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RESEARCH ARTICLE

The Promoter of the Gene Encoding the C_4 Form of Phosphoenolpyruvate Carboxylase Directs Mesophyll-Specific Expression in Transgenic C_4 *Flaveria* spp

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The function of the C_4 mechanism of photosynthesis depends on the strict compartmentation of the enzymes involved. Here, we investigate the regulatory mechanisms that ensure the mesophyll-specific expression of the C_4 isoform of phosphoenolpyruvate carboxylase. We show that 2 kb of the 5′ flanking region of the *Flaveria trinervia* C_4 *PpcA1* gene is sufficient to direct mesophyll-specific expression of the β-glucuronidase reporter gene in transgenic *F. bidentis* (C_4) plants. In young leaves of seedlings, the activity of this promoter is dependent on the developmental stage of the mesophyll cells. It is induced in a basipetal fashion (leaf tip to base) during leaf development. The promoter region of the orthologous nonphotosynthetic *Ppc* gene of *F. pringlei* (C_3) induces reporter gene expression mainly in the vascular tissue of leaves and stems as well as in mesophyll cells of transgenic *F. bidentis* plants. Our experiments demonstrate that during the evolution of the C_4 *Flaveria* species, *cis*-acting elements of the C_4 *Ppc* gene must have been altered to achieve mesophyll-specific expression.

INTRODUCTION

The photosynthetic C_4 cycle acts as a pump that concentrates CO_2 at the site of the ribulose bisphosphate carboxylase (RBCS). Photorespiration is thereby largely abolished, and the net photosynthesis rate is increased. The concentration of CO_2 is achieved by the metabolic interaction of mesophyll and bundle sheath cells and is dependent on the correct compartmentation of the enzymes of the C_4 photosynthetic carbon assimilation pathway (reviewed in Hatch, 1978, 1987). It has been shown that this compartmentation is due to differential gene expression (reviewed in Nelson and Langdale, 1992).

The C_4 mechanism of photosynthesis has evolved independently several times during the evolution of angiosperms. This suggests that the changes required for the establishment of a functional C_4 cycle could have been easily accomplished in evolutionary terms. All key enzymes of C_4 photosynthesis, that is, phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase, and NADP malic enzyme, are not unique to C_4 plants but can also be detected in C_3 plants, in which they perform various functions required for basic cell metabolism. These genes have served as the basis for the evolution of the C_4 genes (Moore, 1982; Monson and Moore, 1989).

Closely related C_3 and C_4 species, occurring within a single genus, provide an ideal experimental system for the investigation of the evolution of C_4 genes when a comparative approach is used. Such a strategy is based on the assumption that a non-C_4 isoform gene (hence here referred to as C_3 isoform), which represents the nearest neighbor of a C_4 gene, is still very much like the original C_3 ancestral gene with respect to function and expression properties. Hence, a comparison of such a pair of C_3 and C_4 genes would allow us to identify the changes in gene structure and function that occurred during the evolution of the C_4 gene. A plant system that is well suited for this type of experimental approach is the genus *Flaveria* of the Asteraceae (Powell, 1978). *Flaveria* spp contain C_3 and C_4 species and a large number of C_3/C_4 intermediates that are well characterized by physiological and biochemical experiments (reviewed in Edwards and Ku, 1987). Recently, a transformation procedure has been established for the C_4 species *F. bidentis* of this genus (Chitty et al., 1994). Thus, an analysis of gene

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function and promoter activity using transgenic plants is possible.

We are interested in the regulatory mechanism of the gene encoding the C_4 isoform of PEPC and have chosen the closely related species *F. trinervia* (C_4) and *F. pringlei* (C_3) as our experimental organisms. In these species, PEPC is encoded by a small gene family that can be divided into four different subclasses (designated *PpcA* to *PpcD*), with each characterized by a distinct expression profile (Hermans and Westhoff, 1990). Each class of genes of *F. trinervia* is more similar to the corresponding class of *F. pringlei* than to the other *Ppc* subclasses of *F. trinervia*; hence, they represent pairs of orthologous *Ppc* genes (Hermans and Westhoff, 1992). The *PpcA1* gene of *F. trinervia* (C_4) encodes the C_4 isoform of PEPC. This gene is expressed at high levels in leaves and at very low levels in stems and roots. The corresponding orthologous *PpcA1* gene of *F. pringlei* (C_3) is expressed in a more or less constitutive fashion, with low levels being expressed in leaves, stems, and roots. This pair of orthologous genes with their different expression profiles provides a unique experimental system to determine how the high level of mesophyll-specific expression of the C_4 *PpcA* gene is achieved in the C_4 species of the genus *Flaveria*.

Experiments with a homologous transient expression system using maize protoplasts revealed that the mesophyll-specific expression of the C_4 *Ppc* gene in maize is at least partially due to regulation of transcription (Schäffner and Sheen, 1992). The maize C_4 *Ppc* promoter has also been shown to drive the β-glucuronidase (*GUS*) reporter gene expression preferentially in mesophyll cells of transgenic rice (C_3) plants (Matsuoka et al., 1994). These studies, as well as promoter studies performed with the *PpcA1* gene of *F. trinervia* in tobacco (Stockhaus et al., 1994), indicated that the initiation of transcription plays a major role in the mesophyll-specific expression of the C_4 *Ppc* genes.

Cell separation techniques have been used to demonstrate that in *F. trinervia*, the C_4 *Ppc* gene is almost exclusively expressed in mesophyll cells, whereas there was no significant *Ppc* expression detected in bundle sheath cells (Höfer et al., 1992). Our goal was to localize the *cis*-acting regulatory elements of the C_4 *Ppc* gene that are responsible for its characteristic expression pattern. In this study, we address the cell specificity of the promoters of the orthologous *PpcA1* genes of *F. pringlei* (C_3) and *F. trinervia* (C_4) by using stably transformed *F. bidentis* (C_4) plants. It is important to note that *F. trinervia* and *F. bidentis* are so closely related that they can be crossed (Powell, 1978).

The use of transgenic plants allows us to follow promoter activity in different organs at different developmental stages of the plant. Based on a comparison of the expression analysis of the two *Ppc* promoter–*GUS* chimeric genes obtained with the tobacco C_3 plant and with *F. bidentis* C_4 plants, we discuss the evolutionary aspects of the switch from a constitutively expressed nonphotosynthetic *Ppc* gene to a C_4 *Ppc* gene that is highly expressed in mesophyll cells.

RESULTS

Chimeric *PpcA1* Promoter–*GUS* Genes and *F. bidentis* Transformation

Figure 1 shows the two chimeric genes *PpcA1-L-Ft–GUS* and *PpcA1-L-Fp–GUS* used in this study. (Ft is the abbreviation for the *F. trinervia* C_4 plant, and Fp stands for the *F. pringlei* C_3 plant.) These are the same constructs that were integrated into the genome of the tobacco C_3 plant (Stockhaus et al., 1994). The 3′ borders of the promoter fragments are located just 5′ of the AUG translation initiation codon. The lengths of the *PpcA* gene fragments relative to the AUG codon are 2185 bp for the *PpcA1-L-Ft–GUS* gene and 2583 bp for the *PpcA1-L-Fp–GUS* gene. Recent sequence analysis of other members of the *Ppc* gene family in *Flaveria* spp revealed the presence of one intron in the 5′ untranslated region of the *Ppc* genes (Ernst and Westhoff, 1997). The position of this intron is indicated in Figure 1. The untranslated leader sequences included in the *PpcA1-L-Ft–GUS* construct and in the *PpcA1-L-Fp–GUS* construct are 234 and 248 bp long, respectively. In Figure 1, the gray boxes indicate regions of similarity between the *PpcA1* genes of *F. pringlei* and *F. trinervia* (>60% identical residues in a window of 15 bp; Hermans and Westhoff, 1992). The constructs were transformed into the Agrobacterium strain AGL1 (Lazo et al., 1991). The Agrobacterium strains were analyzed for the integrity of the chimeric genes and used for *F. bidentis* transformation. Transgenic *F. bidentis* plants were subjected to DNA gel blot analysis. The results of these experiments demonstrated that the integrated chimeric genes were intact (data not shown).

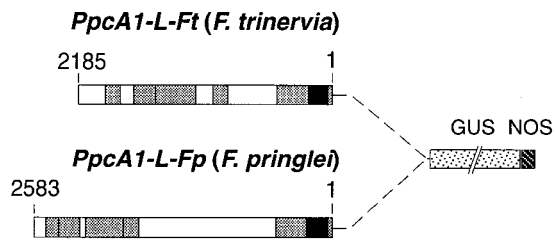

Figure 1. The *PpcA1–GUS* Chimeric Genes Used for *F. bidentis* Transformation.

The end points of the *PpcA1* gene fragments are indicated in the numbers of nucleotides relative to the translation initiation codon. Regions of sequence similarity between the *PpcA1* genes of *F. pringlei* and *F. trinervia* (>60% identical residues in a window of 15 bp; Hermans and Westhoff, 1992) are indicated by gray boxes. Introns in the 5′ untranslated region of the *PpcA1* genes are indicated by black boxes. The introns extend from nucleotides 40 to 208 in the *F. trinervia PpcA1* gene and from nucleotides 33 to 211 in the *PpcA1 F. pringlei* gene (relative to the AUG translation initiation codon). GUS, *GUS* coding region; NOS, nopaline synthase polyadenylation signal.

Table 1. GUS Activities Detected in Extracts of Leaves, Stems, and Roots of Different Transgenic *F. bidentis* Plants

Plant	Leaf (pmol MU mg^{-1} min^{-1})[a]	Stem (pmol MU mg^{-1} min^{-1})[a]	Root (pmol MU mg^{-1} min^{-1})[a]
PpcA1-L-Ft1[b]	147,000	33	1
PpcA1-L-Ft2[b]	76,000	48	4
PpcA1-L-Ft3[b]	4,460	23	14
PpcA1-L-Ft4[b]	43,600	26	4
PpcA1-L-Ft5[b]	103,700	18	4
PpcA1-L-Fp1[c]	426	220	4
PpcA1-L-Fp2[c]	4,600	6,640	13
PpcA1-L-Fp3[c]	1,722	2,240	2
PpcA1-L-Fp4[c]	3,730	14,400	3
PpcA1-L-Fp5[c]	3,740	6,770	30
F. bidentis WT[d]	4	1	1

[a] GUS activities are expressed in picomoles of the reaction product 4-methylumbelliferone (MU) generated per milligram of protein per minute.
[b] Mean value ± SE (pmol MU mg^{-1} min^{-1}): leaf, 74,952 ± 54,704; stem, 30 ± 12; root, 5 ± 5.
[c] Mean value ± SE (pmol MU mg^{-1} min^{-1}): leaf, 2843 ± 1715; stem, 6054 ± 5456; root, 10 ± 11.
[d] WT, wild type.

Expression Levels of *PpcA1–GUS* Genes in Leaves, Stems, and Roots of Transgenic *F. bidentis* Plants

GUS activities of five independent transgenic T_0 plants for each construct were determined in extracts of leaf, stem, and root tissues by using the fluorometric GUS assay. These experiments were repeated at least once, and the results did not vary by >5%. The results of these experiments are presented in Table 1. The *PpcA1-L-Ft* promoter is highly active in leaves, whereas in stem and root extracts, GUS activity is close to the limit of detection at approximately three orders of magnitude (stem) and four orders of magnitude (root) lower than in leaf extracts. This result demonstrates that in *F. bidentis*, the *PpcA1-L-Ft* promoter is almost exclusively active in leaf cells. The GUS expression induced by the *PpcA1-L-Fp* promoter is 20- to 60-fold lower in *F. bidentis* leaves than the expression induced by the *PpcA1-L-Ft* promoter (Table 1). In stems, the expression level induced by the *PpcA1-L-Fp* promoter appears to be higher than in leaves, but the difference is not very large. In roots, however, the measured GUS activities are close to the level of detection, as in the case of the *PpcA1-L-Ft* construct, and slightly higher than background levels found in wild-type plants (Table 1).

Cell Specificity of Expression

The expression of the *GUS* reporter gene can be detected by histochemical methods that lead to a blue staining of cells expressing the *GUS* gene. The results summarized here are based on the examination of a large number of sections of various plant organs of the five independent transgenic plants for each construct presented in Table 1. The incubation times of the sections varied from 5 min to 16 hr to obtain reliable information about the cell specificity of expression induced by both promoters. In Figure 2, an analysis of two transgenic T_0 plants is shown. The histochemical analysis was repeated with T_1 plants carrying the different promoter and *GUS* fusions. In T_0 and T_1 plants, there were no differences observed in the cell specificity of GUS expression. Figures 2A and 2B show that in leaf sections of the transgenic *PpcA1-L-Ft–GUS* plant, the mesophyll cells were intensely stained. In several transgenic plants, mesophyll-specific staining could be observed after only 5 to 10 min of incubation. In these sections, there was no staining observed in bundle sheath cells, vascular tissues, and epidermal cells of the leaf. Incubation times >1 hr led to a diffusion of the blue dye into the bundle sheath cells. In this case, the staining became visible in the area of the bundle sheath cells neighboring the heavily stained mesophyll cells (Figures 2A and 2B).

It is interesting that the mesophyll cells did not stain with equal intensities. The highest intensity of staining was observed in mesophyll cells surrounding the bundle sheaths (Figures 2A and 2B). This is more obvious in leaf sections in which the distance between two bundles is wider than a few cell layers. At the underside of the leaf, spongy mesophyll cells, which are not close to bundle sheath cells, did not show any blue staining (Figure 2A). This result is not due to a staining artifact that might have arisen from differences in substrate penetration into these cells. Figure 2D shows a leaf section of a *F. bidentis* plant transformed with a cauliflower mosaic virus 35S–*GUS* gene (Chitty et al., 1994). Here, the different mesophyll cell types and bundle sheath cells are stained.

The specific ringlike staining pattern of the mesophyll cells surrounding the bundle sheaths, induced by the *PpcA1-L-Ft* promoter, appears to be correlated with the presence of large numbers of chloroplasts in the stained cells. The distribution

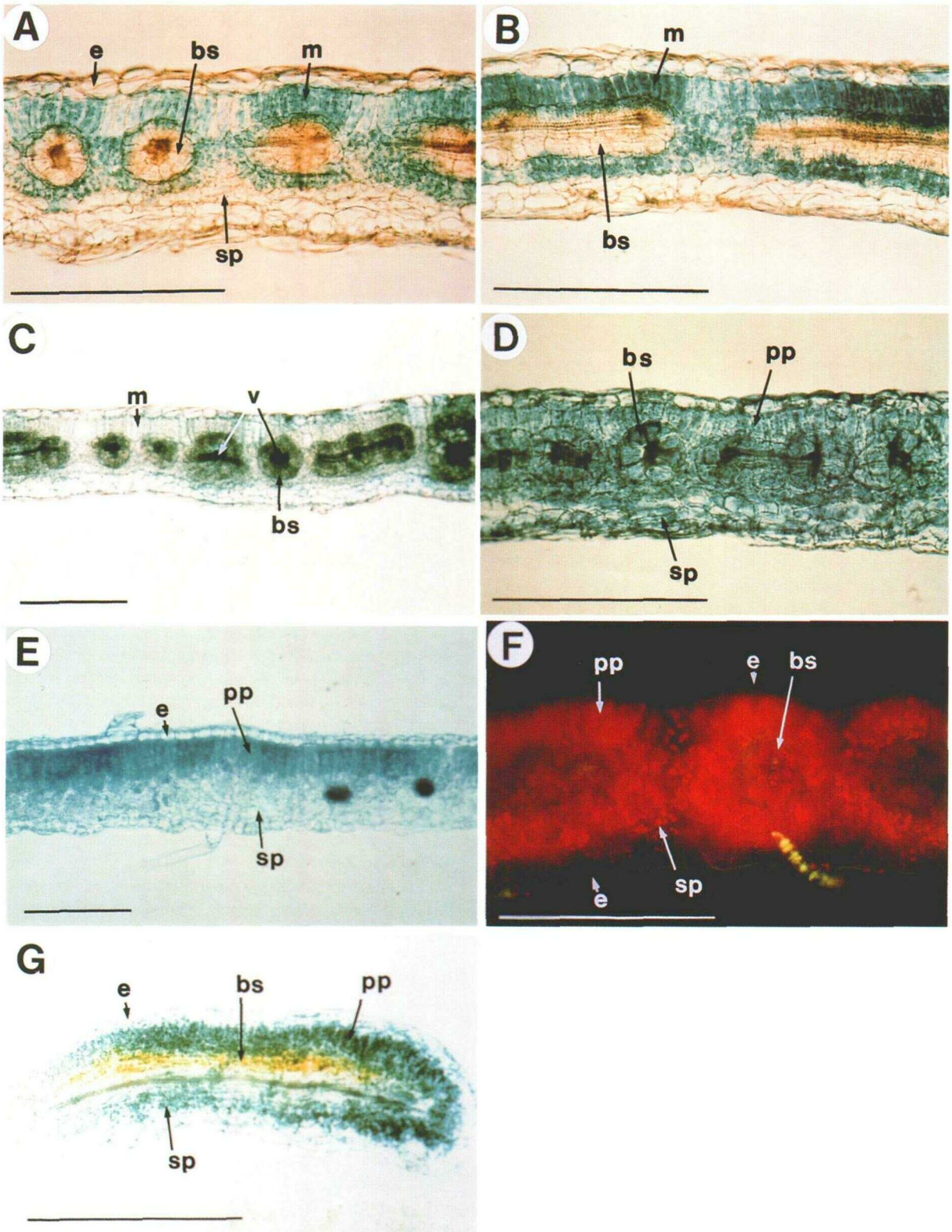

Figure 2. Histochemical Localization of GUS Activity (Blue Staining) in Leaf Sections of Transgenic *F. bidentis* and Tobacco Plants Grown in Soil.

of chloroplasts in leaf sections visualized by chlorophyll fluorescence is shown in Figure 2F. The bundle sheath is surrounded by mesophyll cells containing many chloroplasts, whereas mesophyll cells that are located more than two cell layers apart from the bundle sheath contain very few chloroplasts. Interestingly, in transgenic *F. bidentis* plants, the *PpcA1-L-Ft–GUS* expression could be detected in spongy parenchyma cells that are located close to the bundle sheath. However, in transgenic tobacco (C_3) plants, detectable *PpcA1-L-Ft–GUS* gene expression was restricted to the palisade parenchyma (Figure 2E; Stockhaus et al., 1994).

In leaf sections of *PpcA1-L-Fp–GUS* plants, staining was much weaker and was first visible in cells associated with the vascular tissue (Figure 2C). After incubation times of several hours, staining was observed in mesophyll cells.

In the epidermal cells of the leaf as well as in the guard cells of the stomata, there was no GUS activity detectable for both constructs (Figures 2A to 2C). In some sections, there appeared to be weak staining visible in the epidermis (Figures 2B and 2C). This staining apparently is an artifact of the sectioning of unfixed material at room temperature. When the epidermial layer was removed from the underside of the leaf, there was no staining of these cells detectable even after incubation times of >6 hr (data not shown).

For analysis of the expression pattern in stems, longitudinal sections of the shoot apex as well as cross-sections were examined. Figure 3 shows that in *PpcA1-L-Ft–GUS* plants, there was no staining detectable in stem tissue (Figures 3A, 3C, and 3E). In contrast, the *PpcA1-L-Fp–GUS* construct again resulted in a strong staining signal in vascular tissues of the stem and in cells of the apical meristem (Figures 3B, 3D, and 3F). Figure 3F shows a higher magnification of a vascular bundle in the stem cross-section (Figure 3D). The dark blue staining is located mainly in thick-walled metaxylem parenchyma cells. This staining is extended to the parenchyma cells that surround the vascular bundle. However, there was no GUS activity detectable in the remainder of the interfascicular cambium and in the phloem.

In many experiments, there was no staining of intact roots detected in plants carrying both constructs. In only one experiment was very weak staining detected in root vascular tissues of a *PpcA1-L-Fp–GUS* plant. However, the staining was so weak that we were unable to show this result photographically. As negative controls for these experiments, the same analysis was performed with wild-type *F. bidentis* plants. Even after extended incubation times of >16 hr, there was no staining observed in any tissues of the wild-type plants tested (data not shown).

Developmental Regulation of the *PpcA1-L-Ft* Promoter

In young primary leaves of seedlings, only the tip of the leaf stained blue (Figures 3G and 3H), whereas in older leaves, GUS activity was detectable in the whole leaf blade. Thus, the activity of the *PpcA1-L-Ft* promoter spreads in a basipetal direction (leaf tip to base) during leaf development. Inspection of longitudinal sections of young primary leaves (1 to 2 mm long) revealed that the differentiation of the mesophyll cells progresses in a basipetal direction (Figure 2G). This can be easily followed by the development of the palisade parenchyma. The cells at the base of these leaves are very small and round. Toward the leaf tip, the upper cell layer gradually takes on a rod-shaped appearance and develops to the palisade parenchyma at the leaf tip. GUS-induced staining is first visible in this transition zone, and the intensity of the staining increases toward the leaf tip (Figure 2G). As occurred with sections of adult leaves, the analysis of cell specificity of expression was complicated by the diffusion of the dye from the mesophyll cells into the adjacent bundle sheath cells. An examination of many longitudinal sections and cross-sections of these leaves revealed that as soon as the bundle sheath and mesophyll cells differentiated, the *PpcA1-L-Ft–GUS* construct was expressed in a mesophyll-specific manner.

Analysis of young seedlings showed that the *PpcA1-L-Ft* promoter is switched on at a certain stage during leaf development. How is this promoter regulated in leaves throughout plant life? To answer this question, we determined GUS activities in leaf extracts prepared from 10 consecutive leaves of mature *F. bidentis* plants before the onset of

Figure 2. (continued).

(A) and **(B)** Sections of a fully expanded leaf of a *PpcA1-L-Ft5* plant. Incubation time was 15 min.

(C) Section of a fully expanded leaf of a *PpcA1-L-Fp2* plant. Incubation time was 3 hr.

(D) Section of a fully expanded leaf of a *35S–GUS F. bidentis* plant. Incubation time was 8 hr.

(E) Section of a fully expanded leaf of a *PpcA1-L-Ft* tobacco plant. Incubation time was 6 hr.

(F) Fluorescence microscopy of a section of a fully expanded *F. bidentis* leaf. The chloroplasts are visualized by bright red chlorophyll fluorescence.

(G) Leaf section of the primary leaf (1.5 mm long) of a *PpcA1-L-Ft4* T_1 seedling grown on Murashige and Skoog medium (10 days after germination). Incubation time was 3 hr.

bs, bundle sheath; e, epidermis; m, mesophyll; pp, palisade parenchyma; sp, spongy parenchyma; v, vascular tissue. Bars = 500 μm.

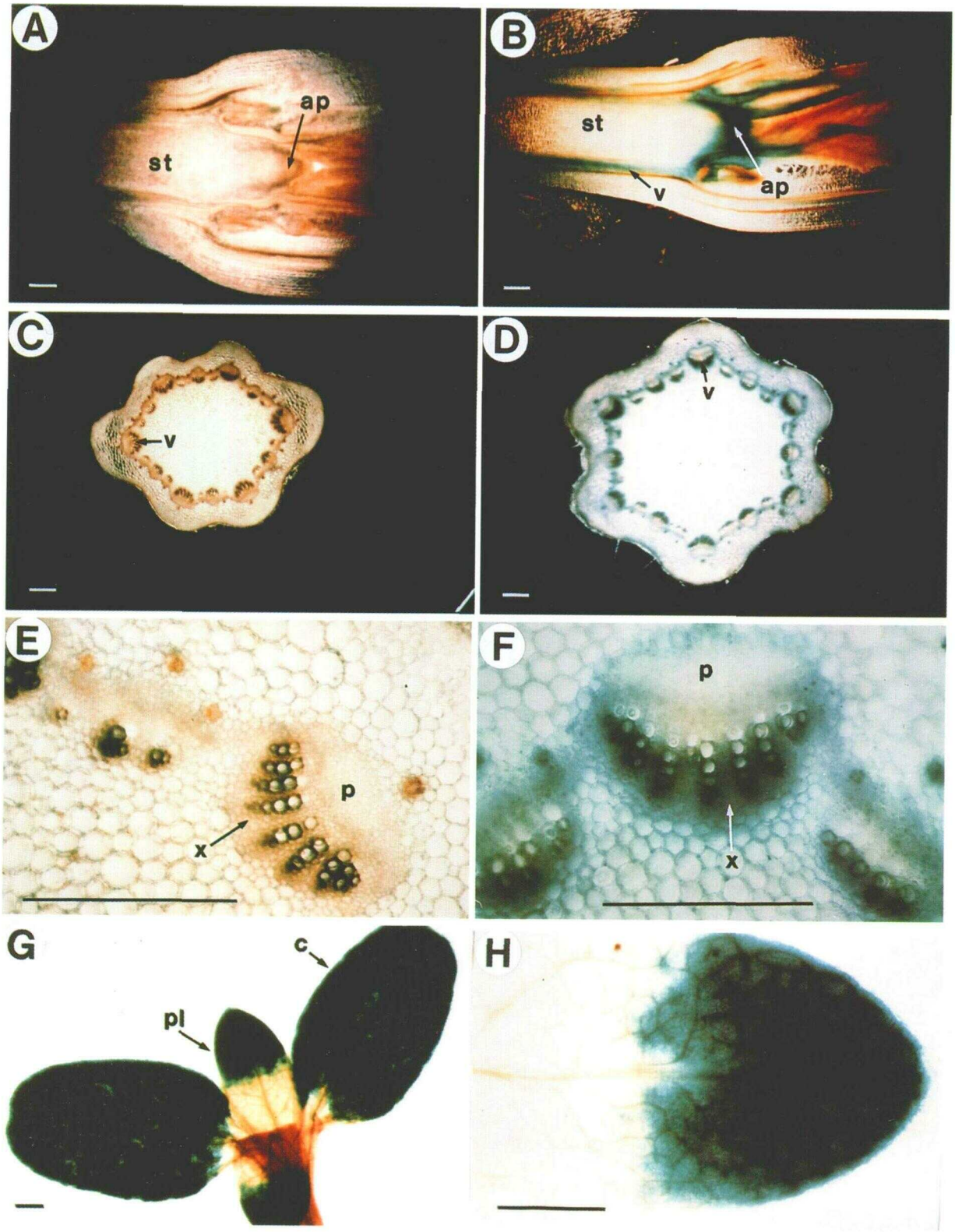

Figure 3. Histochemical Localization of GUS Activity (Blue Staining) in Stem Sections and Seedlings of Transgenic *F. bidentis* (C_4) Plants.

flowering (plant height was 45 to 50 cm). The result of this analysis with a T_1 *PpcA1-L-Ft4* plant is shown in Figure 4A. The leaves are numbered from the top, and the length of each leaf is given in centimeters. GUS activity increased from very low levels in young leaves to a maximum activity in adult fully expanded leaves (leaf 6). In older leaves, GUS activity declined significantly.

To analyze the expression profile of the endogenous *Ppc* gene, RNA was extracted from the same leaves that were used for the GUS activity measurements. One microgram of total RNA of each leaf was dotted onto nylon membrane and hybridized with the cDNA clone of the *PpcA1* gene. The radioactivity bound to the filter pieces was quantified using a scintillation counter. The relative amount of radioactivity is presented in Figure 4B. The amount of detectable *Ppc* steady state RNA increased starting from the youngest leaf and reached a broad peak in leaves 4 and 5. In older leaves, the level of steady state *Ppc* RNA decreased again. These experiments were repeated with an independent T_1 plant, and the expression profiles were very similar to the ones presented in Figure 4.

DISCUSSION

Organ- and Cell-Specific Expression

In this study, our goal was to elucidate the regulatory mechanisms that ensure the correct cell specificity and level of expression of a *Ppc* gene involved in the photosynthetic C_4 cycle. Our results demonstrate that regulatory *cis* elements play a major role in the determination of organ- and cell-specific expression in the *F. bidentis* C_4 plant. The 2-kb *PpcA1* promoter fragment of the *F. trinervia* C_4 plant was sufficient to induce high levels of GUS expression in mesophyll cells; however, in bundle sheath cells, the activity of this promoter fragment was undetectable (Figures 2A and 2B). The difference in expression level, assessed by histochemical staining, was so great that the *PpcA1-L-Ft* promoter could be considered mesophyll specific. This result is in agreement with cell separation studies with *F. trinervia*, demonstrating that the C_4 PEPC gene is expressed in a mesophyll cell–specific manner (Höfer et al., 1992).

We cannot exclude that the *PpcA1-L-Ft* promoter shows some weak activity in bundle sheath cells. However, analysis of this possibility is complicated by the fact that with increasing incubation times, the dye diffuses from the mesophyll cells into the bundle sheath cells. This can be seen in Figure

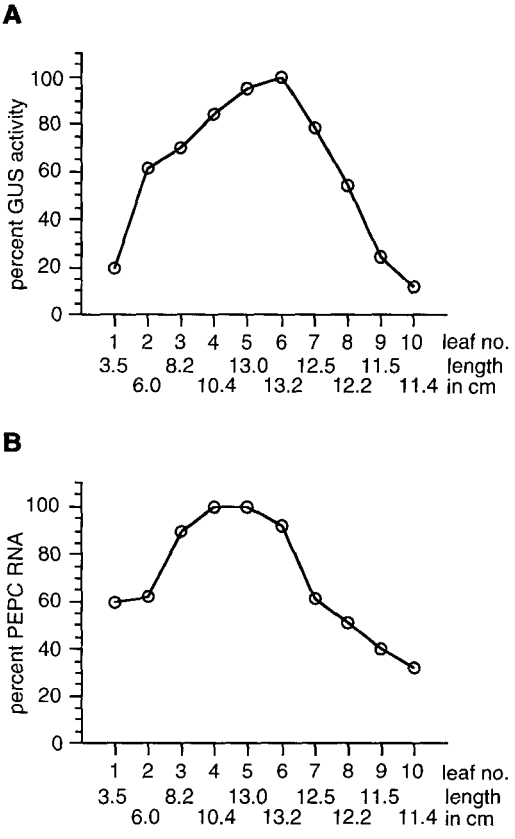

Figure 4. GUS Activities and Steady State mRNA Levels in Leaves of Various Developmental Stages Taken from a Mature *PpcA1-L-Ft4* T_1 Plant.

The leaves are numbered starting from the top of the plant. The length of each leaf is given in centimeters.
(A) Percentage of GUS activities in leaf extracts.
(B) Percentage of C_4 PEPC steady state mRNA levels.

Figure 3. (continued).

Dark-field microscopy was used for **(A)** to **(D)**; bright-field microscopy was used for **(E)** to **(H)**.
(A) Longitudinal section of the shoot apex of an adult *PpcA1-L-Ft5* plant. Incubation time was 6 hr.
(B) Longitudinal section of the shoot apex of an adult *PpcA1-L-Fp2* plant. Incubation time was 3 hr.
(C) and **(E)** Stem sections of an adult *PpcA1-L-Ft5* plant. Incubation time was 6 hr.
(D) and **(F)** Stem sections of an adult *PpcA1-L-Fp2* plant. Incubation time was 3 hr.
(G) T_1 seedling of a *PpcA1-L-Ft5* plant 10 days after germination. Incubation time was 10 hr.
(H) Primary leaf of a *PpcA1-L-Ft5* seedling. Incubation time was 10 hr.
ap, apical meristem; c, cotyledon; p, phloem; pl, primary leaf; st, stem; v, vascular tissue; x, xylem parenchyma. Bars = 500 μm.

2B. The blue dye is visible only in the areas of the bundle sheath cells that are neighboring the heavily stained mesophyll cells; however, there is no dye visible in the sections of bundle sheath cells that are adjacent to the vascular tissue or neighboring mesophyll cells that are not heavily stained.

In the tobacco C_3 plant as well as in the *F. bidentis* C_4 plant, the mesophyll is differentiated into palisade parenchyma and spongy parenchyma. Interestingly, the staining pattern differed between tobacco and *F. bidentis*. In tobacco, the expression of the *PpcA1-L-Ft–GUS* construct was restricted to the palisade parenchyma (Figure 2E; Stockhaus et al., 1994). In *F. bidentis*, the same construct was expressed in the palisade parenchyma cells and in cells of the spongy parenchyma. However, the intensity of the staining appears to be dependent on the distance of the mesophyll cells from the bundle sheath. The highest GUS activities were found in mesophyll cells directly neighboring the bundle sheath, especially in palisade parenchyma cells covering the bundle sheath. With increasing distance from the bundle sheath, the observed GUS activity in mesophyll cells was lower, and at the underside of the leaf, many mesophyll cells were not stained at all (Figure 2A).

This result demonstrates that the mesophyll cells of *F. bidentis* are not only distinguishable by their cell morphology, that is, palisade versus spongy parenchyma type, but also by the expression pattern induced by the C_4 *PpcA1-L-Ft* promoter. Microscopic examination revealed that palisade and spongy mesophyll cells directly adjacent to the bundle sheath cells contain more chloroplasts than do mesophyll cells that apparently are not in direct contact with the bundle sheath cells.

To visualize the distribution of chloroplasts in cross-sections of mature leaves, we used fluorescence microscopy. The mesophyll cells directly adjacent to the bundle sheath contain many chloroplasts. This ringlike structure is clearly visible in Figure 2F. The cells between these "rings" of mesophyll cells as well as spongy parenchyma cells at the underside of the leaf contain only a few chloroplasts. It is tempting to assume that these differences in chloroplast numbers translate into differences in photosynthetic activities. Thus, the activity of the C_4 *PpcA1-L-Ft* promoter in mesophyll cells can be correlated with chloroplast number and hence photosynthetic activity (compare GUS staining intensity in Figure 2A and chloroplast distribution in Figure 2F). Interestingly, very little or no C_4 *PpcA1-L-Ft* promoter activity could be detected in the spongy mesophyll cells adjacent to the lower epidermis. These cells appear to contain the smallest number of chloroplasts of all mesophyll cells. Are these mesophyll cells engaged in C_4 photosynthesis? These cells are at least two to three layers of mesophyll cells apart from the bundle sheath cells and therefore may serve a different function. For instance, they might serve as reflectors that send light back into the palisade parenchyma tissue, thereby increasing the possibility of its absorption for photosynthesis (reviewed in Vogelmann et al., 1996).

The molecular basis of the non-uniform activity of the C_4 *PpcA1-L-Ft* promoter in the mesophyll cells of *F. bidentis* is not known. For the maize C_4 plant, Langdale and Nelson (1991) proposed that a diffusible factor originates in the veins, spreads into the neighboring bundle sheath and mesophyll cells, and influences gene expression patterns of the affected cells. Whether this concept of a positional control of photosynthetic cell type differentiation in the C_4 monocot maize also applies to the differential expression of the C_4 *PpcA1* promoter in the palisade and spongy mesophyll cells of the *F. bidentis* C_4 dicot remains to be shown.

In stems and roots of *F. bidentis*, the *PpcA1-L-Ft–GUS* construct is expressed at levels close to the limit of detection, which is approximately three to four orders of magnitude lower than in leaves. Leaf-specific expression of the *PpcA1-L-Ft–GUS* gene in *F. bidentis* is in perfect agreement with the results of the expression analysis using poly(A)$^+$ RNA isolated from different organs of *F. trinervia* (Hermans and Westhoff, 1990). There is a clear difference in the organ specificity of this construct in *F. bidentis* (C_4) and tobacco (C_3) plants. In tobacco, there is significant GUS expression detectable in roots, whereas expression in stems is rather low (Stockhaus et al., 1994). In addition, in *F. bidentis* leaves, the *PpcA1-L-Ft* promoter induced GUS activities that were one to two orders of magnitude higher than in leaves of the tobacco C_3 plant. This could be due to a basic difference in the set of *trans*-acting factors in C_3 and C_4 plants or to the fact that tobacco is a heterologous plant not closely related to *Flaveria* spp.

When we compare the expression of the orthologous promoters of the C_3 and C_4 *Ppc* genes in *F. bidentis*, we find two significant differences. In evolutionary terms, the C_4 promoter has gained high levels of activity restricted to mesophyll cells, whereas it has lost activity in other tissues, such as in the apical meristem and xylem parenchyma cells (compare Figures 3A and 3B, and 3C and 3D). These differences must be due to changes of the *cis*-acting elements.

This observation is similar to the one made by Matsuoka et al. (1994). These authors have analyzed the promoter of a gene encoding RBCS of maize in transgenic rice (C_3) plants. The *RbcS–GUS* gene is expressed in mesophyll cells of transgenic rice C_3 plants, whereas in the maize C_4 plant, expression of *RbcS* is suppressed in this cell type. The authors conclude that the *RbcS* promoter is not suppressed in mesophyll cells of the rice C_3 plant and that it is very likely that C_4-specific *trans*-acting factors are involved in this process in the maize C_4 plant (Matsuoka et al., 1994). However, it cannot be excluded that post-transcriptional regulatory mechanisms are involved in the suppression of *RbcS* gene expression in mesophyll cells of the maize C_4 plant maize.

The *PpcA1* promoter of the *F. pringlei* C_3 plant is active at low levels in leaves and stems of transgenic *F. bidentis* (C_4), whereas there was no significant GUS activity detectable in roots (Table 1). In the *F. pringlei* C_3 plant, low amounts of transcripts of the *PpcA1* gene were detected in leaves,

stems, and roots (Hermans and Westhoff, 1990). The absense of detectable GUS activities in *F. bidentis* roots could be due either to differences in the set of *trans*-acting factors in the root cells of both plant species or to the possibility that some *cis*-acting elements are missing from the *PpcA1-L-Fp–GUS* construct.

Developmental Regulation of the C_4 *PpcA1* Promoter

Our experiments show that depending on the developmental stage of the leaf, detectable GUS activity can vary by one order of magnitude. This finding reconfirms the importance of the developmental stage for the analysis of physiological processes and gene expression patterns. Measurement of GUS activities in leaves at different developmental stages collected from the same plant revealed that *PpcA1-L-Ft–GUS* gene expression is very low in young emerging leaves and increases during the leaf expansion phase until the maximum size is reached. After the leaf is fully mature, GUS activity decreases. The steady state mRNA level of the C_4 PEPC gene shows a similar increase in expanding leaves. However, the relative level in young leaves is higher, and the maximum of PEPC mRNA accumulation is reached in younger leaves. Although these experiments cannot be directly compared, their results indicate that the GUS activities reflect the developmental regulation of the C_4 PEPC gene.

The developmental regulation of the C_4 *PpcA1-L-Ft* promoter was also apparent when the staining pattern induced by the *PpcA1-L-Ft–GUS* gene in young leaves of seedlings was analyzed. At a certain developmental stage, the leaf tip and the distal parts of the leaf blade were stained, whereas the base of the leaf was not stained at all (Figures 3G and 3H). No *PpcA1-L-Ft* promoter activity was detectable in the developing leaf primordia. GUS staining of the primary leaf shown in Figure 3H appears to be stronger around the veins. We have observed that the staining of palisade parenchyma cells on top of the bundle sheath is much more intense than the staining of palisade parenchyma cells between the bundle sheaths (Figure 2A). By looking from the top onto the surface of the leaf, this phenomenon can explain the impression that staining is stronger around the veins. Analysis of sections of these small primary leaves revealed that even at this early developmental stage, the expression of the *PpcA1-L-Ft–GUS* gene appears to be confined to the mesophyll cell type (Figure 2G).

This result demonstrates that the *PpcA1-L-Ft* promoter becomes activated when the mesophyll cell has reached a certain developmental stage. The induction of this promoter is coupled with the maturation of the leaf that in dicotyledonous plants occurs in a basipetal direction (leaf tip to leaf base) (Dale, 1992). This observation is in agreement with previous studies that revealed the dependence of C_4 gene expression on cell differentiation (Langdale et al., 1988; Langdale and Nelson, 1991). Wang et al. (1993) analyzed RBCS RNA and protein localization in young leaves of the amaranth C_4 plant. They found that in the tip of young leaves, the RBCS RNA and protein are localized in the bundle sheath cells, as occurs in mature leaves, whereas in the base of young leaves, RBCS RNA and protein are found in bundle sheath and mesophyll cells. These results indicate that the specific C_4 gene expression pattern of the *RbcS* genes is dependent on the developmental stage of the leaf.

The nature of the developmental signal(s) that governs the expression of C_4 cycle genes is not known. One approach to identifying these signals would be to localize precisely the *cis* elements that are essential for developmental regulation of the C_4 *Ppc* gene. The isolation of the corresponding *trans*-acting factors will allow us to unravel the signal transduction chain starting from DNA binding factors.

METHODS

Chimeric Genes, Transformation, and Regeneration of *Flaveria bidentis* Plants

The construction of the *Ppc* promoter–GUS fusion genes has been described by Stockhaus et al. (1994).

The two different *PpcA1* promoter–GUS constructs were introduced by electroporation into the *Agrobacterium tumefaciens* AGL1 (Lazo et al., 1991). The transformation of *F. bidentis* plants was performed as described by Chitty et al. (1994). The transformation was performed with hypocotyls of 7- to 9-day-old *F. bidentis* seedlings. The hypocotyls were infected with the corresponding Agrobacterium strain and cocultivated for 2 or 3 days in the dark on CRM medium (Chitty et al., 1994). The explants were washed and cultivated for 25 days on CRM medium containing 200 mg/L timentin and 200 mg/L kanamycin. Developing shoots were cultivated as described by Chitty et al. (1994).

Integration of the chimeric genes into the *F. bidentis* genome was examined by DNA gel blot analysis. Genomic DNA of the transgenic plants and a wild-type control plant was extracted using the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). Ten micrograms of DNA, restricted with HindIII, was subjected to DNA gel blot analysis. The immobilized DNA was hybridized with a ^{32}P-labeled *GUS* probe. The labeled DNA fragments varied in size and were larger than the introduced construct, indicating that the chimeric gene was integrated in the *F. bidentis* genome. To analyze the intactness of the chimeric genes, DNA gel blot experiments were performed with DNA restricted with HindIII and EcoRI. Duplicate blots were hybridized with the respective labeled promoter probe or the labeled *GUS* gene probe. For all plants tested, we observed the expected size of the hybridizing fragments, indicating that the promoter fragment and the intact *GUS* gene are linked in the genomic DNA and that each transgenic plant contains at least one intact copy of the respective chimeric gene.

Measurement of GUS Activity and Histochemical Analysis

Regenerated *F. bidentis* plants or T_1 plants were transferred to soil and cultivated in a greenhouse. Seedlings or mature plants 40 to 50

cm tall before the onset of flowering were used for the measurements of GUS activity at different developmental stages and for the histochemical analysis. Transgenic *F. bidentis* seedlings were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.1% sucrose and 50 mg/mL of kanamycin. The seedlings were cultivated for 10 days at 21°C under a 16-hr-light/8-hr-dark cycle (NL 65 W S white light; Philips, Hamburg, Germany) and then used for the histochemical analysis.

Fluorometric determination of GUS activities (Jefferson et al., 1987; Kosugi et al., 1990) was performed as follows. Approximately 300 mg of plant tissue from leaves, stems, and roots was collected, wrapped in aluminium foil, frozen in liquid nitrogen, and pulverized using a hammer. The tissue powder was transferred to 1.5-mL reaction tubes, and 1 volume of extraction buffer was added (50 mM Na_2HPO_4, pH 7.5, 10 mM β-mercaptoethanol, 10 mM Na-EDTA, 0.1% SDS, 0.1% Triton X-100, 2% polyvinylpyrrolidone, and 20% methanol). This mixture was homogenized using a motor-driven metal pistil that was fitted to the conical part of the reaction tube. The homogenate was centrifuged at 15,000*g*, at 4°C for 5 min, and the clear supernatant was used for measurements of protein content (Bradford, 1976) and GUS activity (Jefferson et al., 1987). The incubation buffer for the measurement of GUS activity was identical to the extraction buffer, except that 4-methylumbelliferyl β-D-glucuronide was added to a final concentration of 2 mM. The fluorescence was measured with a fluorescence spectrophotometer (model F-2000; Hitachi, Tokyo, Japan).

For the histochemical analysis of GUS activity, sections of mature plant organs were cut manually. The sectioning of 1- to 2-mm-long primary leaves of seedlings was performed with a cryomicrotome (Kryostat MTE; SLEE Technik, Mainz, Germany). The seedlings were sectioned at −20°C. The sections were transferred to incubation buffer (50 mM Na_2HPO_4, pH 7.5, 0.1% Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, and 20% methanol). After a brief vacuum infiltration, the sections were incubated at 37°C for 5 min to 16 hr. After this incubation period, the sections were washed with water and then incubated in 70 to 96% ethanol to remove chlorophyll.

RNA Dot Blot Analysis

Leaf RNA was extracted as described by Logemann et al. (1987). One microgram of RNA was glyoxylated in 6% glyoxal, 50% DMSO, 0.005 M sodium–acetate, 0.001 M EDTA, and 0.02 M Mops, pH 7.0, for 45 min at 50°C. SSC buffer (20 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate]) was added to a final concentration of 2 × SSC, and this RNA sample was dotted onto a Hybond$^+$ nylon membrane (Amersham). The filter was incubated at 80°C for 2 hr and hybridized with a random-primed *PpcA1* cDNA probe (Megaprime DNA labeling system; Amersham) in hybridization buffer (250 mM Na_2HPO_4, pH 7.2, 7% [w/v] SDS, and 2.5 mM EDTA) at 65°C for 15 hr. The filter was washed three times for 30 min in 0.1 × SSC at 65°C. The radioactive spots were visualized by fluorography, and the bound radioactivity was quantified by scintillation counting. The RNA quantification experiment as well as the GUS activity measurements were repeated with an independent T_1 plant. The results did not vary by >6%.

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