Plant Molecular Biology **31**: 307–322, 1996.

The maize caffeic acid *O*-methyltransferase gene promoter is active in transgenic tobacco and maize plant tissues

Montserrat Capellades [1], Miguel Angel Torres [1], Ingo Bastisch [1], Virginia Stiefel [1], Florence Vignols [1], Wesley B. Bruce [2], David Peterson [2], Pere Puigdomènech [1] and Joan Rigau [1,*].
[1] *Departament de Genètica Molecular, CID-CSIC, Jordi Girona 18–26, 08034-Barcelona, Spain (*author for correspondence);* [2] *Trait and Technology Development, Pioneer Hi-Bred Intl., P.O. Box 1004, Johnston, IA 50131, USA*

Received 29 August 1995; accepted in revised form 27 February 1996

Key words: Caffeic acid *O*-methyltransferase, *in situ* hybridization, lignification, maize, maize transformation, tobacco transformation

Abstract

The pattern of expression directed by the promoter of the maize caffeic acid *O*-methyltransferase (COMT) gene was studied by histochemical and fluorometric β-glucuronidase (GUS) analysis in transgenic maize and tobacco plants. The COMT promoter directs GUS expression to the xylem and the other tissues undergoing lignification, and it responds to wounding and to elicitors. In transgenic maize plants, expression of GUS corresponds to the pattern of expression of the endogenous COMT gene as determined by northern analysis and *in situ* hybridization. The pattern in transgenic tobacco plants clearly shows that the maize promoter sequence is recognized by tobacco transcriptional factors, in spite of the anatomical differences and the evolutionary distance between these two species. The results suggest that the most significant promoter signals that induce the specific expression of the lignin COMT are conserved in different species.

Introduction

Lignin is a three-dimensional structural component of the plant secondary cell wall. Its presence in the cell wall increases the mechanical strength and resistance of the plant towards biotic and abiotic stresses. Lignin plays an important role in the mechanical support, defence and water retention in plants [37]. 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 [37]. Lignin composition and deposition can also be influenced by wounding, fungal attack, specific enzyme inhibitors, mutations, hormones, metal ions and light [3, 8, 17, 18, 20, 48, 49, 59].

Some of the genes involved in lignin biosynthesis have been cloned. These include those corresponding to phenylalanine ammonia-lyase (PAL) [7, 38, 40, 50, 58], cinnamate 4-hydroxylase [51], 4-coumarate:CoA ligase (4CL) [10, 39, 56], caffeic acid 3-*O*-methyltransferase (COMT, EC 2.1.1.6) [5, 13, 45], caffeoyl-CoA-3-*O*-methyltransferase [60–62] and cinnamyl-alcohol dehydrogenase (CAD) [32, 47]. In addition, genes downstream in the pathway of flavonoid synthe-

sis such as chalcone synthase (CHS) have also been characterized [14, 22, 33].

Production of lignin monomers involves methylation of caffeic acid to ferulic acid by COMT. In angiosperm dicot plants, COMT is bispecific and can also catalyze the methylation of 5-hydroxyferulic acid to sinapic acid. COMTs may therefore act as regulatory steps in the lignin biosynthesis pathway. In tobacco, three distinct COMTs have been isolated and they have indicated different substrate specificities [2, 6, 26, 29]. COMT cDNA was also cloned and described in other species such as aspen [4], poplar [12, 53], alfalfa [17] and almond [16]. In maize, the gene coding for COMT involved in the lignin pathway has been described [5] and the molecular characterization of another putative OMT gene, which could be involved in the suberin pathway, has been reported [25]. The pattern of expression of the COMT gene in maize by RNA blot analysis has previously been published [5]. Recently, it has been proved, by molecular biology means, that the *brown midrib* (*bm3*) mutants in maize have the COMT gene not functional [55].

In this report, we present a study of the expression directed by the maize COMT promoter, fused to the GUS reporter gene, in transgenic maize and tobacco plants, with particular emphasis on expression patterns throughout plant development. As deposition of lignin is an ubiquitous defence in a wide range of monocot and dicot species [9] the response to induction of the COMT::GUS gene by wounding and elicitors has been also studied. To evaluate the patterns obtained by the transgenic plants, an analysis of the mRNA accumulation by *in situ* hybridization was carried out in maize.

Materials and methods

in situ *hybridization*

Experiments were carried out with *Zea mays* (inbred line W64A). Tissue sections (2–3 mm) were fixed in ethanol/acetic acid 3:1 for 30 min at room temperature. The fixative was removed and the samples were stored in 70% ethanol at 4 °C, until their hybridization.

Non-radioactive riboprobes were labelled with digoxigenin-dUTP according to the manufacturer's directions (Amersham). The labelled transcript was hydrolysed by alkali to achieve the desired length range. Pre-treatment and hybridization of samples were carried out as described by Langdale [35]. Digoxigenin hybrids were detected by immunoreaction with alkaline phosphatase conjugated to anti-digoxigenin antibody. The antibody was diluted 1/1000 in TBS and bovine serum albumin fraction V (100 mM Tris-HCl pH 7.5, 400 mM NaCl, 0.5% BSA). The incubation with the antibody was performed for 1–4 h. Tissues were then washed for 3 × 10 min in TBS and BSA. Detection was performed according to the protocol of the manufacturer (Amersham) following incubation overnight. Tissue preparations were photographed using bright-field microscopy on Ektachrome 160 ASA film (Kodak).

Wounding assays

For the northern analysis of the induction of the COMT gene in maize a scalpel blade was used to make longitudinal incisions in roots of 9-day-old plantlets grown in the dark, and transverse incisions in leaves of one-month-old plants grown in a greenhouse. The various parts of the plant were harvested for analysis at different times after wounding. Northern analysis were carried out as described by Montoliu *et al.* [42, 43].

Wounding analysis were also carried out in transgenic plants. In tobacco and maize transgenic plants, grown in a greenhouse, transverse incisions were made with a scalpel on young and not yet expanded leaves (ca. 2–3 cm long), and the histochemical and fluorometric GUS analysis was performed after 12 h. Roots of tobacco plants cultured *in vitro* were split off from the shoot and transverse incisions were made with forceps along the root. After 12 h of horizontal incubation in a Petri dish with an agarized MS medium [44] the histochemical GUS assay was performed. In

6-day-old transgenic maize plants cultured *in vitro*, roots and coleoptiles were lightly wounded with a scalpel and incubated for 12 h in a Petri dish over a wet filter paper before the histochemical GUS assay.

Plasmid construct

The 1963 bp *SphI-XhoI* DNA promoter fragment from the MG18/14 genomic clone [5] of the COMT gene was inserted into *SphI-* and *SalI*-digested pBI101.1 plasmid [30], resulting in a transcriptional fusion of the COMT promoter with the GUS-encoding region of the *uidA* gene of *Escherichia coli* ('GUS') and the 3'-untranslated region of the nopaline synthase gene of *A. tumefaciens* (*Nos-term*) (−1955 construct).

Maize stable transformation

Maize transformation was performed basically as described by Tomes *et al.* [52] with some modifications. Embryogenic callus cultures were initiated from R116 (Hi II, a B73 × A188 derivative, gift from C.L. Armstrong, Monsanto Co.) immature embryos cultured in the dark at 27 °C on a N6-based medium containing 2 mg/l 2,4-D, 690 mg/l proline and 0.85 mg/l silver nitrate (N6 2–6). The calli were subcultured onto fresh N6 2–6 at two-week intervals. After 28-d culture, the Hi II calli were bombarded, using a helium-driven PDS1000 particle gun (DuPont), with tungsten particles coated with DNA encoding bialaphos resistance and the GUS reporter construct. Putative transgenic calli were isolated after 3 months' culture on N6 2–6 containing 3 mg/l bialaphos but not proline. Transgenic somatic embryos were recovered from calli cultured on regeneration medium (MS salts, 100 mg/l myo-inositol, 0.5 mg/l zeatin, 1.0 mg/l IAA, 0.1 mg/l BA, 3 mg/l bialaphos). Plantlets were recovered after transfer of the mature somatic embryos onto MS salts with 100 mg/l myo-inositol and exposure to 16 h light/8 h dark cycle. After regeneration, plantlets were transplanted in sterile soil and grown under greenhouse conditions. Self-pollination were carried out to obtain the T_1 and T_2 progeny.

Tobacco stable transformation

The construct described above with the COMT promoter fused to the GUS and *Nos term* was introduced into DH5α *E. coli* K12 strain and into LBA4404 strain of *Agrobacterium tumefaciens* by transformation. The *Agrobacterium* culture was then used to inoculate sterile leaf discs of *Nicotiana tabacum* L. cv. Petit Havana SR1. Transformed tobacco shootlets were selected in a shoot-inducing medium [54] containing 100 μg/ml kanamycin and 500 μg/ml carbenicillin. Regenerated shootlets were rooted in a root-inducing medium [54] containing 100 μg/ml kanamycin and 250 μg/ml carbenicillin. Plants were grown in a greenhouse and T_1 seeds were collected and germinated in a medium with 200 μg/ml kanamycin. Genomic DNA from transgenic plants was analyzed by Southern blot to determinate the number of incorporated copies of the GUS gene and possible recombinations. This was carried out by digesting genomic DNA by *Hin*dIII and by *Hin*dIII plus *Eco*RI, and hybridizing with the *Hin*dIII-*Sna*BI fragment of the GUS gene as probe.

Histochemical and fluorometric GUS assay

The histochemical localization of GUS in transformed plants was performed essentially as described by Jefferson *et al.* [30], modified by adding methanol to the histochemical reaction mixture. Small pieces of several tissues were immersed in a histochemical reaction mixture containing 1 mg/ml X-Gluc in 50 mM sodium phosphate buffer pH 7, 0.1% Triton X-100, and 20% methanol (v/v). The histochemical reaction was performed in the dark at 37 °C until a blue indigo colour appeared (6–12 h). Tissues were rinsed several times in 50 mM phosphate buffer to stop the reaction, rinsed in 70% v/v ethanol and examined by light microscopy. Staining for lignin

with phloroglucinol 20% (v/v) HCl was performed following the method described by Jensen [31].

The fluorometric GUS assay was performed following the protocol described by Jefferson *et al.* [30], modified by adding methanol to the assay buffer as suggested by Kosugi *et al.* [34]. Measurements were carried out with a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

For the fluorometric GUS assay of the T_2 progeny of a transformed tobacco plant all the plantlets were grown *in vitro* on a MS medium [44]. In very young plantlets, the analysis was carried out on the whole plant, which includes the cotyledons and the primary young root. When the plantlets were 10 and 15 days old, roots and cotyledons (with incipient leaves) were analysed separately. In 25- and 60-day-old plants (subcultured into a fresh medium after 40 days in culture) a separate analysis of the stem, leaves (just expanded) and cotyledons was performed. In 60-day-old plants the petiole of the leaves was also analysed. The analysis was performed using three replicates of 25 seedlings when the plantlets were 5, 10 or 15 days old, and three replicates of five different plants for the 25- and 60-day-old plants.

COMT activation in response to elicitors

The elicitin β-cryptogein isolated from *Phytophthora cryptogea* and kindly provided by Dr Pernollet (INRA Versailles, France), was tested on transformed and untransformed tobacco leaves. Plants were grown in a phytotron at 23 °C, under a 16 h day photoperiod and a photon flux density of 100 μmol $m^{-2} s^{-1}$ provided by Sylvana Grolux fluorescent lamps. Young tobacco leaves which were still expanding were punched with a needle of 0.6 mm. Three to fiver small holes were made in the leaf lamina on both sides of the midrib, and 1 μl of an aqueous of β-cryptogein (0.1 μg/ml), or water for the control, was immediately applied to each hole. Inoculated plants were maintained in the same room for a period of 20 h (6 h light/8 h dark/6 h light) before the leaves were collected for the histochemical and fluorometric GUS assay. At that time, necrotic spots were visible in the leaf lamina.

Results

COMT mRNA accumulation in maize analysed by in situ *hybridization*

It has previously been shown by RNA blot analysis [5] that mRNA accumulation of the COMT gene transcript in maize is higher in tissues such as the maturation region of the root, where lignification occurs. This correlation can be more precisely observed using *in situ* hybridization of different maize tissues. Using non-radioactive probes (digoxigenin-dUTP) in an *in situ* hybridization of longitudinal sections of the root (Fig. 1B), the COMT mRNA was shown to accumulate specifically in the metaxylematic vascular cells of the root. A close-up of Fig. 1B (Fig. 1C) shows this pattern of accumulation around the tracheary elements.

In a cross section of the root, at the level of the differentiating zone, COMT-mRNA was detected in the exodermis and in the metaxylem elements (Fig. 1D). A higher magnification (Fig. 1E) shows more precisely that labelling is specifically detected in the metaxylem cells. On the other hand, in a cross section of young coleoptiles, mRNA accumulation was observed in the vascular bundles of the coleoptile (Fig. 1F and 1G). In cross sections of the leaves, the accumulation was seen in the small and large vascular strands and most clearly in the midrib (see Vignols *et al.* [55]). The results using *in situ* hybridization closely correspond to its mRNA accumulation previously revealed by RNA blot [5]. These figures show a good correlation between lignin biosynthetic sites [37] and COMT mRNA accumulation.

COMT promoter activity in transgenic maize plants

The maize COMT is a single gene that codes for one of the enzymes of the biosynthetic pathway

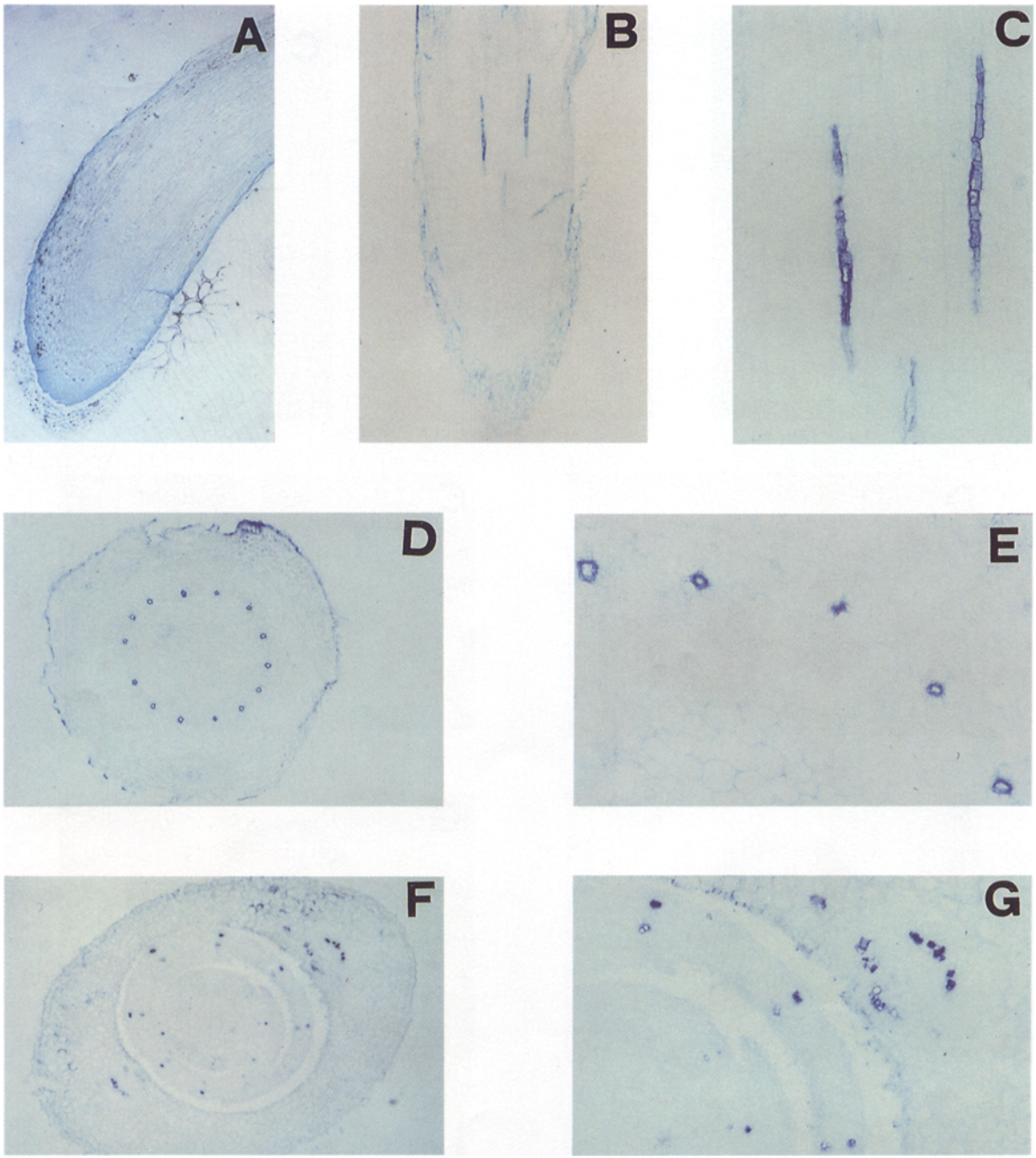
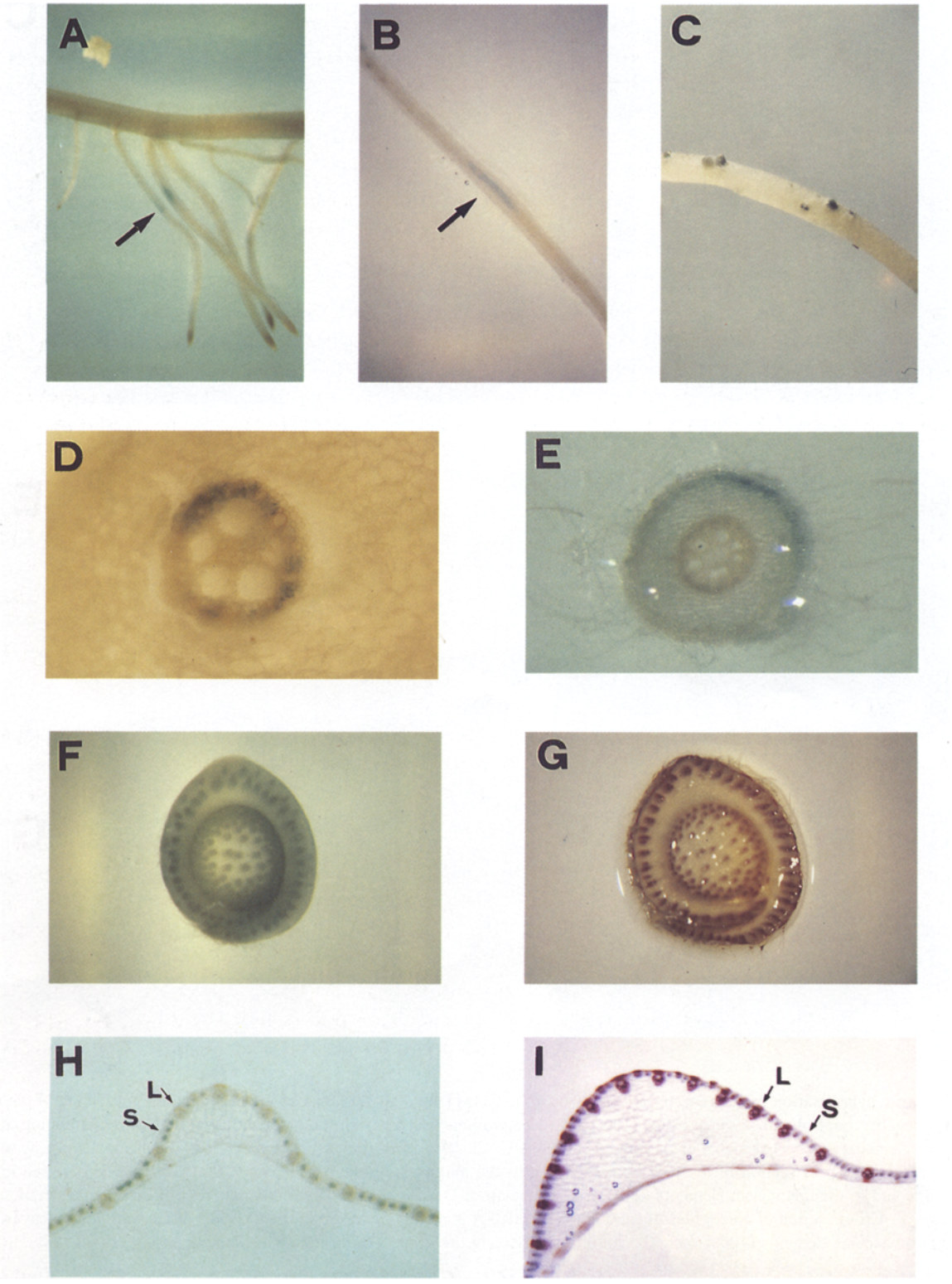


Fig. 1. In situ hybridization in different maize organs with the COMT full-length cDNA probe. A. Longitudinal section of the root tip as a negative control with a sense probe (100 ×). B. Longitudinal section of the root tip showing mRNA accumulation at the tracheary elements and a weak signal in the root cap (100 ×). C. Close-up of B, showing hybridization in the metaxylematic vascular cells (400 ×). D. Cross section of the root corresponding to the maturating zone of the root. COMT mRNA is detected in the exodermis and in the metaxylem elements (200 ×). E. Close-up of D, also showing hybridization only in the metaxylematic cells (1260 ×). F. Cross section of coleoptile. Accumulation of mRNA is mainly observed in the vascular bundles of young leaves and in the two vascular elements (100 ×). G. Close-up of F, showing the expression in the metaxylematic cells (320 ×).


A
B
C
D
E
F
G
H
L
S
I
L
S

of the lignin. The RNA blot [5] and *in situ* hybridization studies showed that the mRNA of this gene was accumulated in tissues where lignification was very active. To test whether the sequences located in the 5′ region of the gene were responsible for the pattern of expression observed, a construct with a 5′ promoter region of the genomic clone was transcriptionally fused to the GUS gene joined to the *Nos term*. The promoter region analyzed ranges from position −1955 of the putative start of transcription to the +7 position [5]. A schematic drawing of the construct is shown as an inset in Fig. 3A.

Maize plants were transformed by microparticle bombardment of embryogenic calli with the COMT promoter-GUS construct and the bar gene as selection marker. We examined several regenerated plants from each of 15 independent transformation events and found T_0 plants from four events that showed detectable levels of GUS by histochemistry. Each of the 15 events was tested for incorporation of COMT::GUS DNA into the maize genome using appropriate primers in a PCR analysis. Results discussed here are derived from the four GUS active events that had the same pattern of expression. Analysis of the T_1 and T_2 progeny of these transformed plants showed GUS expression in different tissues (Fig. 2).

Expression was localized in roots in a well-defined zone corresponding to the maturation region of xylem (Fig. 2A and 2B). High GUS expression was observed in lateral roots that are just emerging through the epidermis (Fig. 2C), and also in tissues surrounding the main root. In a cross section of the xylem maturation zone of the root, expression was found around the metaxylem groups of the stele (Fig. 2D) and also in the exodermis (Fig. 2E). In a cross section of the nodes of the stem, GUS expression was observed in the vascular bundles that extend into the leaf arising from the node (Fig. 2F). To compare these results to the lignin deposition sites, a phloroglucinol-HCl-stained [31] similar section of the stem was examined (Fig. 2G). In a cross section of a young leaf, expression of GUS was observed mainly in the small vascular strands, while little or no expression was observed in the large vascular strands (Fig. 2H). However, cross sections of leaves stained with phloroglucinol-HCl show that the large vascular strands were stained while not all the small strands were stained (Fig. 2I). This may be due to the fact that the large vascular strands were already lignified, while some of the small ones were still undergoing lignification. No GUS activity was detected in pollen grains or in young embryos, in agreement with the results of the mRNA accumulation [5].

COMT promoter activity in transgenic tobacco plants

Tobacco plants were transformed using the Ti plasmid via *Agrobacterium tumefaciens*. The same transcriptional fusion as in maize was used. The *nptII* gene was used as a selection marker. The pattern of expression of this construct in transformed tobacco plants was consistent with most of the qualitative features observed for lignin deposition, and also for the COMT::GUS expression in transgenic maize. The pattern of expression was the same in the 12 independent transformed plants obtained, with different levels

Fig. 2. Histochemical localization of GUS activity in transgenic maize plants. A. Roots of 15-day-old plants showing GUS expression (see arrow) in the xylem maturation region of the secondary roots (10 ×). B. Close-up of A (20 ×), showing that the GUS staining is localized at the vascular system level (see arrow). C. Emerging secondary roots through the main root (20 ×). D. Cross section of the maturating region of root xylem. Expression is visible in the metaxylem groups of the stele (200 ×). E. Cross section of the mature region of the root showing GUS staining at the exodermis (100 ×). F. Cross section of a node of the stem showing GUS expression at the vascular bundles (40 ×). G. Similar section stained with phloroglucinol-HCl showing the lignin deposition sites (40 ×). H. Cross section of a young already expanded leaf from a 20-day-old plant. GUS reaction is observed mainly in the small vascular strands (S) (60 ×). I. Phloroglucinol-HCl stained cross section of the leaf. The large vascular strands (L) are purple stained whereas not all the small strands (S) are stained (80 ×).

of GUS expression depending on the number of integrated copies of the gene and its specific integration site in the genome.

In order to quantify the levels of GUS expression in the different organs of the transformed tobacco plants, fluorometric analysis of the T_2 progeny was performed. The results are shown in Fig. 3B. It appears that, except in the young plantlets, in which a peak of expression was observed in the first few days of germination (see also Fig. 4A), in general, the GUS activity increases with the plant age. While in the young plantlets GUS activity was higher in the root system than in leaves, the reverse occurred in more mature plants which present a high GUS activity in the young already expanded leaves, in the petioles and in the stems.

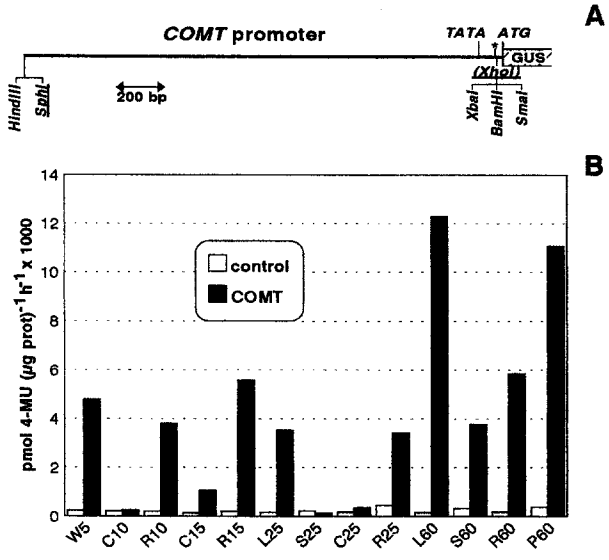


Fig. 3. Scheme of the COMT promoter fused to GUS. Fluorometric GUS analysis of the T_2 progeny of a transgenic tobacco plant. A. Schematic representation of the 1963 bp COMT promoter fragment transcriptionally fused to the *GUS* reporter gene. B. Bar chart of the results of the fluorometric GUS analysis in the transgenic tobacco plants. W corresponds to the whole 5-day-old plantlets, L to the leaves, R to the roots, S to the stems, C to the cotyledons, and P to the petioles. The numbers after letters correspond to the days after germination of the different plantlets. The 60-day-old plantlets were transferred to a new media after 40 days in culture and young leaves already expanded were used for the analysis. The bars are compared to the levels of the untransformed plants (control).

Histochemical analysis of the transformed plants was performed to identify the tissues in which the COMT promoter is active in tobacco. The patterns of GUS expression in transgenic tobacco plants carrying the maize COMT promoter are presented in Figs. 4 and 5. Expression was detected in all the organs of the plant in which formation of xylem and/or biosynthesis of lignin occurs. Figure 4A shows that the COMT promoter activity was detected very soon after germination. In 5-day-old plantlets, GUS staining was observed in the central veins of cotyledons and in the midrib of the transition zone between cotyledons and root. In 25-day-old plants, GUS expression was localized to a short and specific internal zone of the root, where the maturation of xylem takes place (Fig. 4B). This is also illustrated by a cross section of this region of the root (Fig. 4C), where the vascular system was stained. In leaves, GUS expression was clearly observed in the midrib and in all the veins (Fig. 4D). A cross section of the leaf shows that expression is localized in vessels of each vein (Fig. 4E). GUS expression is also well delimited in petioles, particularly in the vascular bundles of the midrib, and also in the small bundles (Fig. 5A). A phloroglucinol-stained section of the petiole shows good correspondence between the expression of the GUS and the lignification sites (Fig. 5B). The immature elements of xylem were GUS stained, while the mature elements were brownish and non-stained. There appears to be an alternation between the immature and mature xylem elements (Fig. 5C). Furthermore, when comparing the GUS stained section of the petiole (Fig. 5C) with its corresponding fluorescence microscopy image (Fig. 5D), it was seen that the immature xylem elements were blue stained while the fluorescence of the lignin [41] was localized to the non GUS-stained mature xylem. In stem sections GUS activity was also clearly observed in the immature xylem elements of the vascular system (Fig. 5E). A more detailed view of this expression is shown in Fig. 5F, which is compared with a similar section observed by fluorescence under UV light (Fig. 5G). GUS activity was confined to the xylem parenchyma and im-

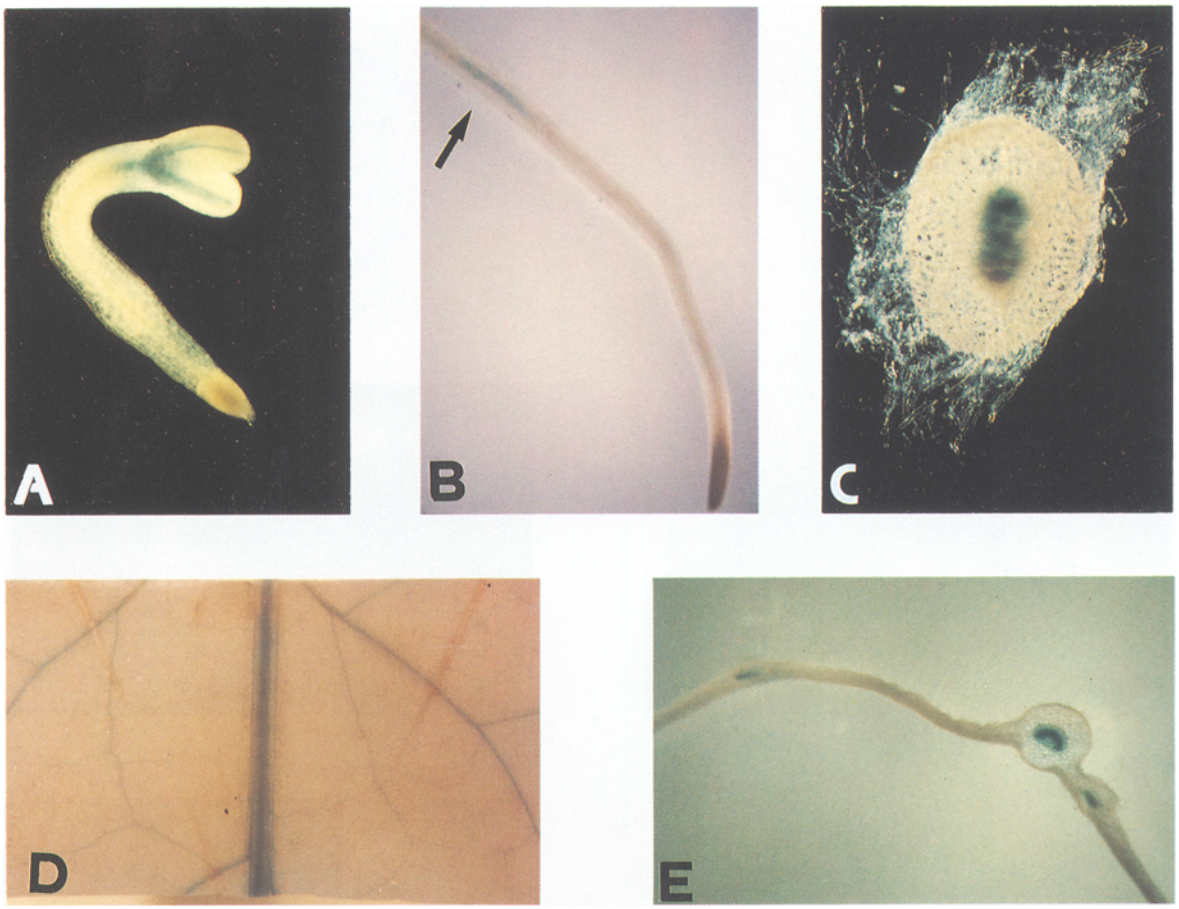


Fig. 4. Histochemical localization of GUS activity of the transgenic tobacco plants in young plantlets and leaves. A. 5-day-old plantlet expressing GUS in the central veins of cotyledons and in the midrib of the transition zone between cotyledons and root (40 ×). B. Root of 25-day-old plantlet showing GUS expression (see arrow) at the maturation zone of xylem (20 ×). C. Cross section of the maturation zone of xylem in the root. The vascular system is blue-stained (100 ×). D. GUS expression in a young leaf just expanded from an adult plant. Blue staining is visible in the midrib and in all the veins (4 ×). E. Cross section of a leaf showing a localized GUS expression in the vascular system of the midrib and of each vein (20 ×).

mature xylem elements, since those cells showing GUS staining were located in a layer next to those stained for lignin, which did not show GUS activity.

Induction by wounding and by elicitors in maize and in transgenic plants

Most of the enzymes of the phenylpropanoid pathway, and particularly PAL [38], are highly inducible in response to biotic and abiotic stresses, like defence reactions after wounding. To test this possibility for the maize COMT, RNA blot analysis of wounded leaves was carried out. Leaves from one-month-old maize plants, grown in the greenhouse, were wounded with the scalpel blade and harvested after 1, 2, 6 and 12 h. A full-length COMT cDNA was used as a probe. There was a seven-fold increase in leaves 6–12 h after injury (Fig. 6A). As a control, uninjured plants (C) were analysed, and also uninjured plants were left to dry in a flow of air (D) in order to determine whether the induction was due to the

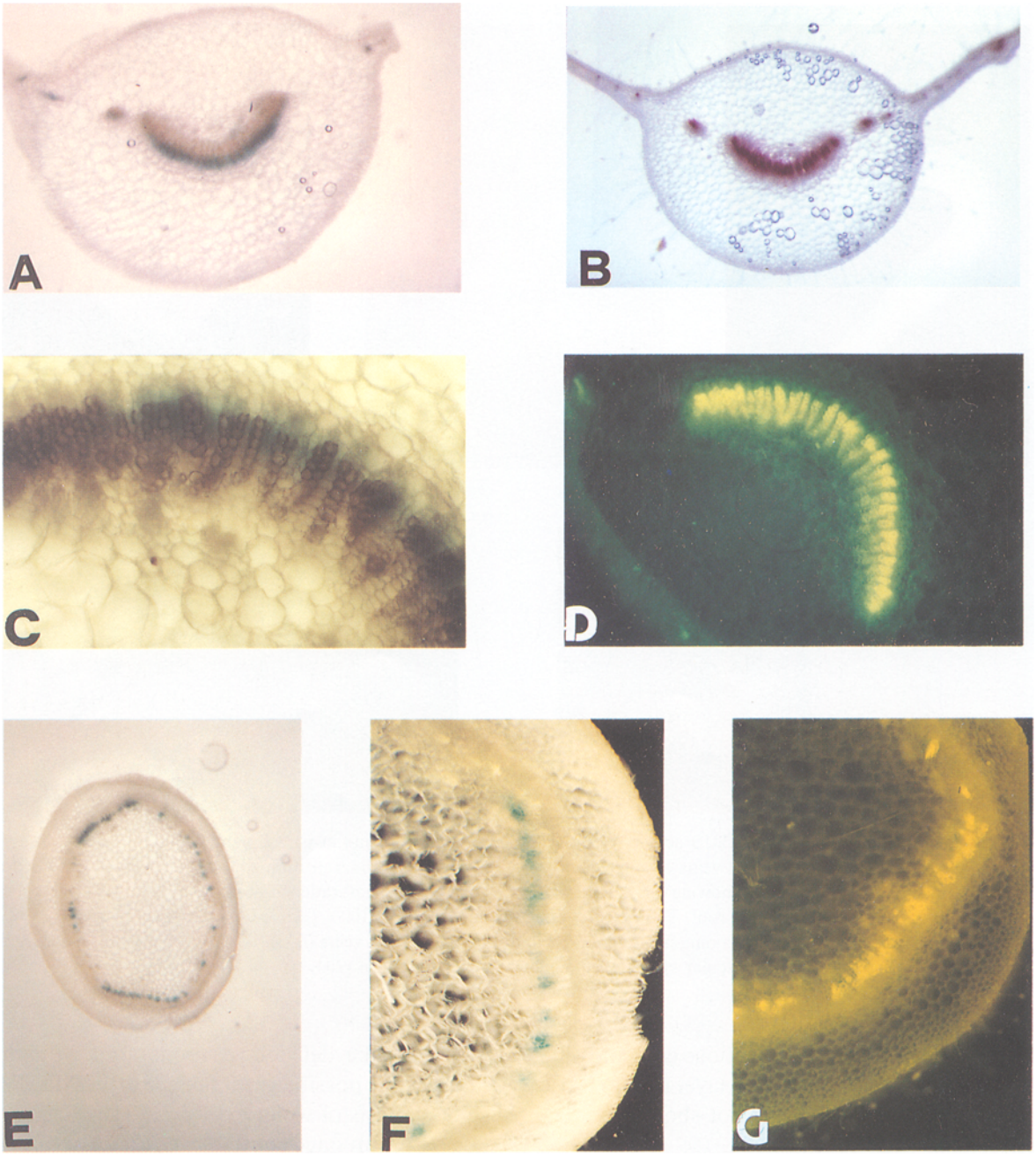


Fig. 5. Histochemical localization of GUS activity in petioles and stems of the transgenic tobacco plants. A. GUS expression in a cross section of a petiole. Blue staining is observed in the vascular bundles of the midrib and also in the small bundles (30 ×). B. Similar section to A stained with phloroglucinol-HCl to localize the sites of lignin deposition (30 ×). C. Close-up of the vascular bundle of the petiole. The xylem parenchyma and the immature xylem elements show GUS activity and are blue stained, while the mature xylem elements are brownish and non stained (250 ×). D. The same section as C observed under UV light microscopy showing lignin depositions (100 ×). E. Cross section of a stem showing GUS activity in the vascular system (20 ×). F. Close-up of a similar section to E in order to visualize the GUS stained cell type. G. Similar section observed under UV light (100 ×), in order to localize the lignified xylem elements. The COMT::GUS expressing cells are placed next to the lignified xylem elements in both petiole and stem.

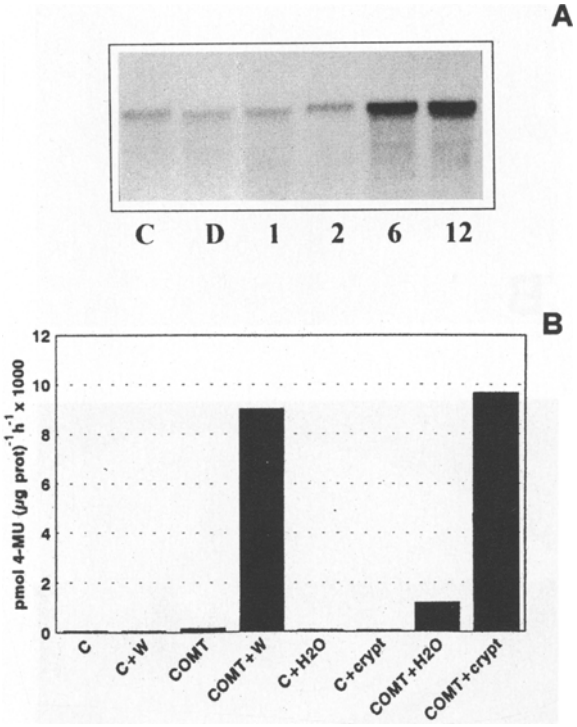


Fig. 6. A. Northern blot analysis of maize leaves after wounding. A full-length COMT cDNA was used as a probe. Leaves from one-month-old plants, grown in the greenhouse, were harvested at 1, 2, 6 and 12 h after wounding. Sample C corresponds to control undamaged root and D to the uninjured plants desiccated in an air flow. B. Fluorometric GUS assay of tobacco leaves harvested 12 h after wounding or 20 h after elicitor (β-cryptogein) treatment (C, untransformed control plant; W, wounded leaf; crypt, punched leaf with cryptogein; H_2O, punched leaf without cryptogein (only water); COMT, transformed plant).

injury or to the desiccation effect produced by the injury. The results show a clear induction of their mRNA accumulation by wounding, especially in leaves.

Since an induction of the COMT gene was observed by RNA blot analysis in wounded leaves in maize (Fig. 6A), it was determined whether wounding stimulates the expression of the COMT promoter construct in the transgenic plants. By histochemical GUS analysis in transgenic tobacco and maize plants, non-systemic induction was also observed around the injured area of leaves and roots after 12 h. In transgenic maize plants, the induction of the GUS activity was more pronounced in the young roots than in coleoptiles and leaves. The expression was localized around the injured areas of the root (Fig. 7A), and very weak or no GUS reaction was observed in wounded young leaves of maize. In contrast, in the tobacco transgenic plants the wounding effect was clearly observed in both leaves and roots but the GUS activity was higher in wounded young, not yet expanded (ca. 2–3 cm long) leaves (Fig. 7B). In tobacco, as well as in maize, induction by wounding was much more intense in young than in mature leaves. In tobacco leaves, GUS activity was observed around the vascular system and in the leaf epidermis and parenchyma which are unlignified tissues in healthy plants. GUS staining was also observed on the trichomes surrounding the injured area of the tobacco leaf (Fig. 7C).

It has been shown [3] that messenger RNAs corresponding to OMT accumulate in tobacco leaves in filtrated with an elicitor isolated from *Phytophthora megasperma*. To analyse the possible induction effect of elicitors in the transgenic plants, 1 μl of β-cryptogein (0.1 μg/ml solved in water), or water alone as a control, was applied to not yet expanded leaves of transformed tobacco plants. In normal conditions at this stage of development these leaves do not show any detectable GUS activity by histochemistry. Untransformed tobacco plants were used as a control of endogenous GUS induction. The histochemical GUS assay revealed that in the leaves of the transgenic tobacco plants treated with β-cryptogein, GUS expression was visible in the midrib, the veins and the leaf lamina surrounding the veins (Fig. 7C). The trichomes present in the GUS expressing tissues were also blue-stained. However, in leaves treated with only water GUS staining was localized just around the hole made by the needle (Fig. 7D). No GUS reaction was observed in untransformed tobacco plants treated with β-cryptogein. No induction of the GUS expression was obtained in the transgenic maize plants, indicating that probably cryptogein is not an appropriate elicitor in this species. Besides the histochemical GUS assay, a fluorometric GUS analysis was carried out on not yet expanded

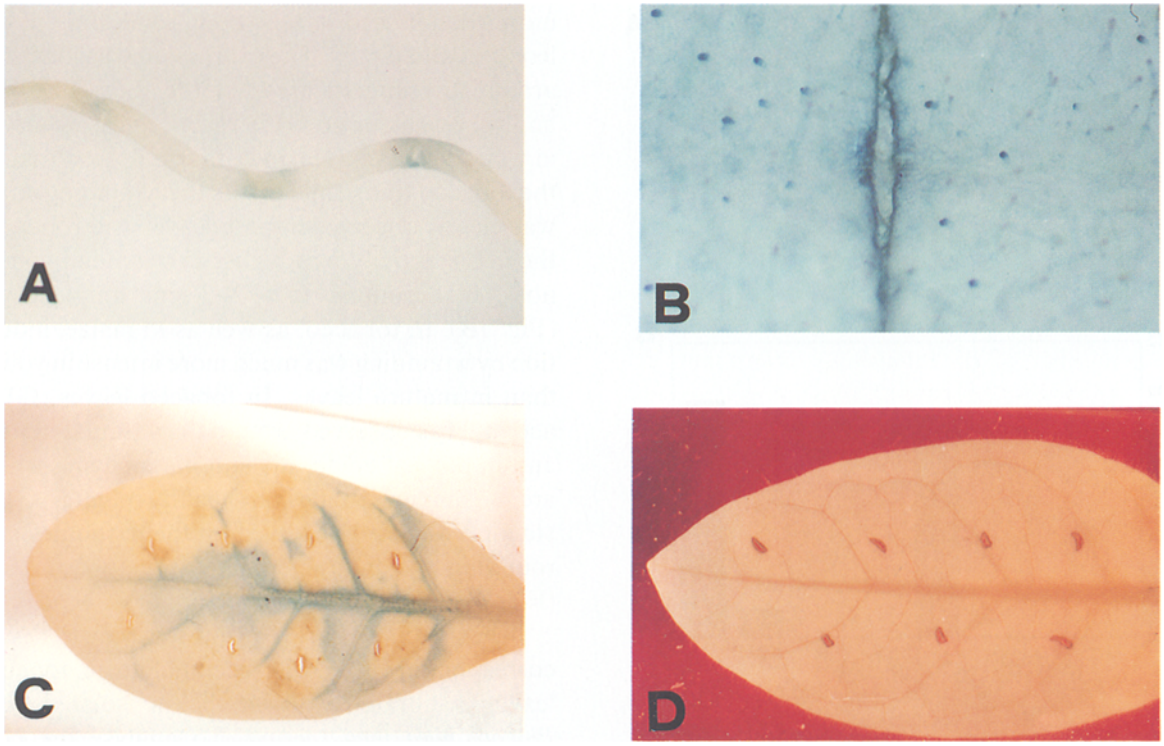


Fig. 7. Histochemical localization of GUS activity in wounded or elicited transgenic plants: A. GUS activity in a 6-day-old wounded root of a transgenic maize plant (20 ×). B. GUS activity in a young leaf, not yet expanded, of a three month old transgenic tobacco plant grown in a greenhouse, lightly wounded with a scalpel. GUS staining is observed on the injured tissues and also in the trichomes surrounding the wounded area (40 ×). C. Punched leaf of a transgenic tobacco plant treated with β-cryptogein solved in water (4 ×). D. Punched leaf of transgenic tobacco plant treated with only water, as a control (4 ×).

leaves injured with an scalpel or treated with β-cryptogein (Fig. 6B). As a control an untransformed plant and uninjured leaves of the transgenic plant were analysed. The results confirm those observed by histochemistry. Induction is also observed in leaves of transgenic tobacco plants punched without cryptogein due to the wounding effect produced by the needle.

Discussion

The patterns of mRNA accumulation of the maize caffeic acid *O*-methyltransferase (COMT) gene have been analyzed in maize by northern blot [5] and by *in situ* hybridization. Here the expression directed by the promoter region was studied in transgenic maize and tobacco plants. The locations where the mRNA accumulation is maximum seem to be the cells which are synthesizing their secondary wall, in accordance with the function of COMT, that it appears to provide precursors for lignification. The COMT mRNA was not detected in regions that were already lignified. This effect was clearly observed by *in situ* hybridization in the root, where the COMT expression was confined to the lignifying xylem of the maturation zone around the tracheary elements and in the exodermis. The COMT mRNA accumulation was also limited to the vascular bundles of the coleoptiles, and in the vascular strands of the leaves.

The pattern of mRNA accumulation found for COMT is similar to that obtained by the histochemical GUS studies carried out in the maize transgenic plants. The COMT promoter driving

the GUS gene, introduced in transgenic maize plants, direct GUS activity to specific cells in the root and leaves of young plantlets (ca. 10-day-old plants), where the highest mRNA accumulation was also observed. In wheat it has been described that the highest levels of COMT and PAL activity also occur within 10 days of germination, at a time when the lignification is still at a minimum [28]. There is also a clear correspondence to the pattern obtained in transgenic tobacco plants, with a number of distinguishing differences. In roots, the mRNA accumulation, as well as the GUS expression was detected specifically in the maturation region of the xylem in both maize and tobacco transgenic plants. In the maize root, in contrast to tobacco, the exodermis is lignified [15], and GUS expression was thus also observed. A correlation between the cells expressing GUS activity and those undergoing lignification was clearly observed in sections of stems, petioles and leaves. The COMT gene in maize and tobacco appears to be tightly controlled in those cells where the synthesis of lignin takes place. In both species, maize and tobacco, the GUS was expressed in the cells surrounding the lignified tissues, as visualized in stems and petioles of tobacco plants stained with phloroglucinol or observed under UV light by fluorescence. As a conclusion, a 2 kb sequence upstream of the COMT gene is sufficient to direct specific expression in cells undergoing lignification.

We have done a comparison between our results, using the maize COMT promoter in transgenic tobacco plants, with those obtained by Jaeck *et al.* [29], using the COMT mRNA of tobacco. Using the OMT-I probe by *in situ* hybridization in petioles and leaves they observed signal in the xylematic parenchyma and in the lignifying cells of the xylem. This results have a clear correspondence with the GUS expressions presented in this paper.

A variety of factors, both environmental and endogenous, have been found to exert an effect upon the process of lignification [18, 37, 57]. Another feature of the COMT genes, shared with genes encoding enzymes of the lignin biosynthetic pathway, is their induction by wounding. The induction was observed in both maize and tobacco plants, especially in young roots and leaves. Such induction produces a lignification in tissues such as the epidermis and parenchyma, which are unlignified in healthy plants. The results presented here clearly show that the COMT maize promoter responds to wounding in both tobacco and maize, but following the specific features of the wound response of each plant species. For instance, the COMT promoter is activated during the lateral root emergence in maize, probably due to the formation of new vascular tissue and/or to the tissue damage incurred during normal root development. This COMT induction could be interpreted as a lignin deposition in the injured area surrounding the site of emergence of the lateral root, as a defence mechanism against pathogen infection through the injured tissues. The late expression of COMT (after 6–12 h) observed after wounding suggests that this enzyme is not involved in the early defence response. COMT induction by elicitors has been seen in transformed tobacco plants, with a clear GUS induction by cryptogein in leaves. We have tested several elicitors in the transgenic maize plants without any positive results, perhaps because the appropriate elicitor was not used. Deposition of lignin is an ubiquitous defence response, and induction of genes encoding enzymes involved in the synthesis of lignin monomers by fungal elicitors has been observed in a wide range of monocot and dicot species [6]. Lignin is highly resistant to attack by most microorganisms and lignified cells walls are considered to be effective barriers to the progression of plant pathogens. In muskmelons, Grand and Rossignol [19] found that enhanced lignification induced by *Colletotrichum lagenarium* was associated with a concerted increase in the activity of the polymer biosynthesis enzymes, including COMT. Dumas *et al.* [11] observed *de novo* synthesis of OMT enzymes during the hypersensitive response to tobacco mosaic virus.

The signals that control the maize COMT promoter in cells accumulating lignin may be widespread in higher plants. Even though the pattern of expression in transgenic tobacco plants is different from that observed in maize, it closely fol-

lows the distribution of cells undergoing lignification. Thus, the factors needed for this control are present in distant species such as tobacco and maize, and in both species these factors are only present in those cells whose wall is becoming lignified. The shared signals of the lignin biosynthetic pathway and the defence reaction are also found in maize and the sequences controlling the induction in the COMT gene appear to be within the 2 kb 5′-upstream region together with the sequences defining the cell-type-specific control already described.

The regulation of phenylpropanoid genes has been an area of active research. A phenylalanine ammonia-lyase gene promoter from *Arabidopsis*, and a 4-coumarate:CoA ligase gene promoter from parsley were studied [23, 46]. The expression in transgenic plants and the length of the regulatory fragments have several similarities with that obtained by other genes of the pathway. Some elements have been described in the promoters of genes involved in the phenylpropanoid metabolism [36, 38, 46] that could be related to their regulation. In the COMT promoter there are similar elements at positions −237 to the transcription start site (ACCTAAC) and at position −304 (ACAACAACCAC), that are homologous to the previously described in *Arabidopsis* and parsley and in a similar positions. The second one coincides with an elicitor-inducible hypersensitive site in parsley. These data are consistent with the hypothesis that these elements could be involved in the regulation of the COMT promoter in maize.

Using the antisense technology with the cinnamyl alcohol dehydrogenase (CAD) genes in tobacco [21, 27, 28], an increase of cinnamaldehyde groups in lignin, but no significant changes in lignin content were suggested. Tobacco plants transformed with the CAD antisense show very low levels of CAD activity and a red-brown colour phenotype in xylem tissues similarly to the *brown-midrib* mutants of maize. When using the antisense of the COMT gene in tobacco [2, 13, 45] a marked decrease of syringyl units and the appearance of 5-hydroxy guaiacyl units were demonstrated. This correlates well with the features characteristic of natural mutants of maize *bm3* where the COMT gene is not functional [55]. All these results indicate that it may be possible to manipulate the processes of lignification by using molecular approaches. Genetic manipulation of genes encoding proteins essential for the plant lignification may enable the regulation of lignin content and composition in agronomically and industrially important crops. The availability of promoters specific of cells involved in lignification may allow a more efficient control of the expression of genes related to these processes.

Acknowledgements

The study was carried out under a grant from Plan Nacional de Investigación Científica y Técnica (BIO 094–734). M.C. was a recipient of fellowships from the Departament d'Ensenyament of the Generalitat de Catalunya. F.V. is a postdoctoral fellow from European Community Human Capital and Mobility Programme. M.A.T. received support from DGICYT (Spain). We thank Dr Gary Huffman (Pioneer Hi-Bred) and Dr Leonor Alegre (Barcelona University) for critically reviewing this manuscript.

References

1. Asada Y, Matsumuto I: Induction of disease resistance in plants by a lignification-inducing factor. In: Nishimura S, Vance C, Doke N (eds) Molecular Determinants of Plant Diseases, pp. 223–233. Springer-Verlag, Berlin (1987).
2. Atanassova R, Favet N, Martz F, Chabbert B, Tollier MT, Monties B, Fritig B, Legrand M: Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. Plant J 8: 465–477 (1995).
3. Baillieul F, Genetet I, Kopp M, Saindrenan P, Fritig B, Kauffmann S: A new elicitor of the hypersensitive response in tobacco: a fungal glycoprotein elicits cell death, expression of defence genes, production of salicylic acid, and induction of systemic acquired resistance. Plant J 8: 551–560 (1995).
4. Bugos RC, Chiang VLC, Campbell WH: cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of aspen. Plant Mol Biol 17: 1203–1215 (1991).

5. Collazo P, Montoliu L, Puigdomènech P, Rigau J: Structure and expression of the lignin *O*-methyltransferase gene from *Zea mays* L. Plant Mol Biol 20: 857–867 (1992).
6. Collendavelloo J, Legrand M, Geoffroy P, Barthelemy J, Fritig B: Purification and properties of the three *o*-diphenol-*O*-methyltransferases of tobacco leaves. Phytochemistry 20: 611–616 (1981).
7. Cramer CL, Edwards K, Dron M, Liang X, Dildine SL, Bolwell GP, Dixon RA, Lamb CJ, Schuch W: Phenylalanine ammonia-lyase gene organization and structure. Plant Mol Biol 12: 367–383 (1989).
8. Diallinas G, Kanellis AK: A phenylalanine ammonia-lyase gene from melon fruit: cDNA cloning, sequence and expression in response to development and wounding. Plant Mol Biol 26: 473–479 (1994).
9. Dixon RA, Harrison MJ: Activation, structure and organization of genes involved in microbial defence in plants. Adv Genet 28: 165–234 (1990).
10. Douglas C, Hoffman H, Schulz W, Hahlbrock K: Structure and elicitor or UV light stimulated expression of two 4-coumarate:CoA ligase genes in parsley. EMBO J 6: 1189–1195 (1987).
11. Dumas B, Legrand M, Geoffroy P, Fritig B: Purification of tobacco *O*-methyltransferases by affinity chromatography and estimulation of the rate of synthesis of the enzymes during the hypersensitive reaction to virus infection. Planta 176: 36–41 (1988).
12. Dumas B, Van Doorsselaere J, Gielen J, Legrand M, Fritig B, Van Montagu M, Inze D: Nucleotide sequence of a complementary DNA encoding *O*-methyltransferase from poplar. Plant Physiol 98: 796–797 (1992).
13. Dwivedi UN, Campbell WH, Yu J, Datla RSS, Bugos RC, Chiang VL, Podila GK: Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. Plant Mol Biol 26: 61–71 (1994).
14. Epping B, Kittel M, Ruhnau B, Hemleben V: Isolation and sequence analysis of a chalcone synthase cDNA of *Mattiola incana* R.Br. (Brassicaceae). Plant Mol Biol 14: 1061–1063 (1990).
15. Esau K: Anatomía vegetal. Ediciones Omega S.A., Barcelona (1990).
16. Garcia-Mas J,Messeguer R, Arus P, Puigdomenech P: The caffeic acid *O*-methyltransferase from *Prunus amygdalus*. Plant Physiol 108: 1341 (1995).
17. Gowri G, Bugos RC, Campbell WH, Maxwell CA, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.). Plant Physiol 97: 7–14 (1991).
18. Grand C, Boudet AM, Ranjeva R: Natural variations and controlled changes in lignification process. Holzforschung 36: 217–223 (1982).
19. Grand C, Rossignol M: Changes in the lignification process induced by localized infection of muskmelons with *Colletotrichum lagenarium*. Plant Sci Lett 28: 103–110 (1983).
20. Gregersen PL, Christensen AB, Sommer-Knudsen J, Collinge DB: A putative *O*-methyltransferase from barley is induced by fungal pathogens and UV light. Plant Mol Biol 26: 1797–1806 (1994).
21. Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier MT, Schuch W: Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. Plant J 6: 339–350 (1994).
22. Harker CL, Ellis THN, Coen ES: Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2: 185–194 (1990).
23. Hauffe KD, Paszkowski U, Schulze-Lefert P, Hahlbrock K, Dangl JL, Douglas CJ: A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell 3: 435–443 (1991).
24. Hauffe KD, Lee SP, Subramaniam R, Douglas CJ: Combinatorial interactions between positive and negative cis-acting elements control spatial patterns of 4CL-1 expression in transgenic tobacco. Plant J 4: 235–253 (1993).
25. Held BM, Wang H, John I, Wurtele ES, Colbert T: An mRNA putatively coding for an *O*-methyltransferase accumulates preferentially in maize roots and is located predominantly in the region of the endodermis. Plant Physiol 102: 1001–1008 (1993).
26. Hermann C, Legrand M, Geoffroy P, Fritig B: Enzymatic synthesis of lignin: purification to homogeneity of three *O*-methyltransferases of tobacco and production of specific antibodies. Arch Biochem Biophys 253: 367–376 (1987).
27. Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T: Increase of cinnamaldehyde groups in lignin of transgenic plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. Biosci Biotech Biochem 59: 929–931 (1995).
28. Higuchi T: Biosynthesis of lignin. In: Higuchi T (eds) Biosynthesis and Degradation of Wood Components, pp. 141–161. Academic Press, Orlando, FL (1985).
29. Jaeck E, Dumas B, Geoffroy P, Favet N, Inze D, Van Montagu M, Fritig B, Legrand M: Regulation of enzymes involved in lignin biosynthesis: induction of *O*-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus. Mol Plant-Microbe Interact 5: 294–300 (1992).
30. Jefferson RA: Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
31. Jensen WA: Botanical histochemistry, principles and practice. In: Beadle GW, Emerson R, Whitaker DM (eds), WH Freeman and Co, San Francisco, CA (1962).
32. Knight ME, Halpin C, Schuch W: Identification and characterization of cDNA clones encoding cinnamyl alcohol dehydrogenase from tobacco. Plant Mol Biol 19: 793–801 (1992).
33. Koes RE, Spelt CE, Mol JNM: The chalcone synthase multigene family of *Petunia hybrida* (V30): differential, light-regulated expression during flower development and UV light induction. Plant Mol Biol 12: 213–225 (1989).

34. Kosugi S, Ohashi Y, Nakajima K, Arai Y: An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci 70: 133–140 (1990).
35. Langdale JA: *In situ* hybridization. In: Freeling M, Walbot V (eds) The Maize Handbook, pp. 165–180. Springer-Verlag, New York (1994).
36. Lawton MA, Clouse SD, Lamb CJ: Glutathione-elicited changes in chromatin structure within the promoter of the defence gene chalcone synthase. Plant Cell Rep 8: 561–564 (1990).
37. Lewis NG, Yamamoto E: Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41: 455–496 (1990).
38. Lois R, Dietrich A, Hahlbrock K, Schulz W: A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J 8: 1641–1648 (1989).
39. Lozoya E, Hoffman H, Douglas C, Schulz W, Scheel D, Hahlbrock K: Primary structures and catalytic properties of isoenzymes encoded by the two 4-coumarate:CoA ligase genes in parsley. Eur J Biochem 176: 661–667 (1988).
40. Minami E, Ozeki Y, Matsuoka M, Koizuka N, Tanaka Y: Structure and some characterization of the gene for phenylalanine ammonia-lyase from rice plants. Eur J Biochem 185: 19–25 (1989).
41. Monties B: Lignins. Plant phenolics. In: Harborne JB (ed) Methods in Plant Biochemistry, vol. 1, pp. 113–157. Academic Press, London (1989).
42. Montoliu L, Rigau J, Puigdomènech P: A tandem of α-tubulin genes preferentially expressed in radicular tissues from *Zea mays*. Plant Mol Biol 14: 1–15 (1989).
43. Montoliu L, Puigdomènech P, Rigau J: The *Tubα3* gene from *Zea mays*: structure and expression in dividing plant tissues. Gene 94: 201–207 (1990).
44. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497 (1962).
45. Ni W, Paiva NL, Dixon RA: Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. Transgenic Res 3: 120–126 (1994).
46. Ohl S, Hedrick SA, Chory J, Lamb CJ: Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2: 837–848 (1990).
47. O'Malley DM, Porter S, Sederoff RR: Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). Plant Physiol 98: 1364–1371 (1992).
48. Pellegrini L, Geoffroy P, Fritig B, Legrand M: Molecular cloning and expression of a new class of ortho-diphenol-*O*-methyltransferases induced in tobacco (*Nicotiana tabacum* L.) leaves by infection or elicitor treatment. Plant Physiol 103: 509–517 (1993).
49. Pellegrini L, Rohfritsch O, Fritig B, Legrand M: Phenylalanine ammonia-lyase in tobacco. Plant Physiol 106: 877–886 (1994).
50. Tanaka Y, Matsuoka M, Yamamoto N, Ohashi Y, Kano-Murakami Y, Ozeki Y: Structure and characterization of a cDNA clone for phenylalanine ammonia-lyase from cut-injured roots of sweet potato. Plant Physiol 90: 1403–1407 (1989).
51. Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Werck-Reichhart D: Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA 90: 4102–4106 (1993).
52. Tomes DT, Ross MC, Songstad DD: Direct DNA-transfer into intact plant cells. In: Gamborg OL, Phillips GC (eds) Plant Cell, Tissue and Organ Culture: Fundamental Methods, Springer-Verlag, Berlin (1995).
53. Tsai CJ, Podila GK, Chiang VL: Nucleotide sequence of a *Populus tremuloides* gene encoding bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. Plant Physiol 107: 1459 (1995).
54. Van Lijsebettens M, Valvekens,D: Tobacco leaf disc infection with *Agrobacterium tumefaciens*. In: Van Montagu M (ed) EMBO Practical Course on Plant Molecular Biology, pp. 15–18, Ghent, Belgium (1987).
55. Vignols F, Rigau J, Torres MA, Capellades M, Puigdomènech P: The brown midrib 3 (*bm3*) mutation in maize occurs in the gene encoding caffeic acid *o*-methyltransferase. Plant Cell 7: 407–416 (1995).
56. Voo KS, Whetten R, O'Malley D, Sederoff RR: 4-coumarate:CoA ligase in xylem of loblolly pine. Plant Physiol 108: 85–97 (1995).
57. Walter MH: Regulation of lignification in defence. In: Boller T, Meins F (eds) Plant Gene Research: Genes Involved in Plant Defence, pp. 327–352. Springer-Verlag, New York (1992).
58. Wanner LA, Li G, Ware D, Somssich IE, Davis KR: The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol Biol 27: 327–338 (1995).
59. Whetten R, Sederoff R: Lignin biosynthesis. Plant Cell 7: 1001–1013 (1995).
60. Ye ZH, Kneusel RE, Matern U, Varner JE: An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell 6: 1427–1439 (1994).
61. Ye ZH, Varner JE: Differential expression of two *O*-methyltransferases in lignin biosynthesis in *Zinnia elegans*. Plant Physiol 108: 459–467 (1995).
62. Zhang XH, Dickson EE, Chinnappa CC: Nucleotide sequence of a cDNA clone encoding caffeoyl-Coenzyme A 3-*O*-methyltransferase of *Stellaria longipes* (Caryophyllaceae). Plant Physiol 108: 429–430 (1995).