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Expression of Class I *O*-Methyltransferase in Healthy and TMV-Infected Tobacco

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Tobacco possesses two distinct classes of *O*-methyltransferases (OMTs; *S*-adenosyl-L-methionine:o-diphenol *O*-methyltransferases; EC 2.1.1.6). Here we report on the cloning and the expression pattern of the class I OMT that is specifically involved in lignin biosynthesis. Near-full-length cDNAs have been isolated from tobacco libraries constructed from leaf and stem poly(A)$^+$ RNA. Sequence analysis demonstrated that the OMT I clones derived from two different mRNA species. The two types of OMT I mRNA were reverse transcribed from total RNA and the cDNAs were amplified by polymerase chain reaction and characterized by restriction analysis. The same proportion of the two transcripts was measured in stem tissue of healthy plants and in leaves reacting hypersensitively to tobacco mosaic virus, indicating a coordinate expression of the two OMT I genes. Consistently, genomic hybridization indicated the presence of two OMT I genes in the amphidiploid genome of tobacco. The pattern of expression of OMT I genes was studied by in situ mRNA hybridization. In stem, petiole, and root tissues, OMT I genes were found to be specifically expressed in vascular cells and epidermis. In healthy leaves OMT I mRNA was only detected in vascular strands, whereas, in leaves bearing tobacco mosaic virus–induced necrotic lesions, a particularly strong accumulation of the labeling was also localized in the upper and lower epidermis.

Additional keywords: Nicotiana tabacum, RT-PCR.

The biological functions of phenylpropanoid derivatives in plants are highly diverse (Legrand 1983; Ride 1983; Hahlbrock and Scheel 1989). Phenylpropanoids serve as pigments, antibiotics, and UV protectants; they also function as complex polymeric constituents of surface and support structures including lignin. In vascular plants, lignin is deposited mainly in the middle lamella and in the secondary cell wall and provides rigidity (Fahn 1982; Monties 1989). Increased lignification has been often observed in response to infection (Kimmins and Wuddah 1977; Vance et al. 1980; Ride 1983; Legrand 1983) and is believed to participate in parasite confinement because lignin represents an undegradable barrier for most microorganisms.

Lignin originates from the oxidative polymerization of hydroxylated cinnamyl alcohols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Gross 1977). The two latter monolignols bear methoxyl groups that are introduced by *O*-methyltransferases (OMTs), which are regulatory enzymes controlling lignin content and composition. Variations in lignin composition are observed in the plant kingdom, depending on species, tissue, developmental stage, and subcellular location (Lewis and Yamamoto 1990).

In Samsun NN tobacco leaves reacting hypersensitively to tobacco mosaic virus (TMV), the activity of the enzymes of the phenylpropanoid pathway is strongly increased (Legrand et al. 1976; Collendavelloo et al. 1983). Three isoforms of OMT (OMT I, II, and III) have been purified to homogeneity (Hermann et al. 1987). OMT I, the major enzyme of healthy plants, is preferentially expressed in vascular tissues (Jaeck et al. 1992). OMT I is specific for phenylpropanoid-type substrates, whereas OMTs II and III, which are barely detectable in healthy tissues, have a broader substrate specificity (Collendavelloo et al. 1981). Upon TMV infection, the three OMT isoforms are strongly stimulated (Legrand et al. 1978; Collendavelloo et al. 1983). These features suggest a role in lignification and defense for OMT I and a specific function in generating ligninlike barriers in infected tissues for OMTs II and III. Recently, OMT clones have been isolated and shown to belong to two distinct classes of OMT genes: class I genes code for OMT I and class II for enzymes II and III (Jaeck et al. 1992; Pellegrini et al. 1993).

It appears, therefore, that the regulation of the lignin biosynthetic genes must integrate specific developmental cues with the pathogenic stress signals perceived upon infection. To study such a complex pattern of expression, class I OMT genes may serve as an useful model. Recently, the analysis of lignin of transgenic tobaccos in which OMT I gene expression had been inhibited has indicated that OMT I controls the synthesis of sinapic acid from 5-hydroxy ferulic acid (Atanassova et al. 1995) Here we report the analysis of full-length OMT I cDNAs isolated from stem and leaf cDNA libraries and demonstrate that the clones are derived from two distinct messengers. Consistently, two OMT genes of class I were detected in the tobacco genome by Southern analysis. Individual expression of OMT I genes was investigated by reverse transcription polymerase chain reaction (RT-PCR) coupled to restriction

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Nucleotide and/or amino acid accession data can be found at EMBL database numbers X74452 (for OMT I-a) and X74453 (for OMT I-b).

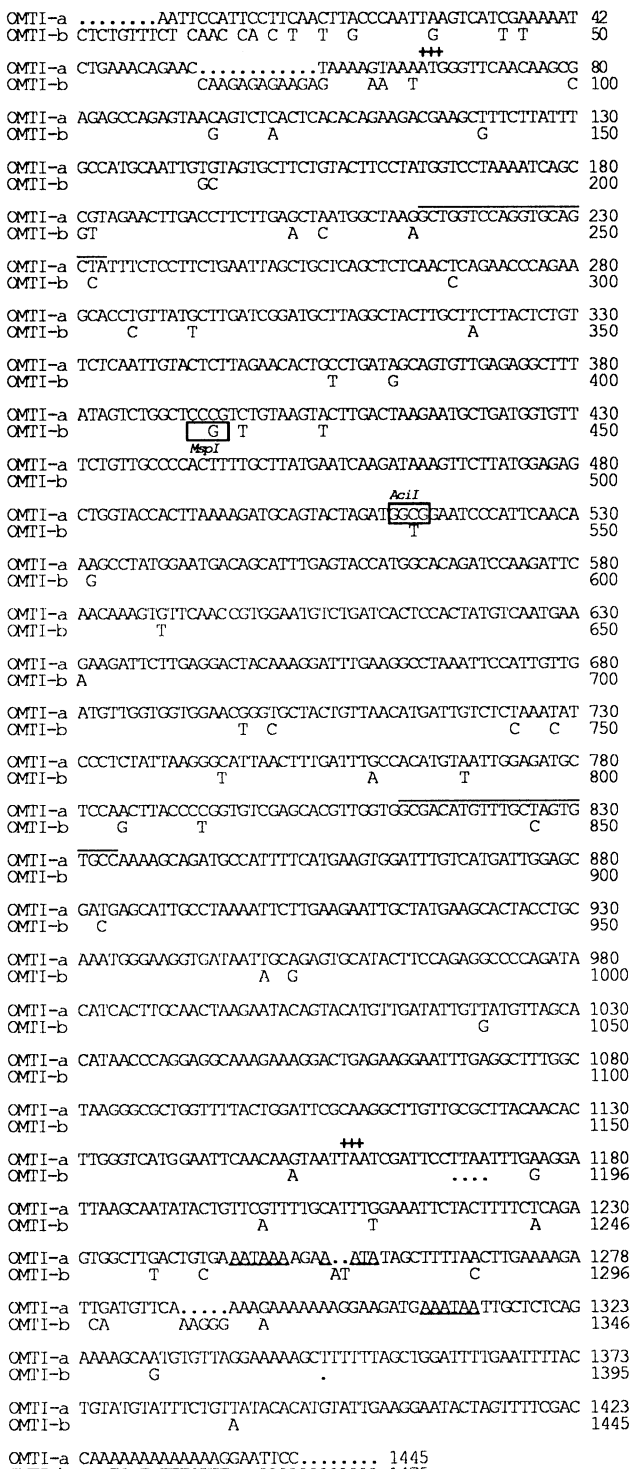

Fig. 1. Nucleotide sequence comparison of *O*-methyltransferase (OMT) I-a and OMT I-b cDNAs. Sequences were aligned and are identical except where indicated; • indicates base missing in OMT I-a or I-b ; +++ denotes translation start and stop; putative polyadenylation signals are underlined; sequence of primers used for polymerase chain reaction is overlined and position of *Aci*I and *Msp*I sites specific to each sequence is indicated.

analysis of PCR products. The two genes were demonstrated to be expressed at a constant ratio in stem tissues and in TMV-infected leaves of tobacco. In situ hybridization experiments demonstrated that OMT I gene expression is preferentially localized in the epidermis, the vascular bundles of stems and petioles, and the tracheary elements of roots. In healthy leaves, OMT I messengers were shown to accumulate in small and large vascular strands. Upon infection of leaf tissues, an additional accumulation of OMT I transcripts was found in the lower and upper epidermis and in midvein parenchyma cells.

RESULTS

Cloning of OMT I cDNAs.

Through the use of primers based on partial sequences of OMT I cDNA and protein (Jaeck et al. 1992) a 620-bp fragment was amplified by PCR from RNA isolated from TMV-infected leaves. After subcloning and sequencing, the fragment was used to screen a tobacco stem cDNA library. Four clones were isolated out of 300,000 and their insert size, measured after amplification by PCR (Rasmussen et al. 1989), was found to be approximately 1.5 kb. Results from partial sequencing were consistent with a unique sequence for all clones. For one clone the complete DNA sequence was determined and is presented in Figure 1 (OMT I-a). Comparison of the OMT I-a sequence with the 620-bp fragment obtained by RT-PCR from infected leaf RNA revealed 19 nucleotide differences (data not shown), suggesting the occurrence of two distinct messengers in stem and leaf (see below). A cDNA library constructed from infected tobacco leaf poly(A)$^+$ RNA (Pellegrini et al. 1993) was screened with a probe synthesized from the OMT I-a sequence and seven additional OMT I cDNA clones were identified. After amplification of the inserts by PCR, five clones were identified as OMT I-a cDNA according to their pattern of digestion, whereas the two others showed a different restriction pattern. One of the latter was sequenced and its nucleotide sequence is shown in Figure 1 (OMT I-b). The OMT I cDNA clones contain the complete open reading frames but have a size shorter than that of the mRNA estimated on Northern (RNA) blots. This may indicate that some of the 5′ untranslated sequences are missing.

Sequence analysis and comparison with OMTs of other organisms.

The two nucleotide sequences are presented in Figure 1 and share 64, 97, and 86% similarity in 5′ noncoding, coding, and 3′ noncoding regions, respectively. Both OMT I cDNAs encode a 364 amino acid protein (calculated M_r 39,437 for OMT I-a and 39,342 for OMT I-b). Three putative polyadenylation sites were found (Fig. 1).

The protein sequences deduced from the cDNAs of OMT I-a and OMT I-b show 97% identity and are presented in Figure 2. They have about 75% identity with other dicotyledon OMTs cloned from poplar (Dumas et al. 1991), aspen (Bugos et al. 1991), and alfalfa (Gowri et al. 1991), indicating that these sequences originated from closely related genes. Moreover, tobacco OMT I sequences, like other plant OMTs (Bugos et al. 1991; Collazo et al. 1992; Pellegrini et al. 1993), show five regions (indicated in Figure 2) that are conserved in animal and bacterial OMTs displaying different phenolic substrate specificities. Regions I, III, and IV have been shown to

be present in many enzymes requiring *S*-adenosyl-L-methionine as a substrate and may be structurally or evolutionarily related (Ingrosso et al. 1989).

Genomic Southern blot analysis.

Total genomic DNA of tobacco was restricted with enzymes that have (*Eco*RI) or have not (*Eco*RV, *Xba*I) recognition sequence within the OMT I cDNA, and was analyzed by Southern blotting (Fig. 3). Only two bands were revealed after digestion with *Xba*I alone (lane 4) or with *Xba*I and *Eco*RV enzymes together (lane 2), suggesting the occurrence of two class I OMT genes in the tobacco genome. This contrasts with what was found for class II OMT genes, which were demonstrated to be organized as a small multigene family of four to six members (Pellegrini et al. 1993). After *Eco*RI digestion, up to four bands of variable intensity were detected (lane 1), in accordance with the presence of an *Eco*RI site in the OMT I coding sequence. Under the high stringency conditions used, a probe derived from a class II OMT cDNA (courtesy L. Pellegrini) hybridized different DNA fragments (data not shown), thus demonstrating the absence of cross-hybridization between class I and class II genes.

Genomic DNA purified from the two ancestral relatives of *N. tabacum, N. sylvestris* and *N. tomentosiformis,* was also restricted with *Xba*I and submitted to Southern blotting (Fig. 3, lanes 3 and 5). In each case, one band was revealed whose molecular size was similar to that of one of the two DNA fragments detected on the tobacco blot (lane 4). These findings indicate that tobacco has inherited one OMT I gene from each of its two parent species.

Expression pattern of tobacco OMT I genes.

By Northern blot hybridization, the OMT I probe has been shown to detect a single mRNA species of 1.7 kb in leaf and stem tissue of tobacco (Jaeck et al. 1992). The high degree of similarity over the whole OMT I-a and OMT I-b sequences precludes the synthesis of specific probes. Therefore, the study of individual expression of OMT I-a and OMT I-b genes

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OMTI-a MGSTSESQSNSLTHTEDEAFLFAMQLCSASVLPMVLKSAVELDLLELMAK 50
OMTI-b     Q  K                 A           L

OMTI-a AGPGAAISPSELAAQLSTQNPEAPVMLDRMLRLLASYSVLNCTLRTLPDS 100
OMTI-b                          I        T          S G

OMTI-a SVERLYSLAPVCKYLTKNADGVSVAPLLLMNQDKVLMESWYHLKDAVLDG 150
OMTI-b             F

OMTI-a GIPFNKAYGMTAFEYHGTDPRFNKVFNRGMSDHSTIMSMKKILEDYKGFEG 200
OMTI-b
           I                  II
OMTI-a LNSIVDVGGGTGAIVNMIVSKYPSIKGINFDLPHVIGDAPTYPGVEHVGG 250
OMTI-b                     H                A
                        III              IV
OMTI-a DMFASVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVIIAECIL 300
OMTI-b
                                  V
OMTI-a PEAPDTSLATKNIVHVDIVMLAHNPGGKERTEKEFEALAKGAGFTGFARL 350
OMTI-b

OMTI-a VALTTLGSWNSTSN 365
OMTI-b           N
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Fig. 2. Comparison of aminoacid sequences encoded by *O*-methyltransferase (OMT) I-a cDNAs. All residues of OMT I-a are shown. OMT I-b residues are identical except where indicated. Sequences conserved in OMTs of different origin are overlined.

was carried out by RT-PCR coupled with restriction analysis of the products (Becker-André and Hahlbrock 1989). The 620-bp amplified region includes two unique restriction sites, one (*Aci*I) specific to OMT I-a cDNA, the other (*Msp*I) only present in OMT I-b cDNA (see Figure 1). The restriction products were analyzed by electrophoresis on agarose gels, blotted, and hybridized with the radioactive probe. The autoradiogram is presented in Figure 4. The experiment was performed with total RNA from TMV-infected tobacco leaves (Fig. 4A) or tobacco stem (Fig. 4B). Increasing amounts of RNA were reverse transcribed and amplified to verify if the method was quantitative under the conditions used. An increasing intensity of the signal was observed (Fig. 4C) and the evaluation of the radioactivity bound to the nylon membrane by scintillation counting demonstrated a linear relationship between the RNA amounts and the radioactivity of the products (data not shown). Total RNA (0.5 µg; Fig. 4C, lane 2) extracted from leaves infected with TMV for 40 h (Fig. 4A) or from tobacco stem (Fig. 4B) was used as template for RT-PCR. The radioactivity of the products obtained with stem RNA (Fig. 4B) was twice as high as that of the DNA amplified from TMV-infected leaf RNA (Fig. 4A) and these findings confirm those

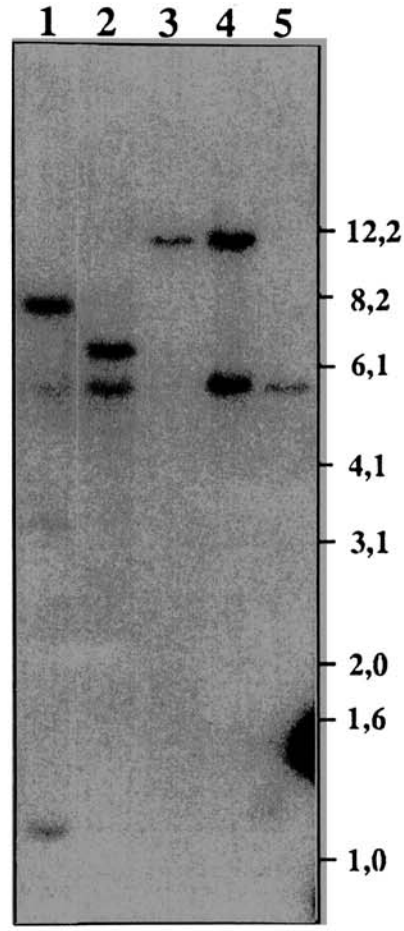

Fig. 3. Southern blot analysis of genomic DNA from different *Nicotianae*. *N. tabacum* DNA (10 µg) was digested with restriction enzymes *Eco*RI (lane 1), *Eco*RV/*Xba*I (lane 2), or *Xba*I (lane 4). Genomic DNA (5 µg) from *N. sylvestris* (lane 3) or *N. tomentosiformis* (lane 5) was digested with *Xba*I. Probe was synthesized from the *O*-methyltransferase I-a cDNA clone. Molecular marker sizes are in kilobases.

obtained previously by RNA blot analysis (Jaeck et al. 1992). Concerning the individual expression of OMT I-a and OMT I-b genes, essentially the same results were obtained with leaf or stem transcripts: when the PCR products were digested with one single enzyme (Fig. 4, Aci or Msp lane) the bands corresponding to the cleavage products of the OMT I cDNA that contains the restriction site are revealed, along with an undigested product corresponding to the other type of OMT cDNA. As expected, the PCR products were digested to completion by a double digestion (Fig. 4, Aci + Msp lane). By counting the radioactivity of the digested and undigested products, a 3/2 ratio between OMT I-a and OMT I-b cDNA amounts was found with either source of RNA (stem or leaf). Since the experiment was conducted under linear conditions this demonstrates that both types of transcripts were present in a constant ratio and indicates that the two OMT I-a and OMT I-b genes of tobacco are co-regulated in these tissues. A similar relative level of expression has been reported for the two genes encoding the tobacco microbial proteinase inhibitors (Heitz et al. 1993) and may reflect a general regulatory strategy of expression of the allotetraploid genome of tobacco.

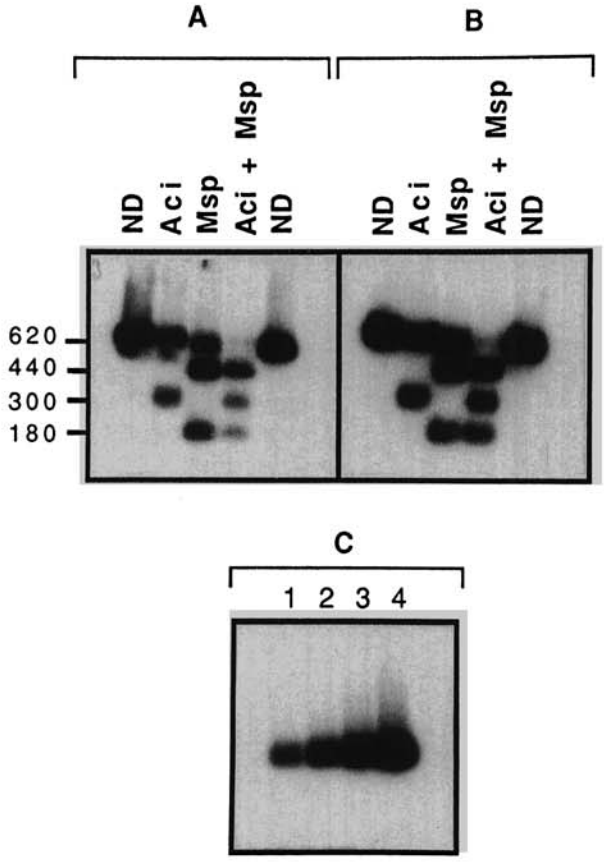

Fig. 4. Analysis of polymerase chain reaction (PCR) products obtained after reverse transcription of total RNA. Total RNA (0.5 µg) from tobacco mosaic virus–infected leaves (**A**) or healthy stems (**B**) was used as template. PCR products were digested either with *Aci*I (Aci) or *Msp*I (Msp) or double digested (Aci + Msp) or not digested (ND). *Aci*I and *Msp*I restriction sites are specific for *O*-methyltransferase I-a and I-b PCR products, respectively (see Fig. 1). In **C**, 0.25 µg (1), 0.5 µg (2), 1 µg (3), or 2 µg (4) of RNA was reverse transcribed. After blotting, hybridization was carried out with a radioactive probe synthesized from the 620-bp PCR product.

In situ hybridization studies.

Studies by RT-PCR and Northern blot analysis (Jaeck et al. 1992) have shown that OMT I mRNAs accumulate in vascular tissue of healthy plants and in leaves bearing lesions induced by virus infection. The spatial distribution of OMT I transcripts within these tissues was examined by in situ hybridization. Sense and antisense riboprobes were synthesized from the 620-bp OMT cDNA fragment obtained by PCR (see Methods).

When a sense probe was used as control, no significant signal was detected as it is in the case of the leaf and petiole cross sections (Fig. 5I,O) shown as examples. In contrast, when the sections were hybridized with the antisense probe a specific labeling was observed in various plant tissues.

In tobacco stem, whose anatomy is shown in a cross section stained with toluidine blue (Fig. 5A), an intense labeling was revealed in the external cortical cells and vascular tissues (Fig. 5B,C). At the highest magnification, it appeared that OMT mRNA accumulated in the young xylem cells but was not detectable in phloem cells (Fig. 5C). Cellular distribution of OMT I mRNA was also studied in vascular tissues of the tobacco petiole whose anatomy is shown in Figure 5D. Cross sections hybridized to antisense OMT I probe revealed the accumulation of transcripts in epidermis cells and in vascular tissues (Fig. 5E). A close-up (Fig. 5F) showed an intense labeling in xylem cells, internal and external phloem cells, and small vascular bundles of the petiole.

In leaf tissues, labeling was detected in the small vascular bundles of the limb (Fig. 5G) and also in the vascular bundles of the midvein (Fig. 5H). When OMT mRNA distribution was examined in TMV-infected leaf tissues, OMT transcripts were found in vascular bundles, as was also observed in healthy material, but an additional accumulation was found in upper and lower epidermis and in parenchyma cells of the midvein (Fig. 5J,K). A close-up of vascular bundles of the midvein (Fig. 5L) showed an intense labeling of external and internal phloem cells. Thus, a larger number of cells accumulate OMT transcripts in infected tissues than in healthy leaf, in accordance with the stimulation of OMT I gene expression in tobacco leaves upon infection (Legrand et al. 1978; Jaeck et al. 1992). However, in the particular case of TMV-infected leaves it is important to mention that class II OMT genes are also expressed upon infection (Pellegrini et al. 1993) and some cross-reactivity of the probe with these transcripts cannot be completely excluded. However, this is rather unlikely because, as already indicated, no cross hybridization between class I and class II OMT genes was observed on Southern blots and because a different pattern of mRNA accumulation (with no labeling of the epidermis, for instance) was revealed by hybridizing cross sections of infected leaves with a class II OMT probe (L. Pellegrini, unpublished). Finally, OMT I gene expression was detected in the epidermis and in the xylem tracheary elements of root tissues (Fig. 5 M,N).

DISCUSSION

Two distinct cDNAs encoding tobacco OMT I, a lignification-related enzyme, have been isolated. Their coding regions are 95% identical and have also about 75% identity with OMTs cloned from other angiosperms, namely poplar (Dumas et al. 1991), aspen (Bugos et al. 1991), and alfalfa (Gowri et

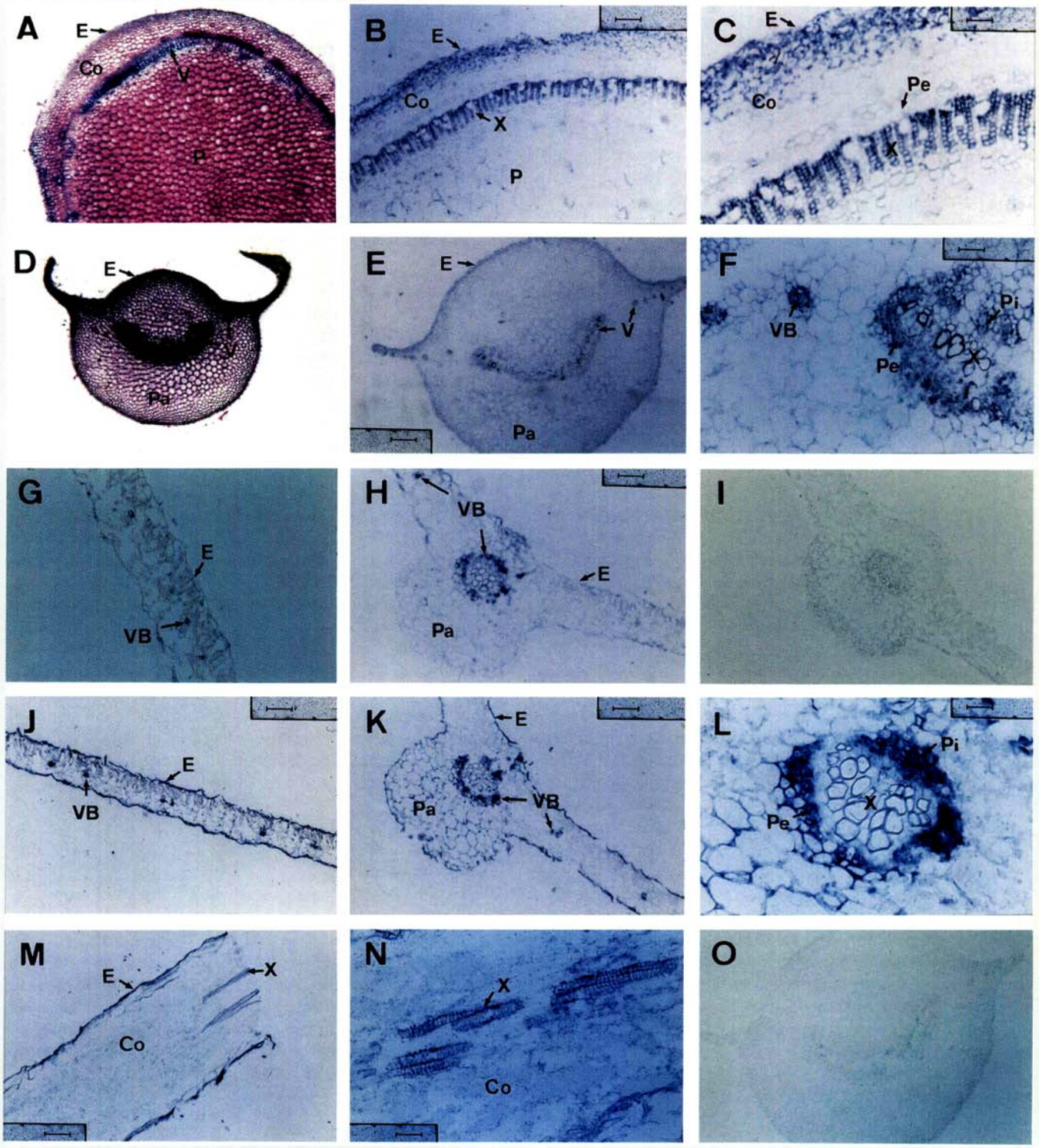

Fig. 5. In situ localization of *O*-methyltransferase I mRNAs in leaves, petioles, stems, and roots of healthy tobacco and in tobacco mosaic virus–infected leaves. **A** and **D,** stained with toluidine blue. **B, C, E, F, G, H, J, K, L, M,** and **N,** hybridized with antisense digoxigenin-labeled riboprobe. **I** and **O,** controls hybridized with sense digoxigenin-labeled riboprobe. **A,** Stem cross section stained with toluidine blue to show cellular anatomy (×25). **B,** Stem cross section (×100). **C,** Stem cross section (×250). **D,** Petiole cross section stained with toluidine blue to show cellular anatomy (×20). **E,** Petiole cross section (×40). **F,** Petiole cross section showing large and small vascular bundles (×250). **G,** Cross-section of healthy limb tissues (×250). **H,** Cross section of limb and the midvein of healthy leaf (×160). **I,** Cross section of infected leaf tissues (control) (×160). **J,** Cross section of infected leaf limb tissues (×160). **K,** Cross section through limb and midvein of infected leaf (×160). **L,** Cross section of infected leaf midvein (×1250). **M,** Longitudinal root section (×400). **N,** Close-up of root tracheary elements (×1250). **O,** Petiole cross section (control) (×60). Abbreviations: Co, cortex; E, epidermis; P, pith; Pa, parenchyma; Pi, inner phloem; Pe, outer phloem; V, vascular tissue; VB: vascular bundle; X, xylem.

al. 1991), and about 55% identity with OMT of maize, a monocotyledon (Collazo et al. 1992). Genomic blot analysis showed the presence of two OMT I genes in the tobacco genome, each one being most probably inherited from one of the two ancestral relatives of the present-day *N. sylvestris* and *N. tomentosiformis*. Two different OMT I transcripts were also characterized by RT-PCR coupled with restriction analysis of the products. The two OMT I genes were shown to be co-expressed in healthy stem and infected leaf in a constant ratio.

We have studied the pattern of OMT I mRNA accumulation by in situ RNA hybridization. In TMV-infected leaves, a high concentration of OMT I transcript was found to accumulate in epidermal cells. Thus, these cells appear to actively synthesize methylated phenolics, which are thought to participate in plant defense. Some of these phenolics are soluble and have been identified as coumarins (Fritig et al. 1972), others are insolubilized in the cell walls but their structure differs from that of lignin (Borg-Olivier et al. 1992).

A similar epidermal-specific localization was observed in parsley leaves for mRNAs encoding phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase and chalcone synthase. These enzymes are regulated by light and are involved in the synthesis of flavonoids that serve as UV-protective agents (Schmelzer et al. 1988). In contrast, PAL, CoA ligases, and other defense-associated genes were shown to be expressed in different cell types of parsley leaves hypersensitively reacting to a nonpathogen fungus (Schmelzer et al. 1989). A uniform distribution of PAL transcripts throughout the leaf tissue was also reported in the case of potato infected with compatible or incompatible races of *Phytophthora infestans* (Cuypers et al. 1988). A similar localization was found for a β-1,3 glucanase induced upon infection in the same host-pathogen system, whereas a quite different picture was obtained for a chitinase that is predominantly constitutively expressed in the epidermis of young potato leaves (Kombrink et al. 1993).

In tobacco, basic isoforms of β-1,3 glucanase and chitinase are localized in epidermal cells of untreated leaves (Keefe et al. 1990). Ethylene treatment was shown to induce a marked, coordinated accumulation of the hydrolases and to change their cell-type-specific distribution: an additional accumulation of these enzymes was found in the mesophyll cells of treated leaves (Keefe et al. 1990). In contrast, no OMT I mRNA was detected in epidermal cells of healthy leaves but upon infection gene expression was revealed in different cell types of the leaf, indicating that most of the leaf tissue is involved in the defense reaction.

Genes involved in the biosynthesis of lignins are actively expressed in vascular tissues, a major site of lignin deposition (Monties 1989). This has been demonstrated by RNA blot analysis in the case of OMTs (Bugos et al. 1991; Jaeck et al. 1992; Collazo et al. 1992; Van Doorsselaere et al. 1993) and cinnamyl alcohol dehydrogenase (CAD) (Knight et al. 1992; Grima-Pettenati et al. 1993). Similarly, the analysis of GUS expression under the control of a PAL promoter from bean has shown that this promoter is very active in differentiating xylem and in xylem parenchyma cells of transgenic plants (Liang et al. 1989; Bevan et al. 1989; Leyva et al. 1992). Concerning the methylation steps catalyzed by OMTs, recent progress has been made by the analysis of transgenic plants with low level of OMT activity (Atanassova et al. 1995) and by studying in vitro differentiating *Zinnia elegans* cells (Ye et al. 1994). An OMT specific for the caffeoyl-coA ester (CCoA-OMT) has been implicated in the first methylation step leading to guaiacyl (G, monomethoxylated) unit of lignin. By tissue print hybridization, Ye and Varner (1995) have shown that CCoA-OMT and OMT I genes are differentially expressed in lignifying *Zinnia* cells, in accordance with the changes in lignin composition that are known to occur during cell differentiation. On the other hand, the inhibition of OMT I gene expression in transgenic tobaccos has been shown to result in a deep decrease in syringyl (S, dimethoxylated) unit content of lignin and in the appearance of a new unit (OH-G) derived from 5-hydroxyferulic acid (Atanassova et al. 1995). These results demonstrate that OMT I controls the second methylation step leading to S unit synthesis. In the present study, OMT I transcripts were shown to accumulate mainly in the epidermis and immature xylem elements of different organs of the plant. In vascular bundles of midribs and petioles, OMT I gene expression was also found associated with internal and external phloem cells. Thus, it appears that distinct cell types are specifically and actively involved in the biosynthesis of syringyl units that are incorporated into the secondary cell walls of lignifying cells (Monties 1989).

PAL transcripts have been shown to accumulate in leaf cells surrounding necrotic lesions and in vascular tissues (Pellegrini et al. 1994). Thus, PAL and OMT genes appear to be expressed in the same cells. In fact, an integrated spatial and temporal program of expression of the whole set of genes encoding lignin biosynthetic enzymes is necessary to regulate phenolic synthesis and to make them available for lignification or defense reactions. By modulating gene expression in transgenic plants it is now feasible to manipulate the phenylpropanoid pathway in order to (i) determine which are the control steps in the metabolism, (ii) unravel how infection affects cell specificity of gene expression, and (iii) investigate the role of phenolics in the impediment of cell-to-cell virus movement in hypersensitively reacting hosts.

MATERIALS AND METHODS

Biological materials.

Experiments were performed with 3-month-old tobacco plants (*Nicotiana tabacum* L. Samsun NN) that were grown in an air-conditioned greenhouse at 22 ± 2°C. For infection with TMV, the first two fully expanded leaves at the top of each plant were inoculated by rubbing with an aqueous suspension of purified TMV (wild-type strain) in the presence of sand. The inoculated plants were incubated in a growth chamber at 22 ± 1°C (16-h photoperiod); under these conditions, local lesions appeared 34 to 36 h after inoculation.

PCR.

The synthesis of the OMT probe was performed by PCR after reverse transcription of total RNA. The antisense primer (ATGGATCCGGCACACTGGCAAACATGTCGC) was derived from the sequence of a partial OMT I cDNA clone (Jaeck et al. 1992) and the sense primer (GCIGGICCIGGIGC IGCIAT) was derived from the sequence of a peptide (peptide 41, AGPGAAISPSELAAQL) isolated after trypsin digestion of the purified OMT I protein (Jaeck et al. 1992). For amplification of the cDNA, the PCR reaction was carried out fol-

lowing conditions previously described (Jaeck et al. 1992), with 2 µg of first-strand cDNA and 0.2 µM of each primer. A 620-bp fragment was obtained, authenticity of this fragment was confirmed by sequencing, and an internal peptide of OMT I, peptide 43 (Jaeck et al. 1992), has been found to match a portion of the 620-bp sequence. Moreover, this sequence is contained in the OMT I-b sequence (see Figure 1). Subcloning was performed in the *Sma*I site of plasmid pKS (Stratagene, Cambridge, UK) according to standard methods.

For the study of gene expression by PCR, the same conditions were used except that the number of cycles was reduced to 22. After extraction with phenol/chloroform (vol/vol) and chloroform, PCR products were precipitated by the addition of 2 vol of cold absolute ethanol in the presence of 200 mM ammonium acetate, pH 4.5. The pellet was washed with 70% ethanol, lyophilized, and dissolved in 25 µl of sterile, distilled H_2O. Five microliters of the PCR products was digested with *Aci*I and *Msp*I enzymes (Biolabs, Hitchin, UK) in 20 µl for the simple digestion (3 h at 37°C) and 40 µl for the double digestion (3 h at 37°C for each enzyme). The digested products were separated on agarose gel (1%), blotted onto Hybond N^+ nylon membrane (Amersham, Little Chalfont, UK); hybridization and washing were carried out according to the manufacturer's instructions. Digested PCR products were detected with the 620-bp fragment ^{32}P-radiolabeled by random priming (Feinberg and Vogelstein 1983).

Library screening.

The λ ZAP II cDNA libraries of tobacco stem (300,000 plaques) and infected tobacco leaf (10^6 plaques) were plated on NZY (NZ amine and yeast extract) media at a density of ~25,000 plaques on a 140 mm petri dish with *Escherichia coli* XL1-blue (Stratagene) as the host strain. The stem cDNA library was kindly provided by W. Schuch (Zeneca Seeds, Bracknell, UK) and the leaf library was constructed by L. Pellegrini (Pellegrini et al. 1993). Duplicate plaque lifts were performed with nitrocellulose filters (Millipore, Molsheim, France). The filters were hybridized at 42°C with an OMT I probe ^{32}P-labeled by random priming (2.5 × 10^6 dpm/ng DNA) (Feinberg and Vogelstein 1983) in 50% formamide (vol/vol), 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution (Sambrook et al. 1989), and 1 mg of denatured salmon sperm DNA per ml, and washed at 42°C in 2× SSC, 0.1% SDS for 10 min, in 1× SSC, 0.1% SDS for 30 min, and in 0.1× SSC, 0.1% SDS for 15 min. Clones hybridizing to the probe were purified and in vivo excision of the plasmid pSK was carried out with R408 helper phage (Stratagene). Size analysis was performed by PCR according to Rasmussen et al. (1989). The plasmid harboring the longest insert was purified on Qiagen columns and sequenced on both strands by the dideoxynucleotide method (Sanger et al. 1977) with M13 universal primers or cDNA specific primers (17 mers).

In situ hybridization.

Experiments were carried out with 3-month-old tobacco plants, *Nicotiana tabacum* L. Samsun NN. Two- to 3-mm sections of tissues from leaves, stem, petiole, and root were fixed in 3:1 ethanol/acetic acid for 30 min at room temperature. Once the fixative was removed, the samples were stored in 70% ethanol at 4°C. The tissues were embedded in paraffin and 8-µm sections were prepared as described by Langdale (1993). Sense and antisense riboprobes synthesized from 1 µg of linearized DNA template were labeled with digoxigenin-dUTP according to the manufacturer's instructions (Amersham). The transcripts were synthesized in vitro from the 620-bp OMT I fragment obtained by PCR. Pretreatment, hybridization, and detection of samples were carried out as described by Langdale (1993). Labeled hybrids were detected by immunoreaction with anti-digoxigenin antibody conjugated to alkaline phosphatase. The antibody was diluted 1,000-fold in 100 mM Tris-HCl pH 7.5, containing 400 mM NaCl, and 0.5% serum albumin (fraction V, Sigma). The incubation with the antibody was performed for 1 to 4 h. Tissues were then washed for 3 × 10 min with the solution used for antibody dilution. Enzymatic incubation was carried out overnight according to the manufacturer's (Amersham) protocol. Photographs were taken with brightfield microscopy and Ektachrome 160 ASA film.

Southern analysis.

DNA was isolated from young leaves of tobacco following the procedure of Bingham (1981) with some modifications in the final steps described by Heitz et al. (1993). Genomic DNA was digested with restriction endonucleases, separated by size in a 0.8% agarose gel, and blotted onto a nylon membrane (Hybond N, Amersham). Hybridization and washing were carried out following the manufacturer's recommendations. OMT I genes were detected with an OMT I-a cDNA probe ^{32}P-labeled by random priming (Feinberg and Vogelstein 1983).

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