biosynthesis. The homology between the maize protein sequence (OMTMA) and the aspen and alfalfa ones is 62% and 59%, respectively. These five homologous protein sequences are presented in the alignment of Fig. 2. The boxes indicate the five most conserved regions previously described by Bugos *et al.* [6].

Pattern of accumulation of the maize OMT mRNA

The levels of accumulation of the maize OMT mRNA were measured using northern and slot blot analysis. It shows, as expected, a higher accumulation of the transcript in the roots of the plants in comparison to the leaves, and it is about 1600 nucleotides long. A northern analysis has been carried out on RNA extracted from differ-

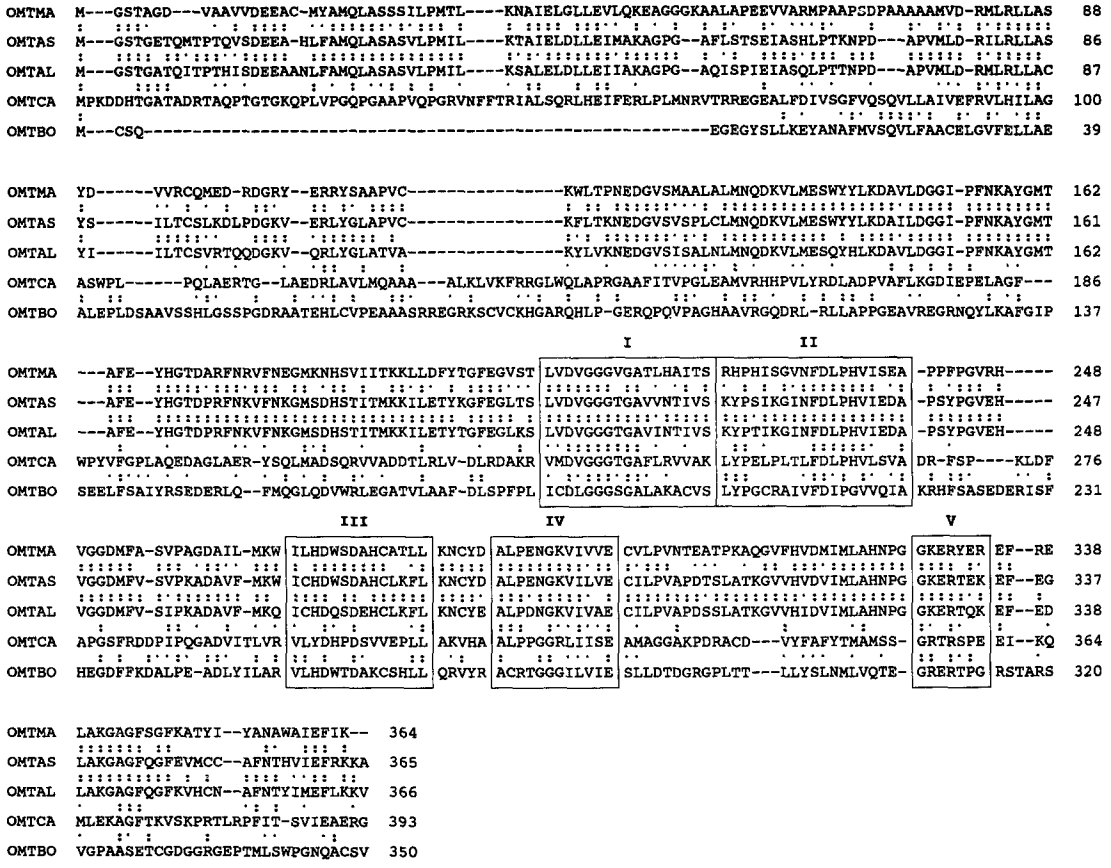

Fig. 2. Alignment of related *O*-methyltransferase protein sequences. The protein sequences are shown in single-letter code. OMTMA corresponds to the COMT from maize, OMTAL to the COMT from alfalfa [20], OMTAS to the COMT from aspen [6], OMTBO to the OMT from bovine pineal glands [26] and OMTCA to the OMT from a purple non-sulfur photosynthetic bacterium [1]. Colons between amino acid residues correspond to amino acid identity and one point to conservative changes. A dash in the sequence indicates a gap introduced in order to maintain a good alignment. The boxes indicate the five most conserved regions.

ent parts of the plant (Fig. 3). It is possible to observe that in all the cases only a single mRNA band was detected. The results also show an increased mRNA accumulation in the elongation zone of the roots. Significant levels of transcription are also observed below the coleoptile node in mesocotyl, a region where cells are actively elongating when the seeds are grown in the dark.

More accurate levels of mRNA accumulation were also measured by slot blot analysis. In Fig. 4 the pattern of accumulation in different tissues and developmental stages of the plant is shown. Besides the high accumulation in the elongation zone of the roots, the mRNA is accumulated at lower but significant levels in the mesocotyl and in the differentiated roots. Low levels are also observed in the node, coleoptile, adult roots, in the meristematic tip of roots, in leaves, flowers (but not in pollen), seeds and embryos during maturation. Figure 4 also shows that the accumulation increases during the first days of growth both in roots and coleoptiles.

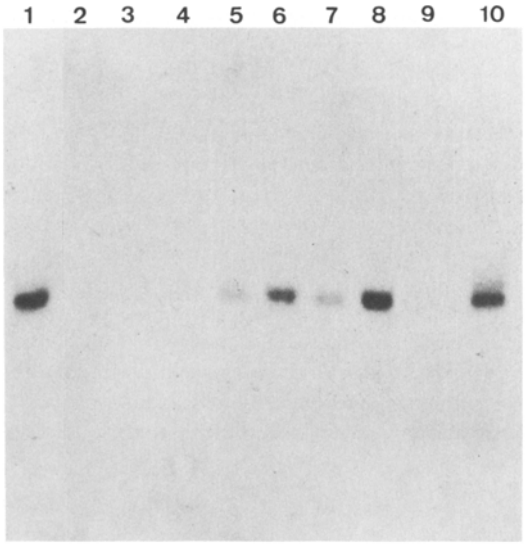

Fig. 3. Northern blot analysis of maize RNA using the MR18 insert as a probe. Each lane contains 10 μg of total RNA from: 3-day-old roots (1), isolated flowers (anthers, stamens and peduncles) (2) and anthers (3) from 120-day-old plants; epicotyls (4), coleoptile nodes (5), mesocotyls (6), differentiating zone of roots (7), elongation zone of roots (8), and root tips (9), from 7-day-old plants; 0.5 μg of poly(A)$^+$ RNA from 3-day-old roots (10).

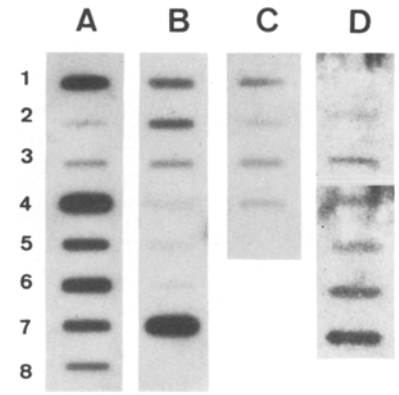

Fig. 4. RNA-slot blot analysis of mRNA accumulation of maize OMT in different organs and developmental stages of the plant. Each slot contains 10 μg of total RNA. A. 3-day-old roots (1), and coleoptiles (2), roots from 6-day-old plantlets manually dissected in three zones, root tips (3), rich in elongating (4), and differentiating (5) cells. The same with coleoptiles dissected in mesocotyl (6), node (7), and epicotyl (8). B. Seeds of 8 (1), 10 (2), and 12 (3) days after pollination; embryos of 18 (4), 30 (5), and 50 (6) days after pollination; 0.5 μg of poly(A)$^+$-RNA of 3-day-old roots (7). 100 pg of an α-tubulin probe as negative control (8). C. 3-month-old roots (1), and leaves (2). Flowers (anthers, stamens and peducles) (3), anthers (4), and pollen (5) from 120-day-old plants. D. 5- (1), 6- (2), and 7-day-old (3) coleoptiles; 4- (4), 5- (5), 6- (6), and 7-day-old (7) roots.

Southern hybridization

To study the number of genes coding for OMT in maize Southern blot hybridization of genomic DNA was carried out. The result is shown in Fig. 5, for DNA prepared from the pure inbred line W64A and the double hybrid E41. The pattern obtained for the five restriction enzymes used with W64A correlates with the sites deduced from the DNA sequence. The map obtained is shown in the same figure. The same pattern is obtained by the *Sac* I analysis in the commercial double hybrid E41. With this latter variety it is possible to detect the same bands already observed in W64A (lane 5) and the two other predicted bands from *Sac* I digestion, which were not detected in the conditions of DNA concentration used for the W64A. This result is compatible with one gene copy present in the maize genome and it shows a low degree of sequence polymorphism.

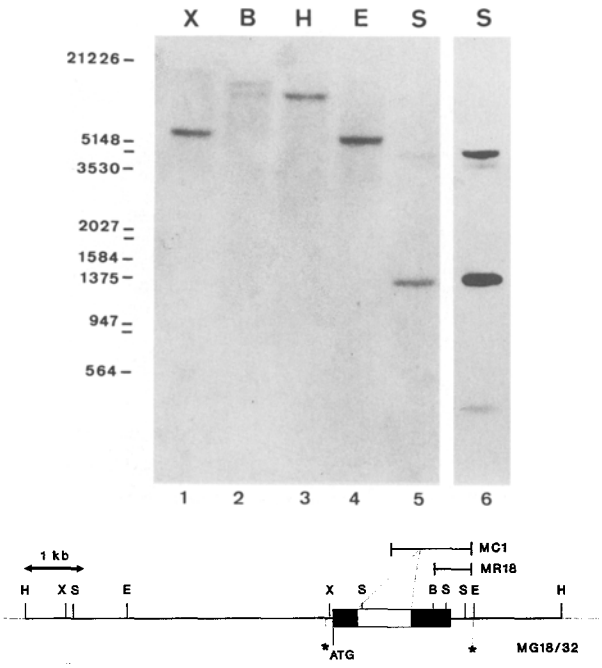

Fig. 5. Southern analysis of genomic OMT sequences homologous to the MC1 cDNA clone of *Zea mays*. Southern blots were prepared from 10 μg of *Zea mays* genomic DNA digested with *Xho* I (X), *Bam* HI (B), *Hind* III (H), *Eco* RI (E) and *Sac* I (S) and hybridized with the 1147 bp *Pst* I fragment from MC1 insert. Lanes 1–5 correspond to W64A pure inbred line and lane 6 to the E41 double hybrid. In the lower part of the figure is shown the map of the MG18/32 genomic clone and the MR18 and MC1 cDNAs. The sequence shown in Fig. 1 corresponds to the zone between asterisks.

OMT activity in bacterial extracts

In the 5′ region of the MC1 cDNA clone two segments homologous to the *E. coli* Shine-Dalgarno ribosome-binding sequences are observed. They are fortuitously present at the appropriate distance from a methionine residue (see Fig. 1). These sequences could act as ribosome-binding sites directing the appropriate translation of the COMT with the MC1 insert. This possibility has already been suggested for aspen and alfalfa COMTs [6, 20] and it offers a way to test the enzymatic activity of the polypeptide encoded by the maize insert.

The MC1 cDNA coding region was inserted in both pBluescript (KS and SK) and pUC plasmids in opposite directions using the unique *Xho* I restriction site located near the 5′ end of the MC1 insert (see Fig. 5). These constructions were introduced into the *E. coli* K12 DH5α strain, which does not need IPTG as inductor of the *lacZ* promoter. With this construction, the OMT protein is out of frame with respect to the *lacZ* polypeptide. The methylation activity of the MC1 insert in extracts of *E. coli* is shown by autoradiography of the thin-layer chromatography silica plates (Fig. 6). Extracts of sonical bacterial cultures were tested for OMT activity. Using these constructs, specific meta-methylation was obtained by adding caffeic acid as a substrate and *S*-adenosyl-*L*-(methyl-^{14}C)-methionine as donor of methyl groups producing ferulic acid (Fig. 6, lane 9). To test which one of these Shine-Dalgarno sequences was responsible for the observed expression, a deleted fragment from the MC1 clone was also analyzed. It was cloned in the *Pst* I site located between these two putative ribosome-binding sites (see Figs. 1 and 5) and it was also out of frame with respect to *lacZ* and in the appropriate orientation. Using this construction no methylation was observed (Fig. 6, lane 10). In the negative controls of bacterial extracts alone with (lane 2) or without (lane 1) plasmid lacking the insert, and in the reaction mixture without bacterial extracts (lane 4), no enzymatic activity was observed. Methylation is not observed either in the absence of caffeic acid (lane 5) or when the insert is in the opposite direction (lane 7). When minimal amounts of bacterial extract are added or with an excess of caffeic acid in the reaction mixture, an additional non-specific methylation occurs producing 3,4-dimethoxycinnamic acid (lanes 6 and 8). With lower substrate concentrations only one meta-specific methylation occurs producing ferulic acid both in bacterial or in plant root extracts (lanes 9 and 11). With plant root extracts in the absence of caffeic acid no reaction is detected (lane 12). Lane 13 shows the chromatographic mobilities of caffeic, ferulic and 3,4-dimethoxycinnamic acids used as markers under UV light. Thus, the bi-methylation pattern might be produced only in *in vitro* conditions with high substrate/enzyme ratios as it has been proposed for OMT from *Populus nigra* [8]. When ferulic

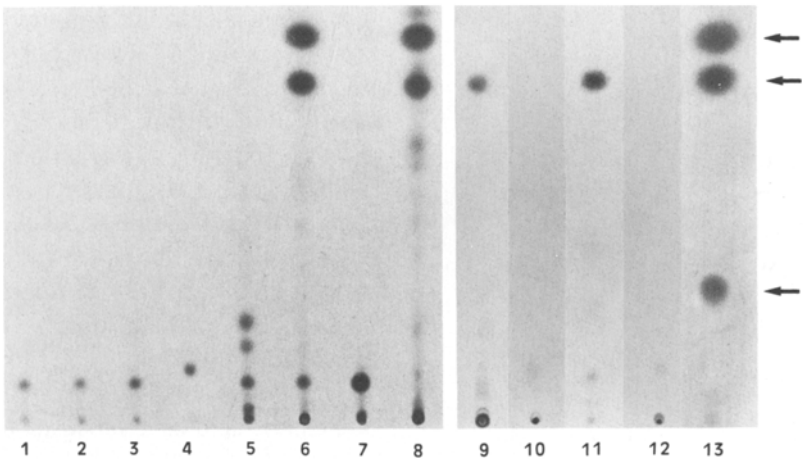

Fig. 6. Autoradiography of thin layer chromatography analysis of OMT activity in *E. coli* extracts with 0.1 μCi of *S*-adenosyl-*L*-(^{14}C-methyl)-methionine and 2.5 mM of caffeic acid. Also shown are negative controls of *E. coli* bacterial extract (without plasmid) with the labelled cofactor, caffeic acid and buffer (1); extract of bacteria with plasmid lacking the insert (2); extract of bacteria with plasmid and an insert unrelated to OMT (3); reaction mixture (with labelled SAM, caffeic acid, and buffer) without extract (4); and extract of bacteria with plasmid and the OMT insert, with labelled SAM but without caffeic acid (5). Extract of bacteria with pUC plasmid and the OMT insert, with caffeic acid and labelled SAM (6). Bacterial extract with pBluescript SK + plasmid and the OMT insert in the opposed orientation (7), and in pBluescript KS + (correctly oriented) (8). Lanes 9 to 12: similar autoradiography using only 0.05 mM of caffeic acid; as in lane 8, but with this lower concentration of caffeic acid (9); the same with a deletion between the two ribosome-binding site strings (10); roots extracts with lower caffeic acid and SAM-[^{14}C] (11); and the same without caffeic acid (12). Lane 13 is a UV pattern of a thin-layer chromatography of the caffeic, ferulic and 3,4-dimethoxycinnamic acids (arrows from top to bottom) as a control to evaluate the RFs of the observed spots.

acid, its isomer isoferulic acid (methylated at para-position), and sinapic acid are added at high concentrations, no methylating activity was observed (data not shown).

Discussion

In this paper we report the characterization of the lignin *O*-methyltransferase gene from maize. Clones corresponding to the cDNA and genomic sequences have been obtained and sequenced. The initial clone MR18 was obtained after differential screening, carried out in order to obtain genes highly expressed in the radicular system. The complete sequence of the protein was obtained from both cDNA and genomic clones and it is identical in all these cases. One of the cDNA clones contains the complete coding sequence and it allows to determine the position of the single intron observed in the gene and to obtain expression of the protein in bacterial extracts.

The identification of the protein coded by clones MR18 and MC1 as a lignin *O*-methyltransferase comes from two different lines of evidence: the sequence homology and the specific activity in extracts from bacteria containing the cDNA insert that has been compared with the corresponding activity in crude plant extracts. A high homology was found with the lignin *O*-methyltransferase sequences recently reported from alfalfa and aspen [20, 6]. These genes, both from dicotyledonous species, have 86% of similarity between themselves. With the sequence of maize, a monocotyledonous plant, the level of homology is about 60%. We were also able to detect 20% identity with bovine OMT and OMT from *Rhodobacter capsulatus*. In contrast, no significant homology levels, even within the boxes of conserved sequences, were observed with the rat or human catechol *O*-methyltransferase [41, 3] or with the alfalfa cafeoyl-CoA 3-*O*-methyltransferase [43]. These differences could partially be explained by the substantially lower molecular

weights of these proteins [6]. The alignment among the five homologous sequences produces a number of conserved domains as it is shown in Fig. 2. Boxes I, III and IV are probably involved in the interaction with SAM as can be deduced by a homology comparison among different enzymes requiring this cofactor [25]. Box II probably represents an extension of the conserved region included in box I. Box V is a hydrophilic domain also conserved in this alignment of *O*-methyltransferases.

By northern analysis it can be concluded that the maize OMT mRNA is preferentially accumulated in the elongation zone of the roots during development, but minor accumulation is observed in other parts of the plant except in pollen, where no transcripts were detected. The accumulation of the mRNA is related to the elongating tissues of the plant as it is observed in the corresponding part of the root and mesocotyl. In such tissues lignification of the vascular system is very active. This is in agreement with a correlation between lignin biosynthesis and the accumulation of mRNA from this gene.

From Southern blotting results of plant genomic DNA a single gene copy seems to be present in maize genome. In other plants, for instance in tobacco, two COMT isoforms of 38.5 and 39.5 kDa were reported, but no genetic data are available [24]. In cabbage, also two isoforms of 42 ± 2 kDa were described but no sequences were reported [11]. In agreement with this, two isoforms of 41 kDa have also been described in alfalfa and from Southern analysis at least two genes seem to be present in the genome [48]. In aspen, only one protein band of about 45 kDa has been obtained after purification [5] but an OMT gene family of about three genes has been suggested from Southern analysis [6]. If other OMTs exist in maize they should be quite different in sequence and therefore with low homology with the one reported here.

Extracts from bacteria transformed with the MC1 cDNA, cloned in the appropriate orientation in pUC and pBluescript, show activity in the methylation of caffeic acid to ferulic acid and 3,4-dimethoxycinnamic acid. Such activity is detected although the insert is out of frame with respect to the *lacZ* polypeptide. The same was described for the two homologous genes previously reported from aspen and alfalfa [6, 20]. In both cases it was suggested that the inserts have their own active ribosome-binding sites, but the possible signals implicated in this phenomenon were not described. In the nucleotide sequence of both genes it is possible to observe purine-rich sequences located in the equivalent position with respect to the functional Shine-Dalgarno string of the maize sequence and also near methionine residues in the correct frame. In the maize 5′-coding region of the cDNA sequence two purine-rich regions (AGGAGG) can be found, which are identical to the *E. coli* consensus ribosome-binding site. Both are at the correct distance to a corresponding methionine residue located in the coding frame (Fig. 1). A deletion of the cDNA clone located between the two putative ribosome-binding sites was done having the insert also out of frame and in the appropriate orientation with respect to the *lacZ* promoter. With such construction no enzymatic activity was detected in the bacterial extracts. Therefore, only the first Shine-Dalgarno sequence in the $5' \rightarrow 3'$ direction could explain the functional translation of the enzyme. If it is the case, the lack of 18 residues in the *N*-terminal end does not have any apparent effect on the correct activity of the enzyme.

At a high substrate concentration (2–3 mM caffeic acid), both meta- and para-*O*-methylation is detected producing ferulic acid and 3,4-dimethoxycinnamic acid, respectively, as previously reported [8]. With lower amounts of substrate in the reaction mixture (10–50 μM) only an analogous pattern of meta-specific *O*-methylation is observed, identical in both bacterial or plant tissue extracts. This is in agreement with the meta-specificity of the protein and suggests that the same enzyme is acting in both cases. Moreover, in 9-day-old coleoptile extracts (not shown) substantially lower levels of methylation producing ferulic acid were detected, in agreement with the lower expression of the gene observed in this tissue by slot blot analysis. In plant extracts without adding caffeic acid, no methylated compound

is detected, indicating that only low amounts of free caffeic acid are present in plant cells. Therefore, in appropriate conditions ferulic acid is the main product of the reaction, and it seems that in physiological conditions only the single meta-specific *O*-methylation of caffeic acid occurs.

In conclusion, the caffeic acid *O*-methyltransferase gene from maize has been cloned and its sequence and pattern of expression has been obtained. The cloned gene may be a useful system to study the process of lignification in monocotyledonous plants and the control of genes preferentially expressed in the radicular system. The expression of the protein in bacterial extracts may also be useful to study the specificity of the enzyme.

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