Regulation of Enzymes Involved in Lignin Biosynthesis: Induction of O-Methyltransferase mRNAs During the Hypersensitive Reaction of Tobacco to Tobacco Mosaic Virus

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The mRNAs encoding orthodiphenol-O-methyltransferases (OMTs; EC 2.1.1.6), which are involved in the biosynthesis of lignin precursors, are highly induced in tobacco leaves during the hypersensitive reaction to tobacco mosaic virus (TMV). OMT messengers were fractionated on a sucrose gradient and translated *in vitro*. Protein A-Sepharose columns adsorbed with specific antisera raised against purified OMTs were used to select translation products, and the translatable activity of OMT mRNA was measured at different stages of infection. Oligonucleotides derived from peptide sequences of purified OMT I were used to prime polymerase chain reactions; total RNA was used as template to allow the isolation of an OMT I clone. RNA blots, hybridized with the OMT I probe, revealed a unique messenger of 1.7 kb. The kinetics of accumulation of OMT I mRNAs during the hypersensitive reaction to TMV parallels the kinetics of translation and suggests that an increase in mRNA controls the increase in the rate of enzyme synthesis. In healthy plants, RNA blot hybridization showed that the steady-state level of OMT I mRNA is very high in vascular tissue compared to the level measured in leaves.

Additional keywords: cDNA cloning, *Nicotiana tabacum*, PCR.

In plants, the phenylpropanoid pathway provides common precursors for isoflavonoids, coumarins, stilbenes, lignins, insoluble esters, and various forms of other phenolics (Legrand 1983; Ride 1983; Hahlbrock and Scheel 1989; Lewis and Yamamoto 1990). These compounds are derived from phenylalanine and have often been implicated in plant resistance to pathogens. Many of them are antibiotics termed phytoalexins (for review see Bailey and Mansfield 1982). In addition, lignification or esterification of phenolics participates in cell wall thickening, which is a common response to infection (Vance et al. 1980; Ride 1983; Legrand 1983). The deposition of lignin or ligninlike compounds in the cell wall might interfere with fungal penetration, because these polymers represent an undegradable mechanical barrier for most fungi (Vance et al. 1980; Ride 1983; Lewis and Yamamoto 1990). In the case of virus infection, phenylpropanoid metabolism is highly activated (Fritig et al. 1972; Legrand et al. 1976), and an increased deposition of phenolics in the cell wall has also been implicated in the localization of virus close to the penetration site (Kimmins and Wuddah 1977; Massala et al. 1987).

Lignin is a three-dimensional polymer built from three monomers called monolignols. These three building units are p-coumaryl, coniferyl, and sinapyl alcohols, which are polymerized in lignin *via* free radical production catalyzed by peroxidases (Gross 1978). Methyl groups, with an important role in free radical stability, are introduced at the level of caffeic and 5-hydroxyferulic acids by O-methyltransferases (S-adenosyl-L-methionine: o-diphenol-O-methyltransferase, OMT; EC 2.1.1.6). The resulting products, ferulic acid and sinapic acid, respectively, are then reduced to the corresponding alcohols. The lignin content and the monomeric composition of lignins can vary with the plant family, the tissue, the developmental stage, and even the subcellular location (Lewis and Yamamoto 1990). OMTs are, therefore, good candidates as regulatory enzymes of lignin biosynthesis.

In tobacco, three different OMTs can methylate caffeic and 5-hydroxyferulic acids (Legrand et al. 1978). The three enzymes were isolated (Hermann et al. 1987) and showed different substrate specificities (Collendavelloo et al. 1981). OMT I, which is present at a steady-state level in healthy plants, displays a strict specificity for phenylpropanoid-type substrates. The other enzymes (OMTs II and III) are barely detectable in healthy material and have a broader substrate specificity. Upon infection by tobacco mosaic virus (TMV), all three enzymes are stimulated (Dumas et al. 1988). These results indicate that OMT I is specifically involved in lignification, whereas the major function of OMTs II and III is to generate ligninlike barriers in infected tissues.

From labeling experiments with deuterated water (Collendavelloo et al. 1983) or radioactive precursors (Dumas et al. 1988), an increase in *de novo* synthesis of the three

OMTs was demonstrated in TMV-infected tobacco leaves. We have undertaken a study of the molecular mechanism regulating the synthesis and activity of tobacco OMTs. Here, we describe the use of specific antibodies directed against the purified enzyme proteins to characterize the *in vitro* translation products of the mRNAs that encode OMTs and to measure the changes in their translatable activity during TMV infection. Oligonucleotides derived from peptide sequences of OMT I were used to prime polymerase chain reactions (PCR); total RNA was used as template. A 698-bp PCR product was used as a probe to measure OMT I transcript accumulation in TMV-infected tobacco leaves. A parallel increase in translatable activity was observed, suggesting that the increase in *de novo* synthesis of OMTs upon infection is regulated at the transcriptional level.

MATERIALS AND METHODS

Plant growth and virus inoculation. Experiments were performed with 3-mo-old tobacco plants, *Nicotiana tabacum* L. 'Samsun NN', which were grown in an air-conditioned greenhouse at $22 \pm 2°$ C. The first two fully expanded leaves at the top of each plant were inoculated by rubbing with an aqueous suspension of highly purified TMV (wild-type strain) in the presence of Celite. The inoculated plants were incubated in a growth chamber at $22 \pm 1°$ C (16-hr photoperiod), and, under these conditions, local lesions appeared 34–36 hr after inoculation.

Preparation and purification of RNA. Total RNAs were extracted from healthy or TMV-infected leaves (20 g) after primary and secondary veins were removed, or from vascular tissues isolated from the stems of healthy plants. RNAs were prepared according to Howell and Hull (1978) with some modifications (Verwoerd *et al.* 1989). After the last extraction with 1 vol of chloroform, the aqueous phase was mixed with 1 vol of 4 M LiCl. RNAs were precipitated overnight and collected by centrifugation. The pellets were dissolved in 250 μl of water, 0.1 vol of 3 M sodium acetate, pH 5.2, was added, and the RNAs were precipitated with 2 vol of ethanol. After centrifugation, the RNA pellets were washed with 70% ethanol and dried. Poly(A)$^+$ RNAs were purified by two cycles on an oligo(dT)-cellulose column (type 7, Pharmacia, Uppsala, Sweden) according to the procedure of Aviv and Leder (1972). The procedure used for poly(A)$^+$ fractionation on sucrose gradients was described by Commère *et al.* (1986).

In vitro **translation of mRNAs and immunoselection of the products.** A cell-free translation system prepared from rabbit reticulocytes (Amersham, Little Chalfont, England) was used for *in vitro* translation. Poly(A)$^+$ RNA was incubated with 10 vol of reticulocyte lysate in the presence of 1 μCi/μl of ^{35}S-L-methionine (900 Ci/mmol; Amersham). The reaction was stopped by the addition of 25 μl of H$_2$O containing 10 mM L-methionine, 50 μg/ml of RNase A (Sigma Chemical Co., St. Louis, MO), and 0.1 M EDTA. The translation products were analyzed by electrophoresis on 10% polyacrylamide gels (5% stacking gel) under denaturing conditions (Laemmli 1970) and were detected by autoradiography. *In vitro* translation products were immunoselected on affinity columns prepared as follows. Five

milligrams of preswollen Protein A-Sepharose CL-4B (Pharmacia) was poured into a plugged pipetman tip (Treff 1000, Switzerland), and 100 μl of a mixture of sera directed against all three OMTs was loaded onto the column. Similarly, preimmune columns were prepared with preimmune sera. The production of preimmune and specific anti-OMT sera has been described (Hermann *et al.* 1987). The columns were equilibrated with 20 mM phosphate buffer, pH 7.6, containing 150 mM NaCl, 3 mM KCl, and 0.05% Tween 20. After centrifugation at 7,500 × g for 30 min, the translation mixture was diluted with 0.5 ml of column equilibration buffer. The solution was loaded onto a preimmune column, and the flow-through was directly loaded onto an anti-OMT column. The columns were washed with 15 ml of equilibration buffer made in 1 M NaCl and dried by centrifugation at 7,500 × g for 5 min. The gel was transferred into Eppendorf tubes and resuspended in 20 μl of H$_2$O containing 4% (v/v) β-mercaptoethanol and 5% (w/v) sodium dodecyl sulfate (SDS). The mixture was boiled for 3 min, and after centrifugation the protein solution was analyzed by gel electrophoresis (Laemmli 1970) after 0.25 μg of each purified OMT as carrier was added.

Preparation of protein extracts. Leaf material (2 g) was ground with a pestle and mortar, in the presence of Polyclar AT (10%), 0.6 g of quartz, and 5 ml of ice-cooled 0.1 M phosphate buffer, pH 7.5, containing 15 mM β-mercaptoethanol. The mixture was filtered through a double layer of cheesecloth, and the filtrate was centrifuged at 20,000 × g for 20 min. The supernatant was desalted by gel filtration on Sephadex G-25 (coarse), and soluble protein was determined by the method of Bradford (1976).

Immunoblotting. The basic procedure of Towbin *et al.* (1979) was used with modifications described previously (Geoffroy *et al.* 1990). The relative intensity of immunoreactive bands was evaluated by scanning blots with a CS-930 densitometer (Shimadzu Co., Kyoto, Japan) under the reflection mode.

Purification of tobacco OMT I, isolation of tryptic peptides, and microsequencing. Protein extract (from about 100 g of leaves) was fractionated successively by precipitation with (NH$_4$)$_2$ SO$_4$, desalting on Sephadex G-25, and anion exchange chromatography on DEAE-cellulose, which separated the three tobacco OMTs (Hermann *et al.* 1987). OMT I fractions were purified by affinity chromatography on adenosine agarose as described by Dumas *et al.* (1988). After the purified protein was concentrated on a Centricon 30 microconcentrator (Amicon, Beverly, MA), it was digested with 1 μg of trypsin in 0.1 M Tris-HCl, pH 8.0, for 4 hr at 37° C. The peptide mixture was loaded on a C4 reversed-phase column equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted by a linear gradient of acetonitrile (0–70% in 0.1% aqueous TFA). Selected peptides were spotted on a precycled Polybrene-coated glass filter and microsequenced with a gas-phase sequenator (Model 470 A, Applied Biosystems, Foster City, CA).

Synthesis of OMT I probes by PCR. PCR was performed following the method of Saiki *et al.* (1985) with some modifications (Frohman 1990; Lee and Caskey 1990).

For first-strand cDNA synthesis, 10 μg of total RNA was heated at 65° C for 3 min, cooled on ice, and incubated in 50 μl of 50 mM Tris-HCl, pH 8.3, containing 75 mM

KCl, 1 mM DTT, 15 mM MgCl$_2$, and 1 mM of each dNTP in the presence of 40 pmol of the antisense primer. Antisense primers were degenerate primers derived from the sequence of peptide 16 (hereafter called antisense 16) (CCG/ATT-IGCIGGIAG/AIGCT/CTCA/GTA) or peptide 43 (antisense 43) (TAIGCIGGIGCA/GTCICCIATIACA/GTGIGG) or from an oligo(dT)-adaptor primer (GCATTC GATCTCGAGGTACC(T)$_{17}$) that contains the ClaI, XhoI, and KpnI recognition sites. After the addition of 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Arlington Heights, IL), the reaction mixture was incubated at 42° C for 2 hr. After the mixture was heated for 5 min at 95° C, it was 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 50 μl of sterile distilled H$_2$O.

For amplification of cDNA, the PCR reaction was performed in 100 μl of buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM MgCl$_2$, 0.01% (w/v) gelatin, 200 μM of each dNTP, and 0.8 μM sense and antisense primers. Different sense primers were used, depending on the procedure used for first-strand synthesis. The different couples of primers were antisense 16 with sense 43, antisense 43 with sense 16, and the oligo(dT)-adaptor with sense 43. After 5 min of denaturation at 94° C, the solution was cooled, and 5 U of Taq DNA polymerase (Beckman, Fullerton, CA) was added. Thirty cycles of amplification were carried out automatically with a step program (94° C, 1 min; 50° C, 2 min; 72° C, 3 min) followed by a 15-min final extension.

RNA blot hybridization analysis. Total RNA was extracted at various times after TMV inoculation and was fractionated (12 μg per lane) on formaldehyde agarose gels as described by Lehrach et al. (1977); an RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used as a size marker. The RNAs were transferred onto Hybond N nylon membrane (Amersham); hybridization and washing conditions were according to the manufacturer's instructions. Tobacco OMT I mRNA was detected with a radioactive probe prepared from the 698-bp OMT I cDNA by random priming (Feinberg and Vogelstein 1983). After various exposure times, autoradiograms were scanned, and band intensities were measured by densitometry in the absorbance mode.

RESULTS

Characterization of OMT mRNAs by immunoselection of the translation products. Previous experiments showed that OMTs are present in minor amounts in tobacco leaves (Hermann et al. 1987). Therefore, OMT mRNAs were expected to be rare, and OMT mRNAs were enriched by fractionation of poly(A)$^+$ RNAs on a sucrose gradient under native conditions that allowed the poly(A)$^+$ RNAs to be translated in vitro without purification. Polypeptides with molecular mass values in the range of OMT subunits (39 kDa for OMT I, 42 kDa for OMT II, and 43 kDa for OMT III; Hermann et al. 1987) were detected by electrophoretic analysis of the translation products of fractions 5, 6, 7, and 8 (data not shown). Therefore, 5 μg of RNA

from each of these fractions was translated in the reticulocyte system, and the products were analyzed on SDS gels after immunoselection on affinity columns adsorbed with specific anti-OMT antibodies. Most of the translation products passed through the columns (Fig. 1, lane NA). In fractions 6, 7, and 8 (A), two adsorbed bands, in which intensity peaked in fraction 7, were detected. No signal was detected from control columns adsorbed with preimmune sera (data not shown). The mobility of the upper band corresponded to an apparent molecular mass of 42 kDa; the lower band corresponded to 39 kDa. These values are similar to those reported earlier for OMT subunits (Hermann et al. 1987). Thus, the immunoselected proteins correspond to the translation products of OMT mRNAs. Moreover, because the molecular masses found for the translation products are similar to those of the purified enzymes, active OMTs are not synthesized from larger precursors.

Translational activity of OMT mRNAs during the hypersensitive reaction. Because OMT mRNAs can be detected by immunoselection of their in vitro products, we studied the changes in the translational activity of OMT messengers during the hypersensitive reaction of tobacco to TMV. Poly(A)$^+$ RNAs were purified from healthy and infected plants, and the translational activities were assayed in vitro. Figure 2 shows the immunoselected translation products obtained with mRNAs from healthy or TMV-infected plants 42 and 56 hr after inoculation. In the case of infected plants, three bands were revealed after autoradiography of the gel: two bands corresponding to OMTs and one additional minor band of 70 kDa. The messenger of the 70-kDa protein, unlike those of OMTs, was detected in healthy material. The 70-kDa protein may be a subunit of phenylalanine-ammonia lyase (the first enzyme of the phenylpropanoid pathway), which has been shown to cross-react with anti-OMT antibodies (N. Favet, unpublished).
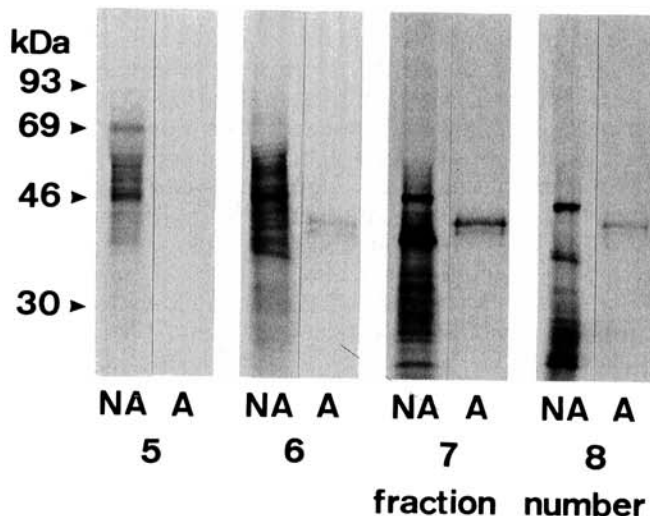

Fig. 1. Immunoselection of tobacco orthodiphenol-O-methyltransferases (OMTs) synthesized in vitro. Five microliters of mRNA from fractions 5, 6, 7, and 8 was translated in vitro in 50 μl of rabbit reticulocyte lysate. In vitro synthesized OMTs were immunoselected on affinity columns (see text). The nonadsorbed products (NA) and the immunoselected products (A) were analyzed on a 10% acrylamide denaturing gel and revealed by autoradiography.

The translational activity of OMT mRNAs was undetectable in healthy plants and was strongly increased during the hypersensitive reaction (Fig. 2). Poly(A)$^+$ RNAs were purified at different times of infection (27, 35, 42, 50, and 56 hr) and translated *in vitro*; the relative amounts of immunoselected translation products were evaluated by measuring the relative intensities of bands on the autoradiogram. Proteins extracted from the same tissues were separated on SDS gels, blotted onto nitrocellulose sheets, and probed with anti-OMT antibodies. After immunospecific staining, the relative amounts of OMTs were evaluated by measuring relative band intensities by reflectance densitometry. The time-course curves of OMT mRNA activity and enzyme synthesis are shown in Figure 3. Translational activity of OMT mRNAs was stimulated just before the appearance of lesions (36 hr post-infection), reached a maximum at about 40 hr, and then decreased. OMT accumulation was detected later and continued unabated until 72 hr post-inoculation. The same kinetics were found for OMT I (Fig. 3A) and for OMTs II and III (Fig. 3B), suggesting that these enzymes are regulated in a similar fashion.

Microsequencing of OMT I and isolation of OMT I cDNAs by PCR. OMT I was digested with trypsin, and the resulting peptides were separated by reversed-phase high-performance liquid chromatography. Figure 4 presents the elution profiles and shows the position of the three OMT I peptides that were microsequenced: peptide 16 (YEALPANGR), peptide 43 (FVLPHVIGDAPAYPG), and peptide 41 (AGPGAAISPSELAAQL). The degeneracy of the genetic code was considered, and mixtures of oligonucleotides, varying in base sequence but with the same number of degenerate bases, were synthesized from peptides 16 and 43 and used to prime the PCR (Frohman 1990). Because the positions of peptides 16 and 43 in the OMT sequence are unknown, both possible couples were tested:
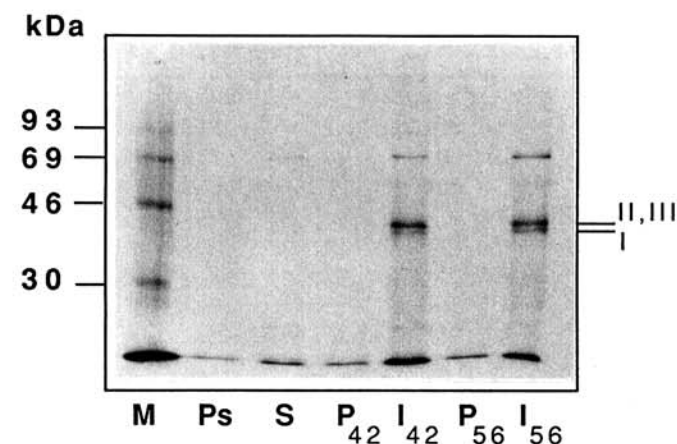
antisense primer 16 with sense primer 43 and antisense primer 43 with sense primer 16. Analysis of PCR products by electrophoresis showed several bands for both couples (Fig. 5, lanes 2 and 3). However, the PCR analyzed with antisense 16 primer and sense 43 primer (Fig. 5, lane 2)
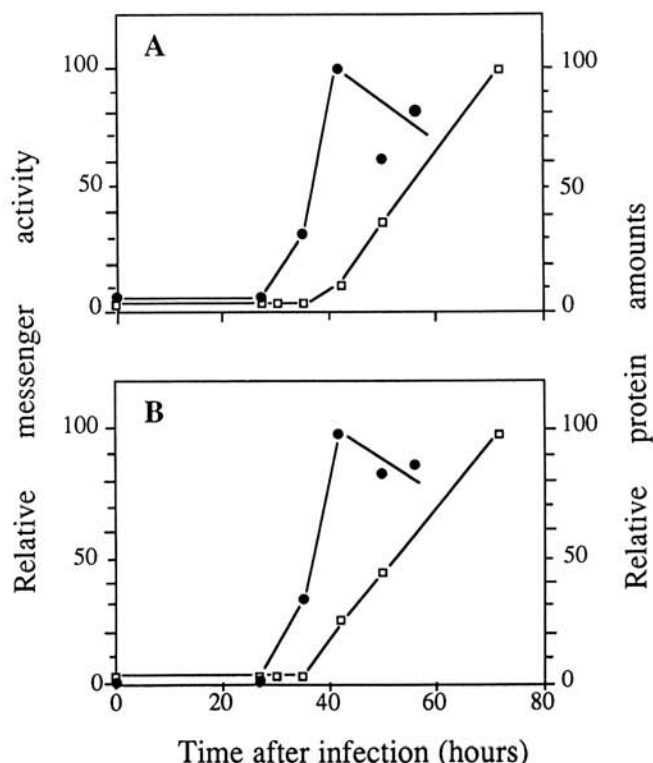
Fig. 3. Time-course of induction of enzymatic proteins and mRNA activities during the hypersensitive reaction of tobacco to tobacco mosaic virus. Messenger RNAs were extracted from healthy (zero time point) and infected leaves after 27, 35, 42, 50, or 56 hr and were translated *in vitro*. Immunoselected products were analyzed by gel electrophoresis and autoradiography. Relative translatable activity of orthodiphenol-*O*-methyltransferase (OMT I) (●-●, A) and of OMTs II and III (●-●, B) was evaluated as described in text. Total leaf proteins were also extracted, separated on sodium dodecyl sulfate gels and blotted onto nitrocellulose. OMT subunits were revealed with OMT antisera, and relative amounts of OMTs were evaluated by densitometry (□-□). A, OMT I; B, OMT II and III.

Fig. 2. Changes in translational activity of orthodiphenol-*O*-methyltransferases (OMTs I, II, III) mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus. Translation products synthesized *in vitro* with 0.5 μg of mRNA from healthy leaves (Ps and S) or leaves infected for 42 hr (P$_{42}$ and I$_{42}$) or 56 hr (P$_{56}$ and I$_{56}$) were immunoselected on affinity columns. Products adsorbed on preimmune antibodies (Ps, P$_{42}$, and P$_{56}$) or selected with anti-OMT antibodies (S, I$_{42}$, and I$_{56}$) were analyzed by electrophoresis on a 10% polyacrylamide denaturing gel and by autoradiography. The molecular masses of markers are indicated on the left (M).
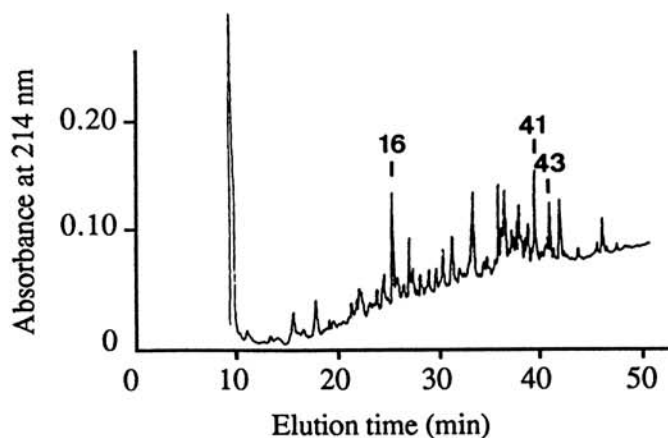
Fig. 4. Reversed-phase high-performance liquid chromatography profile of trypsin-digested orthodiphenol-*O*-methyltransferase (OMT I). The positions of peptides 16, 41, and 43, which were microsequenced, are indicated.

detected a strongly amplified fragment of 176 bp that was not obtained with the other couple of primers (Fig. 5, lane 3). This fragment was purified, subcloned in the *Sma*I site of plasmid pUC9 (Sambrook *et al.* 1989), and sequenced (Sanger *et al.* 1977). The nucleotide sequence and derived amino acid sequence spanned peptide 43 and, thus, identified the PCR product as an OMT cDNA. PCR products obtained with oligo(dT)-adaptor as antisense primer and sense primer 43 were analyzed on agarose gel (Fig. 5, lane 4), blotted onto a membrane, and hybridized with the 176-bp OMT I fragment. A positive fragment of 698 bp was revealed (data not shown), eluted, reamplified under the same conditions, and analyzed (Fig. 5, lane 5). The nucleotide and deduced amino acid sequences are shown in Figure 6; sequences corresponding to those of the sequenced peptides are underlined. Eight of the nine amino acids of peptide 16 are found in the deduced amino acid sequence of the clone, but the ninth is a Lys (position 60; Fig. 6) in place of Arg. This suggests that several OMT I isoforms are present in infected tobacco leaves. The deduced amino acid sequence of the tobacco OMT I appears highly homologous (75–80%) to those of poplar (Dumas *et al.* 1992), alfalfa (Gowri *et al.* 1991), and aspen OMTs (Bugos *et al.* 1991). These enzymes all methylate phenylpropanoid substrates that are precursors of lignin. Our unpublished results indicate a weaker similarity between these enzymes and tobacco OMTs II and III, which have a broader range of *o*-diphenolic substrates. The OMT I cDNA was used to probe OMT I transcripts in northern blot experiments.

OMT I accumulation during the hypersensitive reaction. RNA blot analysis (Fig. 7) was carried out on total RNAs extracted from different tissues. The OMT I probe hybridized specifically to a single mRNA species of about 1.7 kb present in variable amounts in the different samples. This value is similar to that of OMT mRNAs of poplar (Dumas *et al.* 1992), aspen (Bugos *et al.* 1991), and alfalfa (Gowri *et al.* 1991). Relative amounts of transcripts were
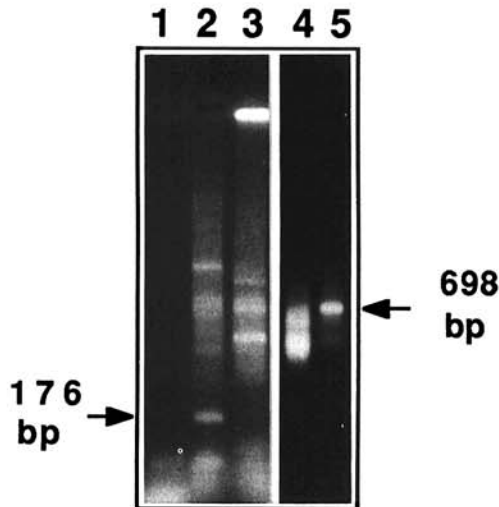
quantified by densitometry. The comparison of lanes X and L in Figure 7 shows that, in a healthy plant, vascular tissues (lane X) contain 10-fold the amount of OMT I transcripts found in leaves (lane L); this indicates constitutive expression of the OMT I gene in these lignified tissues. In tobacco leaves, the level of OMT I mRNA
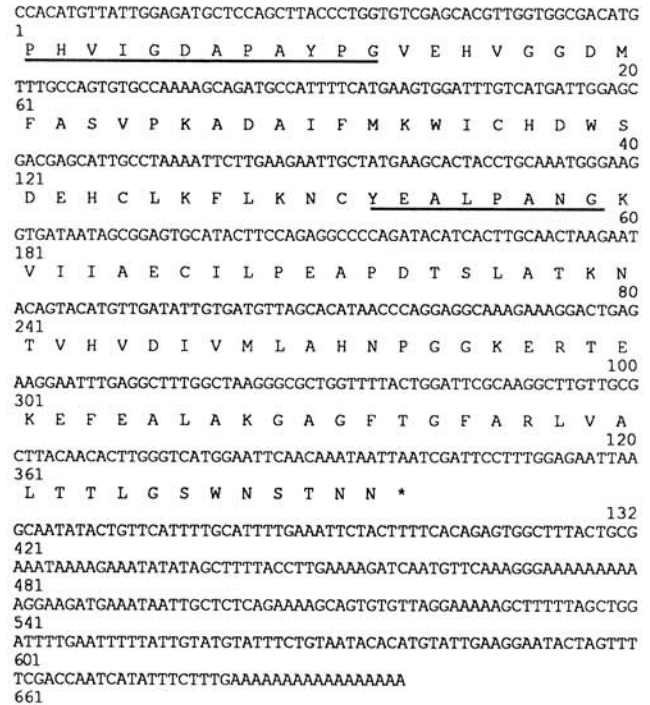
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CCACATGTTATTGGAGATGCTCCAGCTTACCCTGGTGTCGAGCACGTTGGTGGCGACATG
1
 P  H  V  I  G  D  A  P  A  Y  P  G  V  E  H  V  G  G  D  M
                                                          20
TTTGCCAGTGTGCCAAAAGCAGATGCCATTTTCATGAAGTGGATTTGTCATGATTGGAGC
61
 F  A  S  V  P  K  A  D  A  I  F  M  K  W  I  C  H  D  W  S
                                                          40
GACGAGCATTGCCTAAAATTCTTGAAGAATTGCTATGAAGCACTACCTGCAAATGGGAAG
121
 D  E  H  C  L  K  F  L  K  N  C  Y  E  A  L  P  A  N  G  K
                                                          60
GTGATAATAGCGGAGTGCATACTTCCAGAGGCCCCAGATACATCACTTGCAACTAAGAAT
181
 V  I  I  A  E  C  I  L  P  E  A  P  D  T  S  L  A  T  K  N
                                                          80
ACAGTACATGTTGATATTGTGATGTTAGCACATAACCCAGGAGGCAAAGAAAGGACTGAG
241
 T  V  H  V  D  I  V  M  L  A  H  N  P  G  G  K  E  R  T  E
                                                          100
AAGGAATTTGAGGCTTTGGCTAAGGGCGCTGGTTTTACTGGATTCGCAAGGCTTGTTGCG
301
 K  E  F  E  A  L  A  K  G  A  G  F  T  G  F  A  R  L  V  A
                                                          120
CTTACAACACTTGGGTCATGGAATTCAACAAATAATTAATCGATTCCTTTGGAGAATTAA
361
 L  T  T  L  G  S  W  N  S  T  N  N  *
                                                          132
GCAATATACTGTTCATTTTGCATTTTGAAATTCTACTTTTCACAGAGTGGCTTTACTGCG
421
AAATAAAAGAAATATATAGCTTTTACCTTGAAAAGATCAATGTTCAAAGGGAAAAAAAAA
481
AGGAAGATGAAATAATTGCTCTCAGAAAAGCAGTGTGTTAGGAAAAAGCTTTTTAGCTGG
541
ATTTTGAATTTTTATTGTATGTATTTCTGTAATACACATGTATTGAAGGAATACTAGTTT
601
TCGACCAATCATATTTCTTTGAAAAAAAAAAAAAAAAAA
661
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Fig. 6. Nucleotide and deduced amino acid sequence of tobacco orthodiphenol-*O*-methyltransferase (OMT I) clone. Underlined regions indicate the peptide sequences determined directly. The nucleotide sequence is numbered from the 5′ end of the cDNA clone on the left of each line. Amino acid numbers are given on the right side.
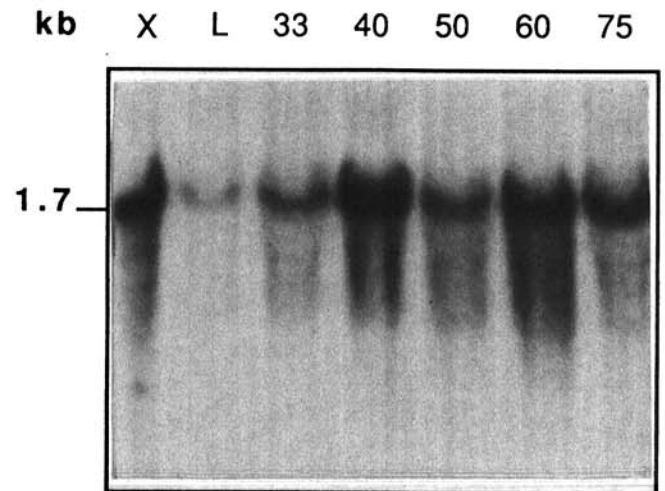

Fig. 5. Electrophoretic analysis of polymerase chain reaction products obtained from total RNA by using various couples of primers: antisense 16 and sense 43 (lane 2), antisense 43 and sense 16 (lane 3), and oligo(dT) and sense 43 (lane 4). The 698-bp product of lane 4 was extracted from the gel and reamplified with the same primers (lane 5). Lane 1 shows a control without template.

Fig. 7. RNA blot analysis. In each lane, 12 μg of total RNA was separated in a 1.2% agarose-formaldehyde gel, transferred to Hybond N membrane (Amersham, England), and hybridized with the orthodiphenol-*O*-methyltransferase (OMT I) 698-bp fragment labeled by random priming. RNA was prepared from vascular tissue of a healthy stem (X), from healthy leaves (L), or from tobacco mosaic virus infected leaves 33, 40, 50, 60, or 75 hr post-inoculation.

increases strongly with infection; 40 hr post-inoculation, OMT mRNAs were 15-fold more abundant in infected leaves than in uninfected leaves (i.e., 1.5-fold the steady-state level of OMT I mRNA in vascular tissues). Moreover, the kinetics of OMT I mRNA accumulation during infection paralleled that of translational activity (Fig. 3). This suggests that the increase in OMT I transcripts in infected leaves accounts for the increase of OMT I messenger activity.

DISCUSSION

OMTs involved in the phenylpropanoid pathway have been purified from various sources (Kuroda et al. 1975; Poulton et al. 1976; Hermann et al. 1987; Dumas et al. 1988; De Carolis and Ibrahim 1989; Pakusch et al. 1991; Edwards and Dixon 1991). In tobacco, three different enzymes are highly induced in leaves during the hypersensitive response to TMV (Dumas et al. 1988). We have investigated the molecular mechanism underlying the increased synthesis of the OMT proteins. OMT mRNAs were fractionated and translated in vitro, and the translation products were identified with specific antibodies raised against the purified enzymes. The in vitro products displayed the same mobility in SDS gels as did the purified enzymes, indicating that no substantial post-translational maturation step had occurred. A large increase in the activity of OMT mRNAs occurred during the hypersensitive reaction. OMT mRNA activity increased just before necrotic lesions appeared and reached a maximum 40 hr post-inoculation, before the rise in enzyme accumulation. Peptides isolated from purified OMT I were microsequenced, and the data were used to synthesize oligonucleotides and prime cDNA synthesis. A PCR product, identified as an OMT I clone by the presence of a peptide sequence in the derived amino acid sequence, hybridized on RNA blots to a 1.7-kb transcript that was detected in TMV-infected leaves and in vascular tissues of a healthy plant. This unique 1.7-kb mRNA species accumulates in TMV-infected leaves with kinetics similar to those observed for OMT I translation. The close correlation among OMT mRNA accumulation, translatable mRNA activity in vitro, and enzyme synthesis in vivo indicates that OMT gene expression is controlled at the transcriptional level. A similar mechanism of regulation has been reported for other enzymes of the phenylpropanoid pathway in response to infection (Fritzemeier et al. 1987) or treatment by elicitors (Kuhn et al. 1984; Edwards et al. 1985).

In vascular tissues from healthy stems, OMT I enzyme activity was 10-fold higher than in healthy leaves, and no OMT II or OMT III activity was detected (data not shown). Similarly, OMT I mRNA was 10 times more abundant in vascular tissues than in leaves. Thus, constitutive expression of the OMT I gene in lignified tissues of tobacco also might be controlled at the transcriptional level. During completion of this work, OMTs of aspen (Bugos et al. 1991), alfalfa (Gowri et al. 1991), and poplar (Dumas et al. 1992) were cloned, and their genes were shown to be preferentially expressed in lignified tissues. These findings support a regulatory role for OMTs in lignification.

Specific inhibition of two enzymes involved in lignifi-cation, namely PAL (Smart and Amrhein 1985; Massala et al. 1987; Moerschbacher et al. 1990) and cinnamyl alcohol dehydrogenase (Grand et al. 1985b; Moerschbacher et al. 1990), reduced lignification. However, the specificity of this approach remains questionable. Today, recombinant DNA technology makes the modulation of lignification feasible at the level of gene expression. OMTs are potential targets, and their importance in the control of lignification is well-illustrated in brown-midrib (bm_3) corn mutants that show a low lignin content due to low OMT activity (Grand et al. 1985a; Lapierre et al. 1988). Moreover, bm_3 hybrids have been obtained and have acceptable agronomic value (Gentinetta et al. 1990). Molecular analysis of OMT gene structure and expression is the first step towards manipulating the OMT gene to modulate the production of lignin in plants.

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