The Plant Cell, Vol. 4, 17–27, January 1992

Subdomains of the Octopine Synthase Upstream Activating Element Direct Cell-Specific Expression in Transgenic Tobacco Plants

Halina Kononowicz,[1] Y. Eureka Wang, Lillian L. Habeck, and Stanton B. Gelvin[2]

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Previous work has shown that the octopine synthase (*ocs*) gene encoded by the *Agrobacterium tumefaciens* Ti-plasmid contains an upstream activating sequence necessary for its expression in plant cells. This sequence is composed of an essential 16-bp palindrome and flanking sequences that modulate the level of expression of the ocs promoter in transgenic tobacco calli. In this study, we have used RNA gel blot analysis of RNA extracted from transgenic tobacco plants to show that the octopine synthase gene is not constitutively expressed in all plant tissues and organs. This tissue-specific pattern of expression is determined, to a large extent, by the 16-bp palindrome. Histochemical analysis, using an *ocs-lacZ* fusion gene, has indicated that the 16-bp palindrome directs the expression of the *ocs* promoter in specific cell types in the leaves, stems, and roots of transgenic tobacco plants. This expression is especially strong in the vascular tissue of the leaves, leaf mesophyll cells, leaf and stem guard cells, and the meristematic regions of the shoots and roots. Sequences surrounding the palindrome in the upstream activating sequence restrict the expression of the *ocs* promoter to fewer cell types, resulting in a reduced level of expression of β-galactosidase activity in the central vascular tissue of leaves, certain types of leaf trichomes, and the leaf primordia.

INTRODUCTION

Many eukaryotic genes contain upstream activating sequences or enhancer elements that are essential for gene transcription. These sequences are not promoters in themselves but activate transcription from linked promoters. They are independent of their orientation relative to the promoters and can be either independent (enhancers) or dependent (upstream activating sequences) upon their position relative to these promoters. Enhancers can be tissue specific or inducible. They may also be modular, in that they may be composed of a limited number of basic sequence motifs that interact in a synergistic fashion (for a review, see Wasylyk, 1988).

Upstream activating sequences have been identified for a number of plant genes. Among the best studied of these activators is that of the cauliflower mosaic virus (CaMV) 35S promoter. The CaMV 35S activator has multiple domains that can function either independently or synergistically to activate the 35S promoter in a developmentally and tissue-specific manner (Benfey et al., 1989, 1990a, 1990b). Upstream activating sequences have also been identified among genes of the *Agrobacterium tumefaciens* Ti-plasmid encoded by T-DNA. T-DNA genes containing such elements include those encoding enzymes involved in cytokinin biosynthesis (*tmr*, de Pater et al., 1987), agropine biosynthesis (*ags*, Bandyopadhyay et al., 1989), nopaline production (*nos*, An, 1987; Mitra and An, 1989), mannopine biosynthesis (*mas*, DiRita and Gelvin, 1987; Langridge et al., 1989; Leisner and Gelvin, 1989; Comai et al., 1990), octopine production (*ocs*, Ellis et al., 1987a, 1987b; Leisner and Gelvin, 1988, 1989; Bouchez et al., 1989; Fromm et al., 1989; Singh et al., 1989), and a gene of unknown function encoding a 780-base mRNA (Bruce et al., 1988).

Recently, we (Leisner and Gelvin, 1988, 1989) and others (Ellis et al., 1987b) have shown that the octopine synthase (*ocs*) upstream activating sequence contains a 16-bp palindrome essential for *ocs* activator function. Specific proteins can bind to this palindrome (Bouchez et al., 1989; Fromm et al., 1989; Singh et al., 1989, 1990; Tokuhisa et al., 1990) and presumably are involved in the activation of the *ocs* promoter. In transgenic tobacco calli, however, sequences surrounding this palindrome are necessary for maximal stimulation of *ocs* transcription. These additional sequences include an element with the potential to form a Z-DNA structure (Leisner and Gelvin, 1989). In this study, we have examined the influence of various DNA sequences from the *ocs* transcriptional activating element upon *ocs* promoter expression in transgenic tobacco plants. We show that the complete *ocs*

[1] Current address: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907.

[2] To whom correspondence should be addressed.

activator confers tissue-specific expression upon the *ocs* promoter. Moreover, DNA sequences surrounding the 16-bp palindrome within the *ocs* activator limit the expression of an octopine synthase-β-galactosidase fusion protein to particular cell types.

RESULTS

RNA Gel Blot Analysis of *ocs* RNA from Plant Tissues and Organs

In previous studies (Leisner and Gelvin, 1988, 1989), we investigated the importance of various sequences upstream of the octopine synthase gene in the activation of the *ocs* promoter. These experiments utilized calli derived from transgenic tobacco plants transformed with constructions containing the *ocs* promoter and structural gene and various fragments of the *ocs* upstream activating sequence. In tobacco calli, octopine synthase activity directed by these constructions correlated with the steady-state level of *ocs* mRNA (Leisner and Gelvin, 1988). We wished to extend these studies and investigate the tissue-specific and developmental utilization of these *ocs* activator sequences in mature tobacco plants. We, therefore, regenerated plants from these transgenic tobacco calli and assayed octopine synthase activity in various tissues and organs of these plants. Control experiments indicated, however, that certain plant tissues contained substances that inhibited the octopine synthase assay (data not shown).

Because octopine synthase activity was not a reliable indicator of *ocs* gene activity in mature transgenic tobacco plants, we performed an RNA gel blot analysis of RNA derived from these plants to examine tissue-specific patterns of *ocs* mRNA accumulation. RNA was extracted from leaves (five leaves equally distributed along the stem), stems (three segments), flowers, the root tip, and the root base. Total cellular RNA (20 μg) was fractionated by formaldehyde-agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized with a DNA fragment containing the *ocs* gene. Figure 1A shows the regions of the *ocs* transcriptional activating sequence that were present in the different plants, and Figure 1B shows the different parts of the plant that were examined for *ocs* mRNA. A total of 15 plants harboring the different constructions were analyzed to determine the amount of *ocs* RNA. Figure 1C shows RNA gel blot analyses of RNA derived from plants containing the constructions pEN1, pAlu106, and pPAL16. There was, in general, a relatively high steady-state level of *ocs* mRNA in the leaves and the root tip, a lower level of *ocs* mRNA in the stem (especially the lower stem sections) and flowers, and a very low level of *ocs* mRNA in the basal sections of the root. Plants containing the constructions pENR1, pAlTaR54, and pAlu45 showed similar patterns of *ocs* mRNA accumulation (data not shown). Hybridization of the blots with an rDNA probe indicated that the lanes were equally loaded (data not shown). These data suggest that the *ocs* promoter is differentially expressed in different tissues and organs of mature tobacco plants. The data further suggest that, at the level of resolution examined in this series of experiments, different fragments of the *ocs* transcriptional activating sequence do not alter the expression pattern of *ocs* mRNA. Although the differing steady-state levels of *ocs* mRNA may also be explained by differential *ocs* mRNA stability, similar patterns of expression were observed using an *ocs::lacZ* fusion gene (see below). It is, therefore, likely that the observed steady-state levels of *ocs* mRNA resulted from tissue-specific differences in expression of the *ocs* promoter.

Histochemical Localization of β-Galactosidase Activity in Transgenic Plants Containing *ocs::lacZ* Fusion Constructions

To determine in which plant cell types various portions of the *ocs* transcriptional activator function, we generated transgenic tobacco plants harboring translational fusions of the *ocs* gene and the *Escherichia coli lacZ* gene. These constructions all contained the *ocs* promoter but differed in the extent of the *ocs* transcriptional activator sequences employed. The translational fusion was the same as that used by Teeri et al. (1989). The constructions used are similar to some of those shown diagrammatically in Figure 1A, except that a gene encoding an octopine synthase–β-galactosidase fusion protein substituted for a gene encoding the complete octopine synthase protein.

The use of β-galactosidase activity as a histochemical marker in plant tissues depends upon the ability to distinguish between such activity encoded by the *lacZ* gene and endogenous β-galactosidase activity present in plant tissues. The endogenous plant activity can be eliminated by fixation of plant tissues with glutaraldehyde. We found that to abolish endogenous β-galactosidase activity we had to modify the protocols of Teeri et al. (1989) (see Methods). Using such modifications, we could completely eliminate the endogenous activity from plants containing the construction pOCSΔ2 and, therefore, not expressing an *E. coli lacZ* gene (data not shown). In all further experiments, control plants containing the construction pOCSΔ2 were examined parallel to the experimental plants. Only in those instances in which the control plants showed no endogenous activity did we examine further the experimental plants for *lacZ*-encoded β-galactosidase activity. For each construction, multiple tissues taken from three independent transformants were examined. There were no differences in the patterns of β-galactosidase expression among the individual transformants for a given construction.

Figure 2 shows sections taken through the leaves of transgenic tobacco plants harboring the constructions pEN1, pAlu106, pAlRa45, and pPAL16. The second vertical column (Figures 2B, 2E, 2H, and 2K) shows that, in all constructions,

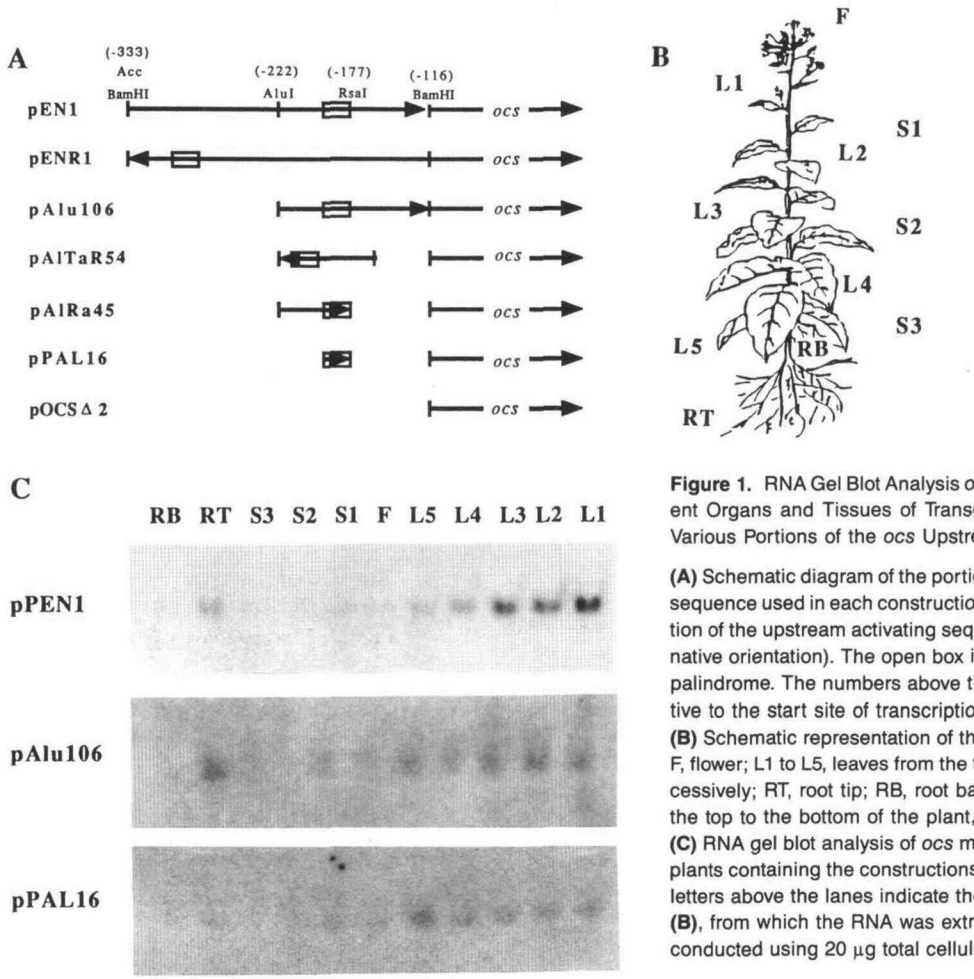

Figure 1. RNA Gel Blot Analysis of *ocs* mRNA Extracted from Different Organs and Tissues of Transgenic Tobacco Plants Containing Various Portions of the *ocs* Upstream Activating Element.

(A) Schematic diagram of the portions of the *ocs* upstream activating sequence used in each construction. The arrow indicates the orientation of the upstream activating sequence fragment (left to right is the native orientation). The open box indicates the position of the 16-bp palindrome. The numbers above the map indicate the position relative to the start site of transcription (+1).

(B) Schematic representation of the tissues assayed for *ocs* mRNA. F, flower; L1 to L5, leaves from the top to the bottom of the plant, successively; RT, root tip; RB, root base; S1 to S3, stem sections from the top to the bottom of the plant, successively.

(C) RNA gel blot analysis of *ocs* mRNA from representative tobacco plants containing the constructions pEN1, pAlu106, and pPAL16. The letters above the lanes indicate the organs or tissues, as defined in **(B)**, from which the RNA was extracted. RNA gel blot analysis was conducted using 20 μg total cellular RNA as described in Methods.

β-galactosidase activity expressed from the *ocs*::*lacZ* fusion gene was present in the leaf mesophyll cells. The activity of the fusion protein in the vascular tissue was strong in those plants harboring the construction pPAL16, weak (and limited to the adaxial phloem cells) in those plants harboring the constructions pAlRa45 and pAlu106, and absent in those plants harboring the construction pEN1. Higher magnification photographs of tangential sections through the leaf blade showed that for plants harboring any of the four constructions the activity of the fusion protein was present in the palisade and spongy parenchyma cells but not in the epidermal cells or in the "stalk" cells of the trichomes. The staining of guard cells could be detected (e.g., Figures 2E, 2H, 2K, and 2L). Subtle differences in the staining patterns of the trichomes could be detected in plants containing the different *ocs* transcriptional activator constructions. Plants containing the construction pPAL16 showed intense staining in the cells in the "head" of both the glandular and nonglandular trichomes (Figures 2K and 2L). Staining in the head of the glandular trichomes was also strong in plants containing the constructions pAlu106 and pAlRa45 but was weak in the heads of the nonglandular trichomes. In plants containing the construction pEN1, only the heads of the glandular, but not the nonglandular, trichomes showed β-galactosidase activity. Such lack of staining in the heads of nonglandular trichomes in pEN1 plants was highly reproducible.

Figure 3 shows the expression of the *ocs*::*lacZ* fusion gene in the guard cells of leaves (Figure 3A) and stems (Figure 3B). This photograph was taken of tissue from a pPAL16 plant; a similar pattern of expression of β-galactosidase activity in the guard cells of pEN1, pAlu106, and pAlRa45 plants was observed (data not shown). Figure 4 shows the histochemical staining of β-galactosidase activity in the stems of transgenic tobacco plants. The first vertical column (Figures 4A, 4E, 4I, and 4M) shows sections at the apical tip of the plant. Successive vertical columns show sections from progressively lower

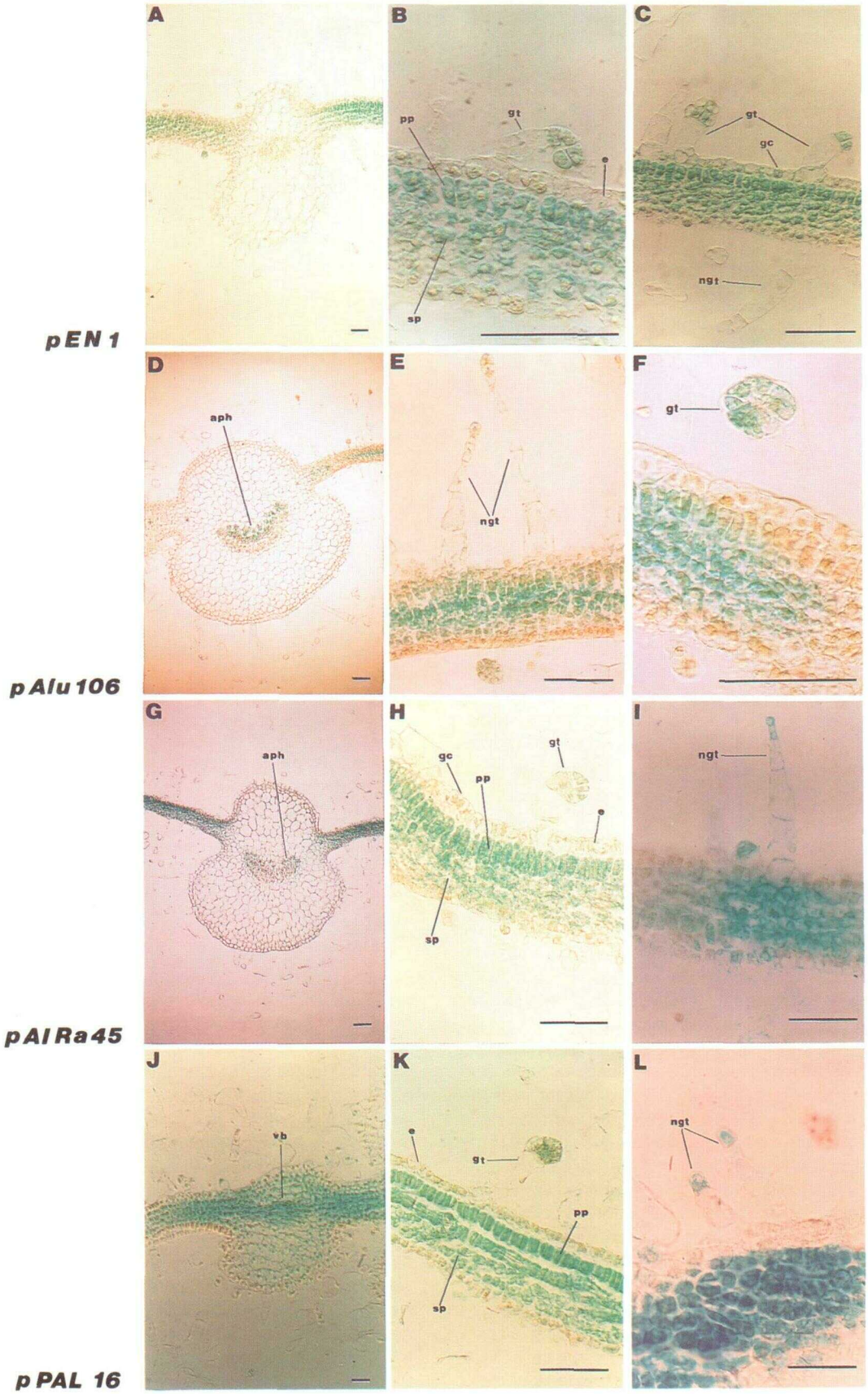

Figure 2. Histochemical Analysis of β-Galactosidase Activity in the Leaves of Transgenic Tobacco Plants Harboring Various *ocs* Upstream Activating Sequence Constructions.

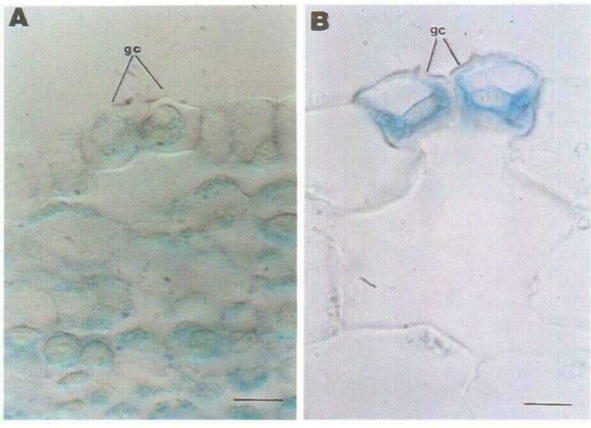

Figure 3. Histochemical Analysis of β-Galactosidase Activity in the Guard Cells of Transgenic Tobacco Plants Harboring the Construction pPAL16.

Tissue from transgenic plants were fixed with glutaraldehyde, stained with X-Gal, embedded, and sectioned as described in Methods. Bars = 10 μm.

(A) Tangential section through the leaf blade of a pPAL16 plant showing guard cells. gc, guard cell.

(B) Section through the stem of a pPAL16 plant showing guard cells. gc, guard cell.

sections of the stem. Activity can be seen in the shoot apex and marginal meristem for all the constructions (Figures 4A, 4E, 4I, and 4M). Only in plants harboring the construction pPAL16, however, could activity be detected in the vascular bundle and the leaf primordium (Figure 4M). Cross-sections of stems from all four constructions showed that the *ocs*::*lacZ* fusion gene was active in both the inner and outer phloem cells but not in the xylem, parenchyma, or epidermal cells (Figures 4B, 4F, 4J, 4L, and 4N). In addition, the fusion gene was active in the leaf gap areas (Figures 4C, 4D, 4G, 4K, and 4O) and in root primordia (Figures 4H and 4P) of plants containing all four constructions.

Figure 5 shows that the expression of the *ocs*::*lacZ* fusion gene in the roots was similar in plants harboring the different constructions. Figures 5A, 5C, 5E, and 5G show that this expression is restricted to the vascular cylinder and is absent in the epidermal and root cap cells. Cross-sections of the root (Figures 5B and 5H) indicate that β-galactosidase activity is detected in the central vascular cylinder but is absent in the epidermis and the cortex. Figure 5F shows that in the upper part of a root (>1 cm from the root tip) expression is limited to the vascular elements. *ocs*::*lacZ* gene activity in an emerging root primordium can be seen in Figure 5D.

DISCUSSION

We have examined the organ and cellular transcriptional specificity conferred upon the octopine synthase gene promoter by various portions of the *ocs* transcriptional activating element. This element consists of a 16-bp palindromic sequence essential for *ocs* gene expression (Ellis et al., 1987a, 1987b; Leisner and Gelvin, 1988, 1989) and surrounding sequences previously shown to be important for determining the quantitative level of expression of the *ocs* gene in tobacco calli (Leisner and Gelvin, 1989). The data presented herein further demonstrate that the 16-bp palindrome is largely responsible for the tissue and cellular specificity of the *ocs* promoter. No β-galactosidase staining was seen in tissue sections from plants containing the construction pOCSΔ2, indicating that the 16-bp palindrome is essential tc direct *ocs* promoter activity. Sequences surrounding the palindrome, however, limit expression of the *ocs* promoter in certain cell types.

Figure 2. (continued).

Leaves of transgenic plants were fixed with glutaraldehyde, stained with X-Gal, embedded, and sectioned as described in Methods. Bars = 100 μm.

(A) Cross-section through the midrib section of a leaf from a pEN1 plant.

(B) Tangential section through the leaf blade of a pEN1 plant showing a glandular trichome. e, epidermis; gt, glandular trichome; pp, palisade parenchyma; sp, spongy parenchyma.

(C) Tangential section through the leaf blade of a pEN1 plant showing both a glandular and nonglandular trichome. gc, guard cell; gt, glandular trichome; ngt, nonglandular trichome.

(D) Cross-section through the midrib section of a leaf from a pAlu106 plant. aph, adaxial phloem.

(E) Tangential section through the leaf blade of a pAlu106 plant showing a nonglandular and glandular trichome. ngt, nonglandular trichome.

(F) Tangential section through the leaf blade of a pAlu106 plant showing a glandular trichome. gt, glandular trichome.

(G) Cross-section through the midrib section of a leaf from a pAlRa45 plant. aph, adaxial phloem.

(H) Tangential section through the leaf blade of a pAlRa45 plant showing a glandular trichome. e, epidermis; gc, guard cell; gt, glandular trichome; pp, palisade parenchyma; sp, spongy parenchyma.

(I) Tangential section through the leaf blade of a pAlRa45 plant showing a nonglandular trichome. ngt, nonglandular trichome.

(J) Cross-section through the midrib section of a leaf from a pPAL16 plant. vb, vascular bundle.

(K) Tangential section through the leaf blade of a pPAL16 plant showing a glandular trichome. e, epidermis; gt, glandular trichome; pp, palisade parenchyma; sp, spongy parenchyma.

(L) Tangential section through the leaf blade of a pPAL16 plant showing nonglandular trichomes. ngt, nonglandular trichome.

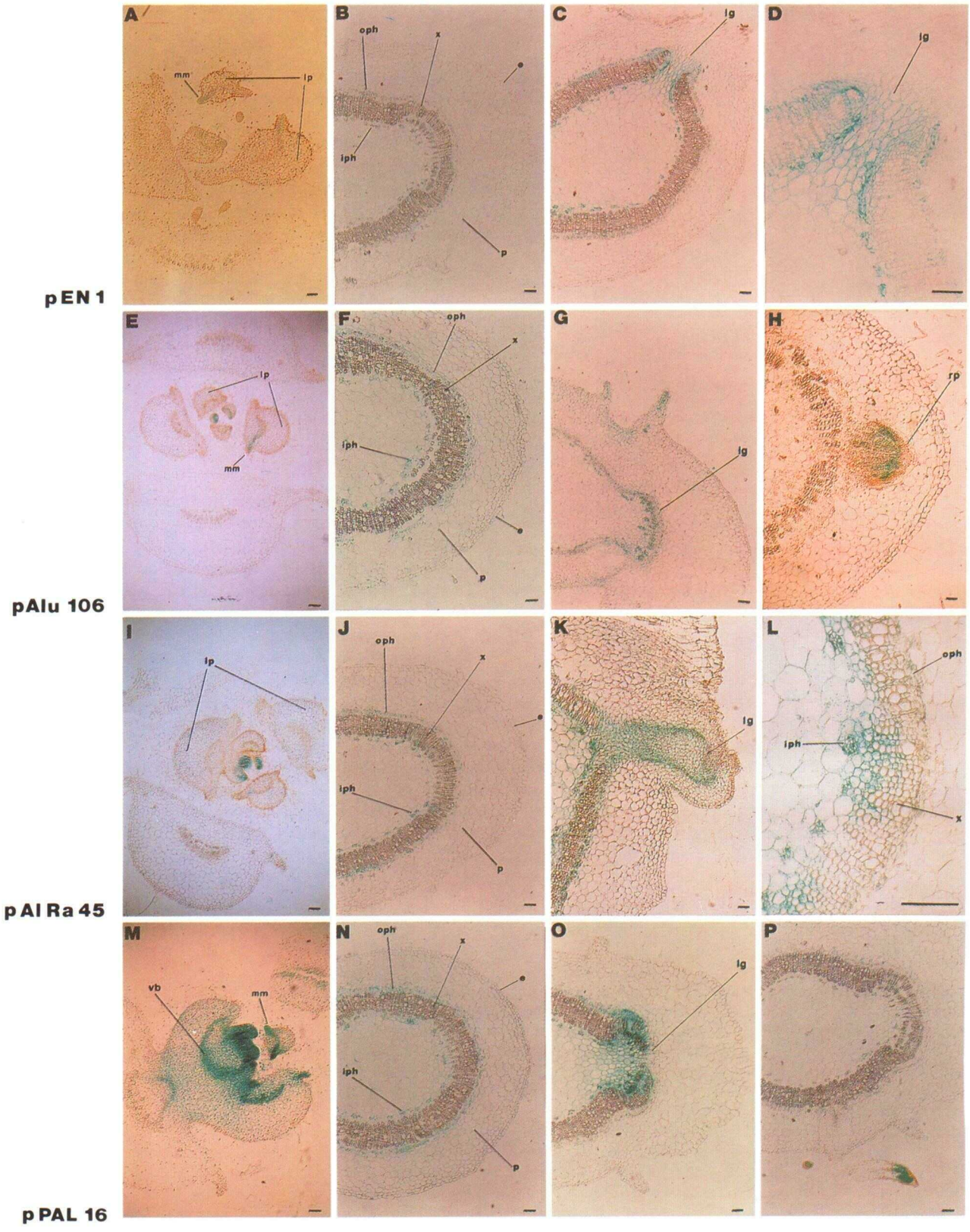

Figure 4. Histochemical Analysis of β-Galactosidase Activity in the Stems of Transgenic Tobacco Plants Harboring Various *ocs* Upstream Activating Sequence Constructions.

In transgenic tobacco plants, the accumulation of *ocs* mRNA is not uniform in different tissues and organs. The expression of the *ocs* gene is greatest in the leaves and root tips, lesser in the stem and the flowers, and very low in the root base. These data suggest that the *ocs* promoter is not active to the same extent in all tobacco cells. The promoter from the nopaline synthase (*nos*) gene, as well as a number of other T-DNA–encoded genes and genes from caulimoviruses, also contain essential elements displaying a high degree of homology to the 16-bp palindrome of the *ocs* upstream activating sequence (Bouchez et al., 1989; Cooke, 1990). The *nos* promoter is not constitutive in tobacco (An et al., 1988). A gradient of activity resulted in higher expression of a *nos*::*CAT* fusion gene in the lower leaves and stem sections than in the upper leaves and stem sections of the plant. The expression of the *nos* promoter was also high in flower sections. Specific sequences in the *nos* promoter region were identified that were responsible for this gradient of activity (Ha and An, 1989).

Our analysis of the expression of the *ocs* gene in tobacco indicates that the *ocs* promoter has a different tissue specificity from that of the *nos* promoter. Using RNA gel blot analysis, we have shown that most of this differential tissue specificity is conferred upon the *ocs* promoter by the 16-bp palindrome portion of the *ocs* upstream activating sequence. RNA gel blot analysis was unable, however, to identify major differences in the tissue-specific pattern of *ocs* gene expression when transcription was directed by different subfragments of the *ocs* transcriptional activating element. That the tissue-specific expression of the octopine synthase gene did not appear to differ among the constructions in these experiments most likely reflects the low resolution of analysis afforded by a technique that relies upon extraction of RNA from tissues with many different cell types. DNA sequences surrounding the homology found within the *ocs* 16-bp palindrome differ substantially among plant promoters, however. Because we have shown that sequences surrounding the 16-bp palindrome of the *ocs* upstream activating sequence can affect the tissue specificity of the *ocs* promoter, we speculate that the different sequences that surround the related element in other plant promoters may likewise affect the tissue specificity of these promoters.

The use of a histochemical staining technique based upon the expression of β-galactosidase activity directed by an *ocs*::*lacZ* fusion gene afforded us a much higher level of resolution of gene expression. This technique allowed us to visualize the specific cell types in which the fusion gene could be expressed. Thus, using a construction that included the entire *ocs* upstream activating sequence, we were able to detect expression of the fusion gene in the mesophyll and guard cells of the leaves, the cells at the tip of glandular trichomes, the internal and external phloem cells and guard cells of the stems, procambial and vascular cells in the root, and cells in meristematic portions of the plant. These meristematic regions included the shoot apex, leaf and root primordia, and cells in the leaf gap area of the stem. Although different constructions incorporating various subfragments of the *ocs* upstream activating sequence did not show greatly differing cellular patterns of gene expression, subtle but reproducible differences were consistently noted. These differences were most noticeable in the vascular tissues of the leaves and apical stem sections and in the leaf primordia. Expression of the *ocs*::*lacZ* fusion gene was undetectable in these cells in plants harboring the construction pEN1 that contains the complete *ocs* upstream activating sequence. Expression in these cells was strong in plants harboring the construction pPAL16 that contains only the 16-bp palindrome portion of the upstream activating sequence. An intermediate

Figure 4. (continued).

For each row, the left-most picture indicates a section through the apical meristem. Pictures progressively to the right in each row show sections taken from successively lower portions of the stem. Tissues were fixed with glutaraldehyde, stained with X-Gal, embedded, and sectioned as described in Methods. Bars = 100 μm.

(A) Section through the apical meristem region of a pEN1 plant. lp, leaf primordium; mm, marginal meristem.
(B) Cross-section through the stem of a pEN1 plant. e, epidermis; iph, inner phloem; oph, outer phloem; p, parenchyma; x, xylem.
(C) Cross-section through a lower portion of a stem from a pEN1 plant showing a leaf gap region. lg, leaf gap.
(D) Higher magnification of the leaf gap region shown in **(C)**. lg, leaf gap.
(E) Section through the apical meristem of a pAlu106 plant. lp, leaf primordium; mm, marginal meristem.
(F) Cross-section through the stem of a pAlu106 plant. e, epidermis, iph, inner phloem; oph, outer phloem; p, parenchyma; x, xylem.
(G) Cross-section through a lower portion of a stem from a pAlu106 plant showing a leaf gap region. lg, leaf gap.
(H) Cross-section through a still lower portion of a stem from a pAlu106 plant showing an emerging root. rp, root primordium.
(I) Section through the apical meristem of a pAlRa45 plant. lp, leaf primordium.
(J) Cross-section through the stem of a pAlRa45 plant. e, epidermis; iph, inner phloem; oph, outer phloem; p, parenchyma; x, xylem.
(K) Cross-section through a lower portion of a stem from a pAlRa45 plant showing a leaf gap region. lg, leaf gap.
(L) Higher magnification of a cross-section through the stem of a pAlRa45 plant. iph, inner phloem; oph, outer phloem; x, xylem.
(M) Section through the apical meristem of a pPAL16 plant. mm, marginal meristem; vb, vascular bundle.
(N) Cross-section through the stem of a pPAL16 plant. e, epidermis; iph, inner phloem; oph, outer phloem; p, parenchyma; x, xylem.
(O) Cross-section through a lower portion of a stem from a pPAL16 plant showing a leaf gap region. lg, leaf gap.
(P) Cross-section through a still lower portion of a pPAL16 stem showing an emerging root.

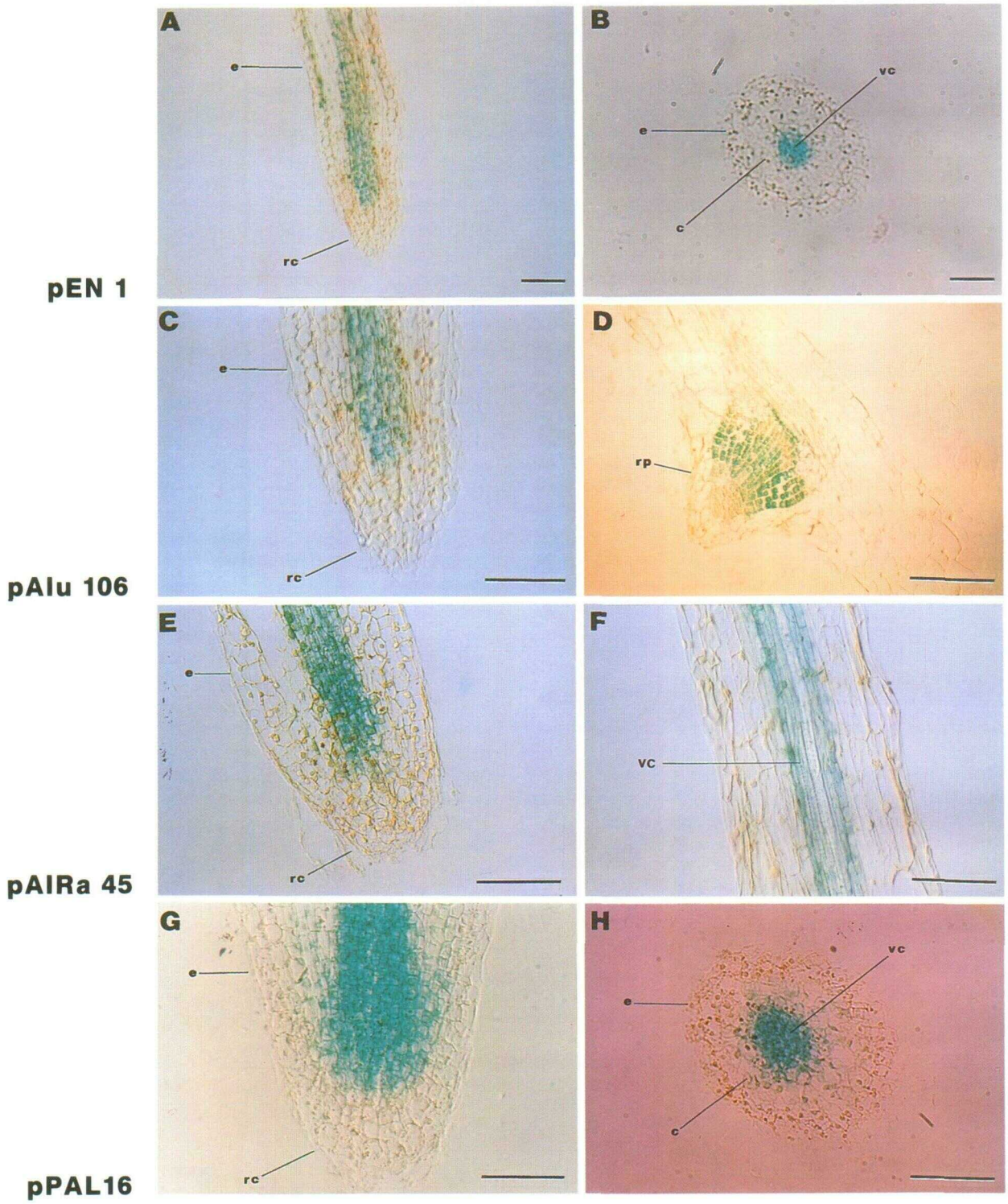

Figure 5. Histochemical Analysis of β-Galactosidase Activity in the Roots of Transgenic Tobacco Plants Harboring Various *ocs* Upstream Activating Sequence Constructions.

Sections were fixed with glutaraldehyde, stained with X-Gal, embedded, and sectioned as described in Methods. Bars = 100 μm.

(A) Longitudinal section through the root tip of a pEN1 plant. e, epidermis; rc, root cap.

(B) Cross-section through the root of a pEN1 plant. c, cambium; e, epidermis; vc, vascular cylinder.

(C) Longitudinal section through the root tip of a pAlu106 plant. e, epidermis; rc, root cap.

(D) Section through the root of a pAlu106 plant showing an emerging root hair. rp, root primordium.

level of expression was seen in the vascular tissues in plants harboring the constructions pAlu106 and pAlRa45 that contain the 16-bp palindrome plus 90 or 29 bp, respectively, of DNA surrounding the palindrome. In addition, β-galactosidase activity was not detectable in the cells at the head of nonglandular trichomes in plants containing the construction pEN1, but expression of the *ocs::lacZ* fusion gene was readily detected in these cells from the pPAL16 plants. It thus appears that DNA sequences surrounding the 16-bp palindrome of the *ocs* upstream activating sequence limit the expression of the linked *ocs* promoter to particular cell types. Silencer sequences that restrict the expression of the *cab* gene in nonroot cells have been identified (Simpson et al., 1986). It should be emphasized, however, that the 16-bp palindrome does not render the *ocs* promoter completely constitutive; expression is still highly specific to particular cell types.

Our data appear to contradict those of Teeri et al. (1989). These authors examined the expression of an *ocs::lacZ* fusion gene in the roots of transgenic tobacco plants. Their analysis showed strong expression of β-galactosidase activity in the epidermal cells of these roots but no expression in the procambial or vascular cells. Our analysis indicated the opposite pattern of expression: we detected β-galactosidase activity only in the procambial and vascular cells but not in the epidermis or in the root cap. The *lacZ* fusion of Teeri et al. (1989) used a position within the *ocs* gene identical to ours. Although it is not possible from the article of Teeri et al. (1989) to determine which portions of the *ocs* upstream activating sequence they included in their construction, our analyses, using the entire upstream activating sequence as well as various portions of this sequence, identified a consistent pattern of expression in the roots that was not dependent upon the region of the upstream activating sequence used in the construction. It is, therefore, unlikely that the differences between our results and those of Teeri et al. (1989) can be attributed to the use of different fragments of the *ocs* upstream activating sequence. The reason for the differences in the results between the two groups cannot presently be explained.

The pattern of expression directed by the *ocs* upstream activating sequence in the roots of transgenic tobacco plants identified in this report resembles the pattern identified by Fromm et al. (1989). These authors were unable, however, to detect expression directed by this upstream activating sequence in the stem or leaves of these plants. We were easily able to detect expression in these tissues. Fromm et al. (1989) used a construction consisting of portions of the *ocs* upstream activating sequence (including the 16-bp palindrome) fused to a truncated CaMV 35S promoter. This promoter/activator combination was affixed to a GUS gene as a transcriptional fusion. Our constructions contained various portions of the *ocs* upstream activating sequence, the *ocs* promoter, and the first third of the *ocs* structural gene. Because we used homologous portions of the *ocs* regulatory and transcribed sequences, our constructions most likely reflect the true pattern of *ocs* gene expression seen in transgenic plants. Indeed, at the level of resolution afforded by RNA gel blot analysis, our histochemical data and our analysis of the steady-state levels of RNA in the tissues of transgenic tobacco plants were consistent. It is possible that the combination of the *ocs* upstream activating sequence and the truncated CaMV promoter used by Fromm et al. (1989) resulted in a novel pattern of expression that differed from that seen using the homologous *ocs* upstream activating sequence and promoter sequences.

The 16-bp palindrome of the octopine synthase transcriptional activating sequence binds the nuclear protein OCSBF-1 (Singh et al., 1989, 1990; Tokuhisa et al., 1990). Analysis of the expression of the OCSBF-1 gene in maize indicated that the gene was most highly expressed in actively dividing and meristematic cells (Singh et al., 1990). Such an analysis has not yet been reported for tobacco. It is possible that the cell-specific expression of the *ocs* gene in tobacco reflects the prevalence of OCSBF-1 in certain cell types. The cell-specific pattern of *ocs* gene expression could then be explained by DNA sequences that regulate OCSBF-1 gene expression rather than DNA sequences that regulate *ocs* gene expression. We feel that although it is possible that this may represent a major level of *ocs* gene regulation in tobacco it cannot be the sole mechanism of *ocs* gene regulation. Sequences surrounding the 16-bp palindrome clearly modulate the expression of the *ocs* promoter both in particular cell types of tobacco plants (this study) and the quantitative level of expression of the *ocs* promoter in tobacco calli (Leisner and Gelvin, 1989). The synergistic interactions of various subdomains of the CaMV 35S promoter to yield novel cell–specific patterns of expression have been documented (Benfey et al., 1990a, 1990b). It is possible that, perhaps to a lesser extent, the *ocs* upstream activating sequence is also composed of subdomains that when deleted or combined with transcriptional activating elements from other genes can confer novel patterns of expression upon linked promoters. Experiments to test this hypothesis are currently underway in this laboratory.

Figure 5. (continued).

(E) Longitudinal section through the root tip of a pAlRa45 plant. e, epidermis; rc, root cap.

(F) Longitudinal section through a region of a root of a pAlRa45 plant >1 cm from the root tip. vc, vascular cylinder.

(G) Longitudinal section through the root tip of a pPAL16 plant. e, epidermis; rc, root cap.

(H) Cross-section through the root tip of a pPAL16 plant. c, cambium; e, epidermis; vc, vascular cylinder.

METHODS

Bacterial Strains and Media

Escherichia coli DH5α was grown in LB medium (Maniatis et al., 1982) at 37°C. *Agrobacterium tumefaciens* LBA4404 and derivatives were grown in either AB minimal medium containing 0.5% sucrose or YEP medium (Lichtenstein and Draper, 1986) at 30°C. Antibiotics were used at the following concentrations (μg/mL): for *E. coli*—ampicillin, 100; kanamycin, 50; for *A. tumefaciens*—carbenicillin, 100; kanamycin, 100; rifampin, 10. Plasmids were mobilized from *E. coli* to *A. tumefaciens* by a triparental mating method (Ditta et al., 1980) using the mobilizing functions of pRK2013 (Figurski and Helinski, 1979).

Plasmid Constructions

Plasmids containing *ocs::lacZ* translational fusions were derived from the previously described plasmids pEN1, pAlu106, pAlRa45, and pPAL16 (Leisner and Gelvin, 1989), which contained the octopine synthase gene and promoter and various portions of the *ocs* transcriptional activating element. pRS414 (Simons et al., 1987), containing a promoterless *lacZYA* sequence, was digested with SnaBI (which cuts in the *lacY* gene) and a HincII fragment was inserted that contains bidirectional polyadenylation signal sequences from the T-DNA 0′ and 1′ genes (coordinates, 21,727 to 22,440; Barker et al., 1983). The resulting plasmid (pRS414-polyA) was linearized at the unique EcoRI site preceding the *lacZ* gene. pEN1, pAlu106, pAlRa45, and pPAL16 were digested with EcoRI (which deletes the carboxy portion of the octopine synthase protein and the *ocs* polyA signal sequence) and individually ligated to pRS414-polyA. The resulting plasmids (pEN1-lac, pAlu106-lac, pAlRa45-lac, and pPAL16-lac) contain various portions of the *ocs* transcriptional activating element linked to the *ocs* promoter and an octopine synthase–β-galactosidase translational fusion.

Nucleic Acid Manipulations

Recombinant DNA procedures were performed according to Maniatis et al. (1982) under P1 containment conditions as specified by the guidelines of the National Institutes of Health.

RNA was extracted from plant tissue (15 plants were examined) by the following procedure: tissue (0.5–4.0 g) was frozen in liquid N_2 and ground to a fine powder using a mortar and pestle. One milliliter of Tris-neutralized phenol was added, and the tissue was ground further while thawing. One milliliter of RNA extraction buffer (0.5 M Tris-HCl, pH 7.0, 100 mM NaCl, 50 mM Na_2·EDTA, and 1.0% SDS) was added, and the tissue was ground until it reached a homogeneous consistency. The mixture was transferred to a 15-mL polypropylene tube and centrifuged at 10,000 rpm for 15 min. The supernatant solution was removed to a fresh tube, and the pellet was reextracted with 1 mL RNA extraction buffer. The aqueous phases were combined and reextracted with 2 mL phenol. The supernatant solution was collected, and nucleic acids were precipitated by the addition of 4 mL ethanol. Following incubation at −20°C, the nucleic acids were collected by centrifugation, dried, and dissolved in 0.5 mL water; then 0.25 mL 6 M LiCl was added. The samples were incubated at 4°C overnight, and the RNA was collected by centrifugation. The RNA was dissolved in H_2O and precipitated by the addition of 0.1 volume 3 M NaOAc and 2.5 volumes ethanol. The RNA was collected by centrifugation and resuspended in H_2O, and the concentration was determined by UV light absorption at 260 nm.

Twenty micrograms of total cellular RNA was fractionated by formaldehyde-agarose gel electrophoresis, blotted to nitrocellulose, and hybridized to a probe containing the *ocs* gene (a 951-bp BstNI fragment; coordinates, 12,602 to 13,553; Barker et al., 1983), as previously described (Karcher et al., 1984).

Plant Transformation and Histochemical Detection of β-Galactosidase Activity

Nicotiana tabacum cv Wisconsin 38 leaf discs were infected with *A. tumefaciens* LBA4404 containing various *ocs::lacZ* fusion constructions. Kanamycin-resistant transgenic plants were regenerated according to Horsch et al. (1985). Sterile plants were maintained in Magenta cubes at 25°C and propagated vegetatively by transferring shoot cuttings. Rooted plants were assayed for β-galactosidase activity 4 weeks following transfer.

Histochemical staining was performed as described by Teeri et al. (1989), with the following modifications. Leaf, stem, and root tissue of transgenic and control plants were fixed with glutaraldehyde in Z buffer (pH 7.4) at room temperature. For each construction, the expression of the *ocs::lacZ* fusion gene was analyzed in a young leaf near the top of the plant and in an older leaf near the base, in the upper and lower parts of the stem, and in the roots. Leaf and stem sections were fixed in 1.0% glutaraldehyde and roots in 0.5% glutaraldehyde for 1 hr. For better penetration of the fixative, the tissue was flash frozen in liquid nitrogen and fixed again in glutaraldehyde for 1 hr. The glutaraldehyde was removed by rinsing the tissue three times in Z buffer; then the tissues were incubated in staining solution (880 μL Z buffer, 50 μL 100 mM $K_3[Fe(CN)_6]$, 50 μL 10 mM $K_4[Fe(CN)_6]$, 8.0% X-Gal) overnight at 28°C. The material was rinsed with Z buffer twice and treated with acetomethanol (1:3) for 1 hr at room temperature to remove chlorophyll. After rinsing in 95% ethanol and absolute ethanol, the tissue was embedded in Tissue Prep 2 medium (Fisher; melting point 56.5°C) and cut into 20-μm sections.

Control plant tissue (a kanamycin-resistant transgenic tobacco plant containing the construction pOCSΔ2) was always prepared parallel to the experimental samples. Experimental samples were analyzed only if the control plant showed no endogenous β-galactosidase activity.

ACKNOWLEDGMENTS

The authors thank Dr. Mary-Alice Webb for useful discussions and Drs. John Hamer and Susan Karcher for critical reading of this manuscript. This work was supported by a grant from the Midwest Plant Biotechnology Consortium.

Received August 19, 1991; accepted November 7, 1991.

REFERENCES

An, G. (1987). A potential Z-DNA–forming sequence is an essential upstream element of a plant promoter. BioEssays **7**, 211–214.

An, G., Costa, M.A., Mitra, A., Ha, S.-B., and Marton, L. (1988). Organ-specific and developmental regulation of the nopaline synthase promoter in transgenic tobacco plants. Plant Physiol. **88,** 547–552.

Bandyopadhyay, R.S., Bruce, W.B., and Gurley, W.B. (1989). Regulatory elements within the agropine synthase promoter of T-DNA. J. Biol. Chem. **264,** 19399–19406.

Barker, R.F., Idler, K.B., Thompson, D.V., and Kemp, J.D. (1983). Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti-plasmid pTi15955. Plant Mol. Biol. **2,** 335–350.

Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8,** 2195–2202.

Benfey, P.N., Ren, L., and Chua, N.-H. (1990a). Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J. **9,** 1677–1684.

Benfey, P.N., Ren, L., and Chua, N.-H. (1990b). Combinatorial and synergistic properties of CaMV 35S enhancer subdomains. EMBO J. **9,** 1685–1696.

Bouchez, D., Tokuhisa, J.G., Llewellyn, D.J., Dennis, E.S., and Ellis, J.G. (1989). The *ocs*-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J. **8,** 4197–4204.

Bruce, W.B., Bandyopadhyay, R., and Gurley, W.B. (1988). An enhancer-like element present in the promoter of a T-DNA gene from the Ti plasmid of *Agrobacterium tumefaciens.* Proc. Natl. Acad. Sci. USA **85,** 4310–4314.

Comai, L., Moran, P., and Maslyar, D. (1990). Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol. Biol. **15,** 373–381.

Cooke, R. (1990). The figwort mosaic virus gene VI promoter region contains a sequence highly homologous to the octopine synthase (*ocs*) enhancer element. Plant Mol. Biol. **15,** 181–182.

de Pater, B.S., Klinkhamer, M.P., Amesz, P.A., de Kam, R.J., Memelink, J., Hoge, J.H., and Schilperoort, R.A. (1987). Plant expression signals of the *Agrobacterium* T-cyt gene. Nucl. Acids Res. **15,** 8267–8281.

DiRita, V.J., and Gelvin, S.B. (1987). Deletion analysis of the mannopine synthase gene promoter in sunflower crown gall tumors and *Agrobacterium tumefaciens.* Mol. Gen. Genet. **207,** 233–241.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980). Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci. USA **77,** 7347–7351.

Ellis, J.G., Llewellyn, D.J., Dennis, E.S., and Peacock, W.J. (1987a). Maize *Adh-1* promoter sequences control anaerobic regulation: Addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. EMBO J. **6,** 11–16.

Ellis, J.G., Llewellyn, D.J., Walker, J.C., Dennis, E.S., and Peacock, W.J. (1987b). The *ocs* element: A 16 base pair palindrome essential for activity of the octopine synthase enhancer. EMBO J. **6,** 3203–3208.

Figurski, D.H., and Helinski, D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans.* Proc. Natl. Acad. Sci. USA **76,** 1648–1652.

Fromm, H., Katagiri, F., and Chua, N.-H. (1989). An octopine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell **1,** 977–984.

Ha, S.-B., and An, G. (1989). *Cis*-acting regulatory elements controlling temporal and organ-specific activity of nopaline synthase promoter. Nucl. Acids Res. **17,** 215–223.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Karcher, S.J., DiRita, V.J., and Gelvin, S.B. (1984). Transcript analysis of T_RDNA in octopine-type crown gall tumors. Mol. Gen. Genet. **194,** 159–165.

Langridge, W.H.R., Fitzgerald, K.J., Koncz, C., Schell, J., and Szalay, A.A. (1989). Dual promoter of *Agrobacterium tumefaciens* mannopine synthase gene is regulated by plant growth hormones. Proc. Natl. Acad. Sci. USA **86,** 3219–3223.

Leisner, S.M., and Gelvin, S.B. (1988). Structure of the octopine synthase upstream activator sequence. Proc. Natl. Acad. Sci. USA **85,** 2553–2557.

Leisner, S.M., and Gelvin, S.B. (1989). Multiple domains exist within the upstream activator sequence of the octopine synthase gene. Plant Cell **1,** 925–936.

Lichtenstein, C., and Draper, J. (1986). Genetic engineering of plants. In DNA Cloning, A Practical Approach, Vol. 2, D.M. Glover, ed (Washington, DC: IRL Press), pp. 67–119.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Mitra, A., and An, G. (1989). Three distinct regulatory elements comprise the upstream promoter region of the nopaline synthase gene. Mol. Gen. Genet. **215,** 294–299.

Simons, R.W., Houman, F., and Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53,** 85–96.

Simpson, J., Schell, J., Van Montagu, M., and Herrera-Estrella, L. (1986). Light-inducible and tissue-specific pea lhcp gene expression involves an upstream element combining enhancer- and silencer-like properties. Nature **323,** 551–554.

Singh, K., Dennis, E.S., Ellis, J.G., Llewellyn, D.J., Tokuhisa, J.G., Wahleithner, J.A., and Peacock, W.J. (1990). OCSBF-1, a maize *ocs* enhancer binding factor: Isolation and expression during development. Plant Cell **2,** 891–903.

Singh, K., Tokuhisa, J.G., Dennis, E.S., and Peacock, W.J. (1989). Saturation mutagenesis of the octopine synthase enhancer: Correlation of mutant phenotypes with binding of a nuclear protein factor. Proc. Natl. Acad. Sci. USA **86,** 3733–3737.

Teeri, T.H., Lehvaslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E.T., Van Montagu, M., and Herrera-Estrella, L. (1989). Gene fusions to *lacZ* reveal new expression patterns of chimeric genes in transgenic plants. EMBO J. **8,** 343–350.

Tokuhisa, J.G., Singh, K., Dennis, E.S., and Peacock, W.J. (1990). A DNA-binding protein factor recognizes two binding domains within the octopine synthase enhancer element. Plant Cell **2,** 215–224.

Wasylyk, B. (1988). Transcription elements and factors of RNA polymerase B promoters of higher eukaryotes. CRC Rev. **23,** 77–120.