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Mesophyll-specific, light and metabolic regulation of the C_4 *PPCZm1* promoter in transgenic maize

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Abstract

To play an essential role in C_4 photosynthesis, the maize C_4 phospho*enol*pyruvate carboxylase gene (*PPCZm1*) acquired many new expression features, such as leaf specificity, mesophyll specificity, light inducibility and high activity, that distinguish the unique C_4 *PPC* from numerous non-C_4 *PPC* genes in maize. We present here the first investigation of the developmental, cell-specific, light and metabolic regulation of the homologous C_4 *PPCZm1* promoter in stable transgenic maize plants. We demonstrate that the 1.7 kb of the 5′-flanking region of the *PPCZm1* gene is sufficient to direct the C_4-specific expression patterns of β-glucuronidase (GUS) activity, as a reporter, in stable transformed maize plants. In light-grown shoots, *GUS* expression was strongest in all developing and mature mesophyll cells in the leaf, collar and sheath. GUS activity was also detected in mesophyll cells in the outer husks of ear shoots and in the outer glumes of staminate spikelets. We did not observe histological localization of GUS activity in light- or dark-grown callus, roots, silk, developing or mature kernels, the shoot apex, prop roots, or pollen. In addition, we used the stable expressing transformants to conduct and quantify physiological induction studies. Our results indicate that the expression of the C_4 *PPCZm1-GUS* fusion gene is mesophyll-specific and influenced by development, light, glucose, acetate and chloroplast biogenesis in transgenic maize plants. These studies suggest that the adoption of DNA regulatory elements for C_4-specific gene expression is a crucial step in C_4 gene evolution.

Introduction

In maize and other C_4 plant leaves, two morphologically and biochemically distinct cell types, the mesophyll cells and the bundle sheath cells, compartmentalize distinct reactions that lead to photosynthetic carbon fixation. CO_2 fixation is first catalyzed in mesophyll cells by phospho*enol*pyruvate carboxylase (PPC) which has no oxygenase activity. The product of that reaction, the C_4 dicarboxylic acid oxaloacetate, is reduced to malate and transported to internal bundle sheath cells, where it is decarboxylated, and the released CO_2 is reassimilated in the Calvin cycle by ribulose-bisphosphate carboxylase (RBC; for reviews see Hatch 1976, 1987; Edwards and Walker, 1983; Furbank and Taylor, 1995). The differentiation of mesophyll and bundle sheath cells that conduct C_4 photosynthesis requires the coordinated expression of genes involved with these pathways (Dengler and Taylor, 2000).

The developmental expression of C_4 photosynthetic genes has been extensively studied (reviewed by Nelson and Langdale, 1989, 1992). The differentiation of C_4 photosynthetic gene expression patterns is thought to be influenced by light as well as cell-specific developmental changes. In dark-grown leaves of maize, both mesophyll and bundle sheath cells express low levels of RBC, but all other C_4 enzymes

are not detectable (Sheen and Bogorad, 1985, 1986, 1987). The compartmentalized expression of RBC to bundle sheath cells, as well as the other cell-specific patterns of C_4 photosynthetic enzymes, is induced upon greening. While light induces a switch of gene expression from C_3 to C_4 patterns, cell position may be more important in the regulation of C_4 photosynthesis (Langdale *et al*., 1988a, b). McCormac *et al.* (1997) studied expression of C_4 genes in photosynthetic and non-photosynthetic leaf regions of a dicot C_4 plant, *Amaranthus tricolor.* Their study shows that developmental loss of photosynthetic capacity in non-photosynthetic leaf segments is associated with loss of C_4 gene expression and cell-specific patterns of mRNA accumulation. A developmental analysis of photosynthetic gene expression during leaf initiation in *Amaranthus hypochondriacus* (Ramsperger *et al.*, 1996) showed that individual C_4 genes are independently regulated during cell type differentiation and that post-transcriptional regulation determines cell-specific patterns of expression very early in leaf development. These studies indicate that C_4 photosynthetic gene expression can be influenced at multiple regulatory levels by alterations in photosynthetic activity or developmental processes.

Several maize *PPC* cDNA and genomic clones have been isolated and analyzed (Harpster and Taylor, 1986; Sheen and Bogorad, 1987; Hudspeth and Grula, 1989; Cushman *et al.*, 1989; Thomas *et al.*, 1990). Schäffner and Sheen (1992) have shown that there are at least three subgroups in the maize *PPC* multigene family, and have demonstrated differential expression of the unique C_4-specific gene, *PPCZm1*, and two non-C_4 specific genes, *PPCZm2* and *PPCZm3A*. *PPCZm1* is unique to C_4 plants and probably evolutionarily derived from C_3 *PPC* genes (Schäffner and Sheen, 1992). At least two types of *PPC* gene expression patterns in various organs have been found. RNA blot analysis and PCR assays demonstrated that *PPCZm2* and *PPCZm3* are expressed in all tissues and developmental stages tested from roots, stems and leaves, whereas *PPCZm1* was exclusively expressed in green leaves. No expression of *PPCZm1* was found in roots or stem tissues.

Differential expression of these three *PPC* genes in maize has been described also by transient expression assays with *PPC-CAT* chimeric gene constructs in maize protoplasts (Schäffner and Sheen, 1992). *PPCZm1-CAT* was expressed exclusively in leaf protoplasts; both *PPCZm2-CAT* and *PPCZm3A-CAT* were expressed in leaf, root and stem protoplasts. Thus, the chimeric gene constructs show the same tissue specificity in their transient expression assay as they are observed *in planta.* Therefore, the tissue-specific expression of these genes appears to be controlled by their distinct promoters and regulated at the level of transcription (Schäffner and Sheen, 1992). Transient expression assays, however, do not provide an accurate means for assessing the developmental component of expression of these genes.

Appropriate developmental expression of C_4 photosynthetic genes is known to be influenced by light and metabolic signals (Sheen, 1990; Nelson and Langdale, 1992). Light induction of the C_4-specific *PPCZm1* gene was examined in transient expression assays on protoplasts isolated from etiolated or illuminated leaves (Schäffner and Sheen, 1992). On the basis of those analyses it was concluded that the light induction of *PPCZm1* relies on light-dependent developmental changes instead of an immediate light activation as found in other maize light-inducible genes. Recently, a unique transcription factor Dof1 was shown to specifically regulate the *PPCZm1* promoter in maize mesophyll protoplasts (Yanagisawa and Sheen, 1998). In contrast, the establishment of tissue-specific patterns of C_4 photosynthetic gene expression in leaves and cotyledons of *Amaranthus* is determined by developmental processes independent of light (Wang *et al.*, 1993). Thus, different C_4 plants might employ different regulatory mechanisms for C_4 gene expression due to their independent evolutionary routes. In addition to light, various metabolites have been shown to influence cell-specific levels of C_4 photosynthetic genes (Sheen, 1990; Jang and Sheen, 1994). Expression of a reporter in stable transgenic plants will provide a useful approach to evaluate inductive stimuli during growth and development.

Experiments using stable dicot transgenic plants, C_4 *Flaveria*, have been conducted to examine the *cis*-acting regulatory elements of C_4-specific PPC genes required for tissue-specific expression. Stockhaus *et al.* (1997) have shown that the orthololegous 2 kb 5′-flanking region of the C_4 PPCA1 gene of *F. trinervia* is sufficient for mesophyll-specific expression in stable transformants of the C_4 dicot plant *F. bidentis*. Studies on this promoter in tobacco had indicated that mesophyll-specific expression is controlled by initiation of transcription (Stockhaus *et al.*, 1994). However, expression of a chimeric gene construct comprising the C_4 PPC promoter from maize fused to the β-glucuronidase (GUS) reporter gene has only been studied in stable transgenic C_3 rice plants

(Matsuoka *et al.*, 1994). Very few studies have been published to date in which transgenic maize plants were used for promoter analysis studies, and this report is the first to analyze the promoter of any C_4 photosynthetic gene in a transgenic C_4 monocot. Stable transgenic plants expressing the GUS reporter allow the activity of the promoter to be assessed developmentally during plant maturation and in a broad variety of tissues and cell types. In addition, transgenic plants provide a convenient means to conduct induction studies on the activity of this promoter *in planta*.

The goal of our study was to investigate the developmental, cell-specific, light and metabolic regulation of the homologous C_4 *PPCZm1* promoter in transgenic maize. To characterize the pattern of homologous C_4 *PPCZm1* expression, we constructed a *PPCZm1-GUS* chimeric gene and transformed cells of immature embryos of maize. Resultant stable transformants were recovered and regenerated to fertile plants. PCR and Southern blot analysis confirmed the presence of both the *BAR* (encoding phosphinothricin acetyltransferase) and *GUS* genes in sixteen independent stable transformants. The expression of the *PPCZm1-GUS* construct in stable transformation events is compared to previously published results from transient expression assays. Our results indicate that the expression of the C_4 *PPCZm1-GUS* fusion gene is mesophyll-specific and influenced by light, glucose, acetate and chloroplast biogenesis in stable transgenic maize plants.

Materials and methods

Construction of chimeric genes

The construction of a cauliflower mosaic virus *35S* promoter-*BAR* fusion gene (Figure 1a, pDPG165) as selectable marker has been previously described (Gordon-Kamm *et al.*, 1990). The 1.7 kb promoter of the maize *PPCZm1* gene was recovered from a partial *Nco*I digest, and ligated with the coding region of the *Escherichia coli UIDA* (*GUS*), as a reporter, fused with a *NOS* 3′ terminator (Figure 1b as pDPG522). Both constructs were isolated by large-scale plasmid preparations on Quiagen columns and used at a concentration of 1 μg/μl in a Tris-EDTA buffer.

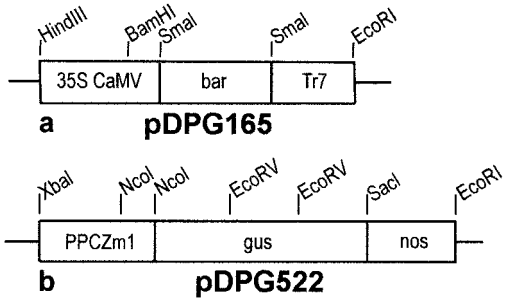

Figure 1. Schematic representation of gene constructs used in transformation experiments. a. pDPG165: CaMV 35S-BAR fusion. b. pDPG522; *PPCZm1-GUS* fusion.

Stable transformation of maize

Immature embryos (1.5–2.0 mm in length) were excised from surface-sterilized, greenhouse-grown ears of High II germplasm (A188 H B73) 12 days after pollination and cultured on a modified N6-based medium containing 1 mg/l 2,4-D and 2% sucrose (Gordon-Kamm *et al.*, 1990). Four hours before bombardment the embryos were transferred to the same medium but containing 0.25 mM sorbitol and 0.25 mM mannitol for osmotic pre-treatment. Gold particle preparations (0.6 μm; BioRad, Hercules, CA) and bombardment with the PDS-1000/He Particle Delivery System (Bio-Rad) was performed according to Kausch *et al.* (1995). Both vectors (pDPG 165 and pDPG 522) were used as intact supercoiled plasmid DNA and co-precipitated on gold microprojectiles used in all transformation experiments. Equal amounts of each plasmid DNA (10 μg) were used for the co-precipitation onto 2.1 mg gold microprojectiles. Embryos were placed adaxial side down and arranged in a circle with a 2 cm diameter to avoid excessive tissue damage during bombardment. The bombarded tissues were incubated for 24 h at 27 °C in darkness and then transferred to selection medium, a modified N6-based medium containing 1 mg/l 2,4-D, 2% sucrose, and 1 mg/l bialaphos for 3 weeks still in darkness at 27 °C. Finally stable transformants were selected by transferring the embryos to the same medium containing 3 mg/l bialaphos. Transformed calli appeared 6–10 weeks after bombardment. This biphasic selection scheme resulted in no escapes. Bialaphos-resistant colonies were expanded and confirmed by PCR and Southern blot analysis to contain the *BAR* gene. PCR and Southern blot analysis confirmed the co-integration of the *GUS* gene. Fertile transgenic plants were regenerated according to Gordon-Kamm *et al.* (1990).

R_1 plant material

The R_0 plants were crossed to a proprietary inbred line (CV), and resultant R_1 plant progeny were used for further analysis. Segregating R_1 plants were grown under several different conditions and evaluated for *GUS* expression. The co-segregating *BAR* and *GUS* minus plants were used as controls in the histological GUS evaluations. Seeds were germinated on germination paper moistened with 0.5% Liberty (to select *BAR*-positive segregating individuals) and 0.1% Domain (a commercial fungicide) at 100% relative humidity and grown in darkness or in dim light conditions, according to the protocol used by Schäffner and Sheen (1992), for three days. Bialaphos-resistant co-segregating seedlings were then either (1) grown in darkness for 5–7 days, (2) planted in soil and transferred to a greenhouse and sampled throughout development for histological analysis, (3) grown hydroponically, or in Perlite containers, in half-strength Clark's medium in a growth chamber in the dark at 27 °C for 5–7 days and used for light induction studies, or (4) planted in Perlite and watered with half-strength Clark's medium (see text for details). Experiments on light induction were conducted by moving plants from the dark to 150 μE m^{-2} s^{-1} for 0–24 h.

GUS assays

Histological GUS assays were performed according to Jefferson (1987) and Stomp (1992). Samples of fresh tissue were dissected in 4% formaldehyde made fresh from paraformaldehyde in 50 mM potassium phosphate buffer pH 7.0, vacuum-infiltrated and fixed for 10 min at 4 °C, rinsed three times for 5 min each, with the same buffer and incubated in GUS reaction mix for 3–8 h (Stomp, 1992). GUS reaction product accumulation was visually monitored such that samples were 'developed' in the GUS reaction mix to achieve proper staining. Time exposure for each GUS reaction was carefully documented. Alternatively, fresh samples were vacuum-infiltrated and incubated in GUS reaction mix for 3–8 h and then fixed. Pre- and post-fixation has a significant effect on the reduction of diffusible GUS reaction product (Stomp, 1992). Reacted and fixed tissues were then dehydrated and cleared (to remove chlorophyll) in an ascending ethanol series and processed for paraffin embedding by standard histological procedures.

Fluorometric determination of GUS activities was performed as follows (Jefferson, 1987). About 1–5 g of plant tissue was collected and frozen in a Falcon tube in liquid nitrogen, and pulverized. Extraction buffer (10 mM Na-EDTA, 0.1% SDS, 0.1% Triton X-100, 2% polyvinylpyrrolidone, 20% methanol) was added to the frozen tissue powder and vortexed to homogeneity. The homogenate was centrifuged at 15 000 $\times$ *g* for 5 min and the supernatant was used to quantify protein content (Bradford, 1976) and to measure GUS activity (Jefferson, 1987).

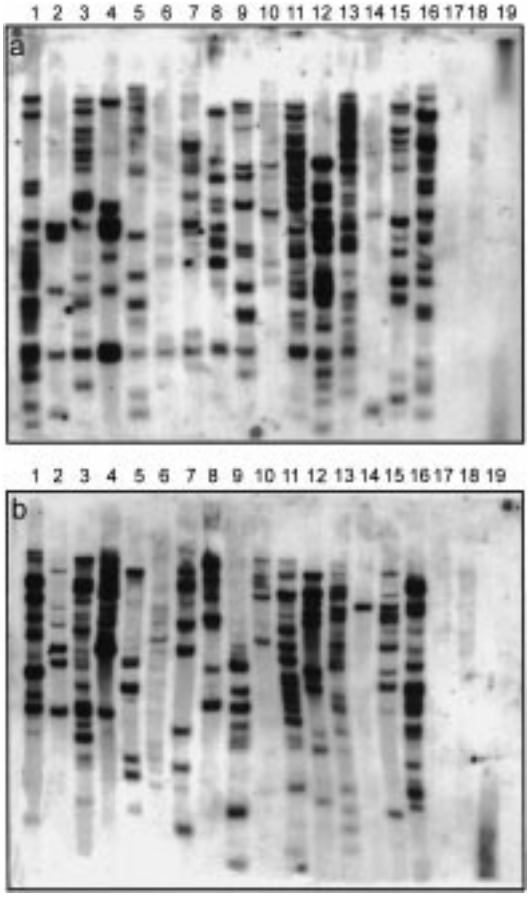

Figure 2. Southern blot analysis of stable transformants PCR positive for *PPCZm1-GUS*. a. Genomic DNA digested with *Eco*RI and probed with the *Nco*I-*Sac*I GUS fragment from pDPG522. b. Genomic DNA digested with *Nco*I-*Sac*I and probed with the *Nco*I-*Sac*I GUS fragment from pDPG522.

Results

The PPCZm1-GUS *construct and maize transformation*

To test whether the homologous C_4 *PPCZm1* promoter was capable of directing C_4 expression patterns

Table 1. Co-transformation and expression efficiency of *PPCZm1-GUS* in stable transgenic plants regenerated from bialaphos-resistant colonies.

Clone	R_0 plantlets	Plants to GH	Total R_1 seed	Transgene copy number
01	9	4	820	<10
02	5	5	293	6
03	5	4	0	<10
04	10	9	2178	<10
05	6	5	53	5
06	8	8	2069	3
07	5	4	144	8
08	5	4	38	<10
09	5	5	459	<10
10	5	5	615	5
11	4	4	305	<10
12	3	2	331	<10
13	1	0	—	<10
14	0	—	—	1
15	5	5	776	<10
16	0	—	—	<10
17	0	—	—	0

in maize, we constructed a *PPCZm1-GUS* chimeric gene. This construct was co-introduced into maize with a selectable marker gene construct, *BAR*, the expression of which confers bialaphos resistance on the host cell. Figure 1 shows a schematic representation of the *BAR* gene construct, pDPG165 (Gordon-Kamm *et al.,* 1990), used for selection of transgenics (a), and the *PPCZm1-GUS* construct, pDPG522, that was co-bombarded as a separate plasmid (b). Sixteen individual clones were recovered from bombardment experiments with 116 immature embryos as target tissue (Table 1). Plants were regenerated from thirteen clones which expressed GUS in R_0 seedlings and twelve matured to female fertile plants in the greenhouse. Co-transformation frequency was about 80% for the PPCZm1-GUS construct. Seed recovery from the transformed plants was normal in comparison to untransformed greenhouse-grown plants of this genotype. Five of the clones produced transgenic plants that were male-sterile. All sixteen bialaphos-resistant callus lines were PCR-positive for the *BAR* genes and all thirteen regenerated clones were PCR-positive for both the *BAR* and *GUS* genes.

Integration patterns and transgene copy number estimations of the *GUS* gene construct were determined from genomic DNA prepared from individual transformed lines. Southern blot analysis was conducted on the sixteen bialaphos-resistant colonies that had been determined to be PCR-positive for both *GUS* and *BAR*. Genomic DNA was isolated from transgenic cell lines and untransformed controls. Of each DNA sample 10 μg was digested with *Eco*RI (Figure 2a), which cleaves once within the pDPG522 plasmid, and subjected to DNA gel blot analysis. The immobilized DNA was hybridized with a ^{32}P-labeled *Nco*I-*Sac*I *GUS* fragment from pDPG522 to estimate copy number of the integrated chimeric gene. *GUS*-encoding DNA fragments were present in all sixteen transformants. The labeled fragments varied in size and intensity, and were of higher molecular weight than the introduced construct, indicating that the *GUS* gene integrated into the genome, mostly in multiple copies. The negative controls (lanes 17 and 18) did not contain any fragments that hybridize to the *GUS* probe. A second DNA gel blot was conducted on the same genomic DNA samples cleaved with *Nco*I-*Sac*I and probed with the same ^{32}P-labeled *Nco*I-*Sac*I *GUS* fragment from pDPG522 (Figure 2b). Single-copy insertions should yield a single hybridizing fragment. The majority of transformants are multiple-copy insertion events. It is not known from this analysis how many of the copies are incomplete and/or truncated

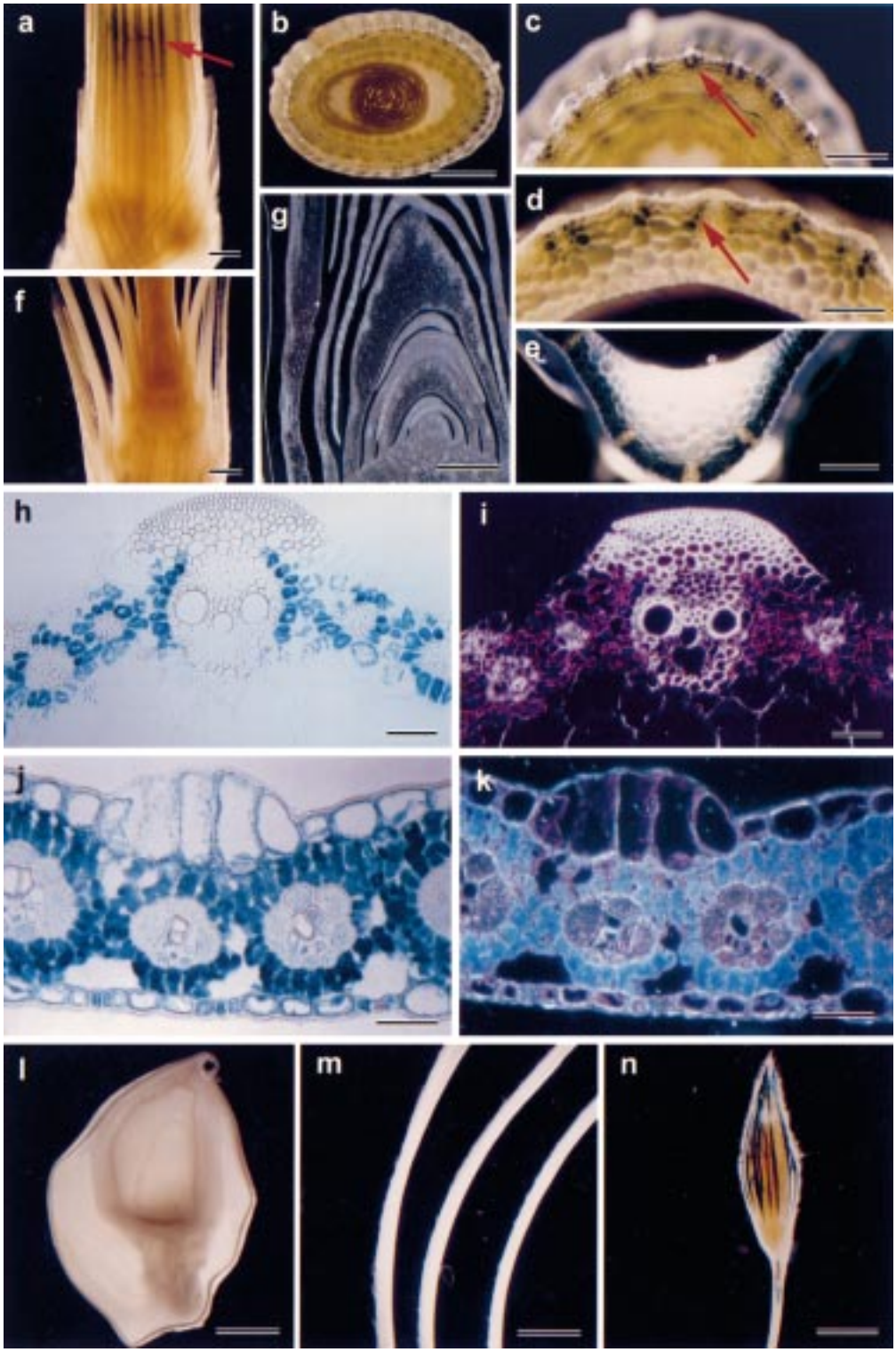
a
b
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Figure 3. Development- and cell-specific analysis of *PPCZm1-GUS* expression by histological GUS localization in R_1 plants selected for bialaphos resistance. a. GUS staining (arrow) in leaf sheath above the intercalary meristem at the base of a 14-day old greenhouse-grown plant. Incubated in GUS reaction solution for 4 h. Bar=0.5 cm. b. Cross-sectional view through the region indicated by the arrow in a. Incubated in GUS reaction solution for 4 h. Bar=0.5 mm. c. Higher magnification of the GUS-positive cells (arrow) shown in b reveals they are pre-mesophyll. Incubated in GUS reaction solution for 4 h. Bar=0.1 mm. d. GUS-positive pre-mesophyll cells in developing leaf sheath. Incubated in GUS reaction solution for 4 h. Bar=0.1 mm. e. GUS activity in cross-section through a mid-vein of the third leaf of a 9-day old greenhouse-grown seedling associated only with the mesophyll cells. Incubated in GUS reaction solution for 6 h. Bar=0.5 mm. f. Freehand longitudinal section through stem apex of an 11-day old greenhouse-grown plant stained for GUS. Incubated in GUS reaction solution for 8 h. Bar=0.5 mm. g. Dark-field microscopy of a longitudinal paraffin section of stem apex of a 14-day old greenhouse-grown plant stained for GUS. Incubated in GUS reaction solution for 8 h. Bar=0.1 mm. h, i. GUS reaction product in a cross-section through the mid-vein of the third leaf of a 9-day old greenhouse-grown seedling (h), and (i) corresponding dark-field microscopy of h. Incubated in GUS reaction solution for 4 h. Bar=50 μm. j, k. GUS reaction product in a cross-section through the blade of the mature leaf blade of a greenhouse-grown plant (j), and (k) corresponding dark-field microscopy of j. Incubated in GUS reaction solution for 3 h. Bar=50 μm. l, m. Absence of GUS in a kernel (l) developing 21 days after pollination, and mature silk (m). Incubated in GUS reaction solution for 8 h. Bar=0.5 mm. n. Positive GUS staining in glumes of male inflorescence. Incubated in GUS reaction solution for 8 h. Bar=0.5 mm.

inserts. Therefore, it is also not known how many of the labeled fragments are capable of expression. The only single-copy event is shown in lane 14 (Table 1). The R_0 plants were crossed to a proprietary inbred line (CV), and R_1 plants were used for further analysis. Of the plants that showed *GUS* expression, via the histochemical GUS assay, all five were found to be high-copy-number insertion events. Transformants 04, 07, and 12 (lanes 4, 7, and 12, respectively) were used extensively in the remainder of this project.

Development- and cell-specific GUS expression

Development- and cell-specific expression of the *PPCZm1-GUS* construct, pDPG522, in stable transgenics was determined by assays for the function enzyme. Histological GUS localizations were conducted on transformed callus, and all major tissues and organs throughout the maize life cycle, including germinating seeds and seedlings, mature vegetative plant parts, as well as the male and female inflorescence of R_0 plants and R_1 progeny. All dark- and light-grown callus from transformed cell lines were negative for histochemical GUS localization. GUS activity in embryogenic callus was not light-inducible. Expression of GUS was first observed in greening regenerating R_0 shoots, but the remainder of the investigation concerned expression patterns exclusively in R_1 plants.

Developmental analysis for tissue-specific GUS expression was conducted on R_1 plants throughout plant growth, including all tissues of germinating seeds and seedlings, 9–21-day old greenhouse-grown plants and mature vegetative and flowering plants (40–66 days old). Histological GUS reactions were performed on all developing and mature plant structures in comparison to untransformed controls. All tissues of germinating seeds and seedlings were negative for GUS activity (see Table 2). As indicated by the presence of GUS reaction product, the *PPCZm1-GUS* fusion gene was expressed at high levels only in the leaves, collars, leaf sheaths and ligules of the *PPCZm1-GUS*-positive transgenic plants. GUS reaction product was observed initially in development in greening portions of the first and second emergent leaves. In light-grown shoots, the GUS activity was strongest in developing and mature mesophyll cells in the leaf, collar and sheath. GUS staining was observed in leaf sheaths above the intercalary meristem at the base of 9–21-day old greenhouse-grown plants (Figure 3a). Expression of *PPCZm1-GUS* in young leaves was indicated by blue staining in all developing and mature mesophyll cells. In cross-sectional views through developing leaves in the shoot (Figure 3b, c, d), GUS activity occurred only in the outermost greening leaves. GUS reaction product is localized to developing mesophyll cells in leaf sheaths and increased in more mature portions of leaves. Figure 3e shows GUS reaction product in a cross section through the mid-vein of the third leaf of a 9-day old greenhouse-grown seedling associated only with the mesophyll cells. GUS activity was not observed in the collenchyma or vascular tissue of the mid-vein. No histological GUS activity was observed in any of the tissues of the stem apex (Figure 3f, g), or intercalary meristems of immature leaves or leaf primordia. Mesophyll-specific expression at the mid-vein is most clearly visualized in histological paraffin sections (Figure 3h, i). In young leaf tissues from greenhouse-grown seedlings at the three-leaf stage, GUS was localized specifically to the mesophyll. In the blade of more mature leaves, however, a low level of GUS reaction product was observed in nearly all cells, except xylem, but appeared strongest in the mesophyll cells (Figure 3j, k). Sections from the fifth leaf of a ten-leaf greenhouse-

Table 2. The distribution and relative levels of histological GUS expression in three transformants.

Transformant	04	07	12
a. Germinating seeds and seedlings			
germinating seeds	–	–	–
coleoptile	–	–	–
mesocotyl	–	–	–
first leaf	++++	++++	++
second leaf	++++	++++	+++
primary roots	–	–	–
b. Mature vegetative plants			
apical meristem	–	–	–
stem	–	–	–
leaves	++++	++++	++++
leaf sheath	++	++	++
ligule	+	+	NA
roots	–	–	–
prop roots	–	–	–
c. Male inflorescence			
anther	–	–	–
pollen	–	–	–
palea	+	+	NA
lemma	+	+	NA
rachilla	–	–	–
d. Female inflorescence			
kernel	–	–	–
silk	–	–	–
cob	–	–	–
husk leaves (inner)	–	–	–
husk leaves (outer)	+	+	+

grown plant were similar to those of later stages in development (not shown). Activity was highest in the mesophyll, present in other cell types, but absent in the xylem and the bundle sheath (not shown). Dark-field microscopy showed low levels of GUS reaction product as light blue or red (Figures 3i, k). The dark-field images depict low levels of GUS reaction product as faint red in the cytoplasm of the bulliform cells and other cells in the epidermis of the section shown in Figure 3k in comparison to higher levels of GUS reaction product that appear light blue only in the mesophyll cells in the same section. We did not observe histological localization of GUS activity in mature kernels (Figure 3l), silk (Figure 3m), dark-or light-grown primary roots, secondary roots, or prop roots. GUS activity was, however, observed in mesophyll cells in the outer husks of ear shoots (not shown) and in the outer glumes of staminate spikelets (Figure 3n). We did not observe GUS reaction product in any of the tissues of inner husk. A visual comparison was made for the intensity of GUS staining in the major tissues and organs throughout the maize life cycle and ranked on a relative scale of 0–4 where 4 is highest. Table 2 summarizes the distribution and relative levels of histological GUS activity in transformed maize plants.

Various tissues from mature plants of transformant 04 were sampled to quantify the levels of GUS activity using the methylumbelliferone (MU) assay (Figure 4). High levels of GUS activity, at ca. 160 000 pmol/min

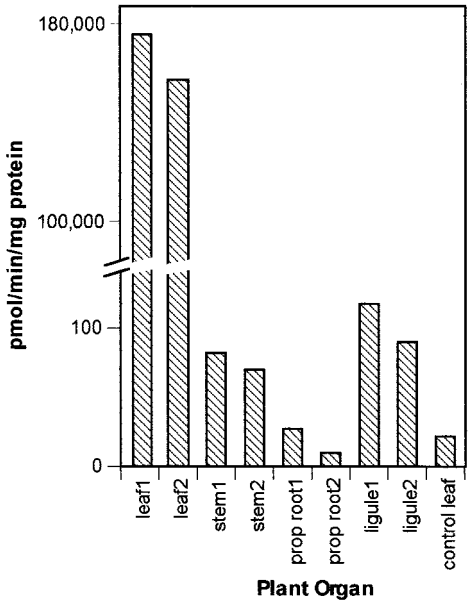

Figure 4. GUS activities detected in protein extracts of mature plant tissues of transgenic maize plants. GUS activities are expressed in picomoles of the reaction product 4-methylumbelliferone (MU) generated per minute per milligram protein.

per mg protein, were observed in the mature leaves (Figure 4). Other tissues from mature transformed plants did not show activity significantly above levels measured in untransformed control leaf tissue. These results demonstrate that the *PPCZm1* promoter is capable of driving GUS expression at a high level exclusively in leaf tissue.

Plant growth for induction studies

To compare inductive stimuli that affect expression of the *PPCZm1* promoter, we first evaluated conditions for transgenic seedling growth. Maize seedlings germinated and grown in complete darkness develop as etiolated shoots. However, when maize seedlings are germinated under low light (40 μE m^{-2} s^{-1}) in the first three days of growth and then transferred to darkness, the second leaf expands normally from the whorl. Seedlings grown this way are similar to those used for isolating protoplasts in transient expression assays (Schäffner and Sheen, 1992). Since the first leaf may be pre-exposed to light, while the second leaf is early in development and deeply buried within the whorl, coleoptile, and first leaf, our analyses are primarily concerned with activity in the second emerging leaf.

Transformants (R_1) were grown from seeds on germination paper moistened with 0.5% Liberty, a formulation of glufosinate (for selection of *BAR*-positive plants), and 0.1% Domain (a commercial fungicide) at 100% relative humidity in a sealed plastic bag in dim light (40 μE m^{-2} s^{-1}) conditions for three days. These BAR-selected germinated seedlings were transferred to hydroponics or Perlite in 7.6 cm pots and watered with half-strength Clark's medium in a Shearer incubator at 60% relative humidity in the dark at 27 °C for 4 days and then exposed to 150 μE m^{-2} s^{-1} of light for 12 h. Entire seedlings were then stained for GUS activity (Figure 5a, right). Alternatively, seedlings were grown in continual darkness under the same conditions (Figure 5a, left) and then exposed to 150 μE m^{-2} s^{-1} for 12 h. The GUS reaction product was most intense at the leaf margins of both the first and second leaves regardless of exposure to low light during the first three days after germination. The intensity of GUS reaction product increased basipetally during prolonged exposure to light. GUS staining was more intense when seedlings were grown under these conditions and transferred to 150 μE m^{-2} s^{-1} of light for 24 h (not shown). These results also indicate that neither bialaphos nor fungicide treatment affect the pattern of GUS activity. Seedlings used for induction studies, therefore, were grown according to the conditions described for the plant shown in Figure 5a, right side, unless otherwise indicated.

Light induction studies

To evaluate the time necessary to induce GUS activity driven by the *PPCZm1* promoter, we constructed a time course of light induction. Expression of the GUS reporter gene in response to the duration of light was examined in transformed seedlings. Induction was conducted by removing plants from darkness and transferring them to 150 μE m^{-2} s^{-1} of light for 0–24 h. Light induction was stopped by removing plants from the light and staining them for GUS activity (Figure 5b). Histological GUS reaction product was clearly visible after 5 h exposure to light. A time course of light exposure on the first leaf of plants shows a similar light induction requirement (Figure 5c). A low level of GUS activity was observed in the first leaf of some dark-grown controls, often in longitudinal sectors. Care was taken in subsequent experiments to choose bialaphos-resistant plants whose coleoptile protruded the seed coat 1–2.5 cm to obviate this background. Dark-grown seedlings without the 3-

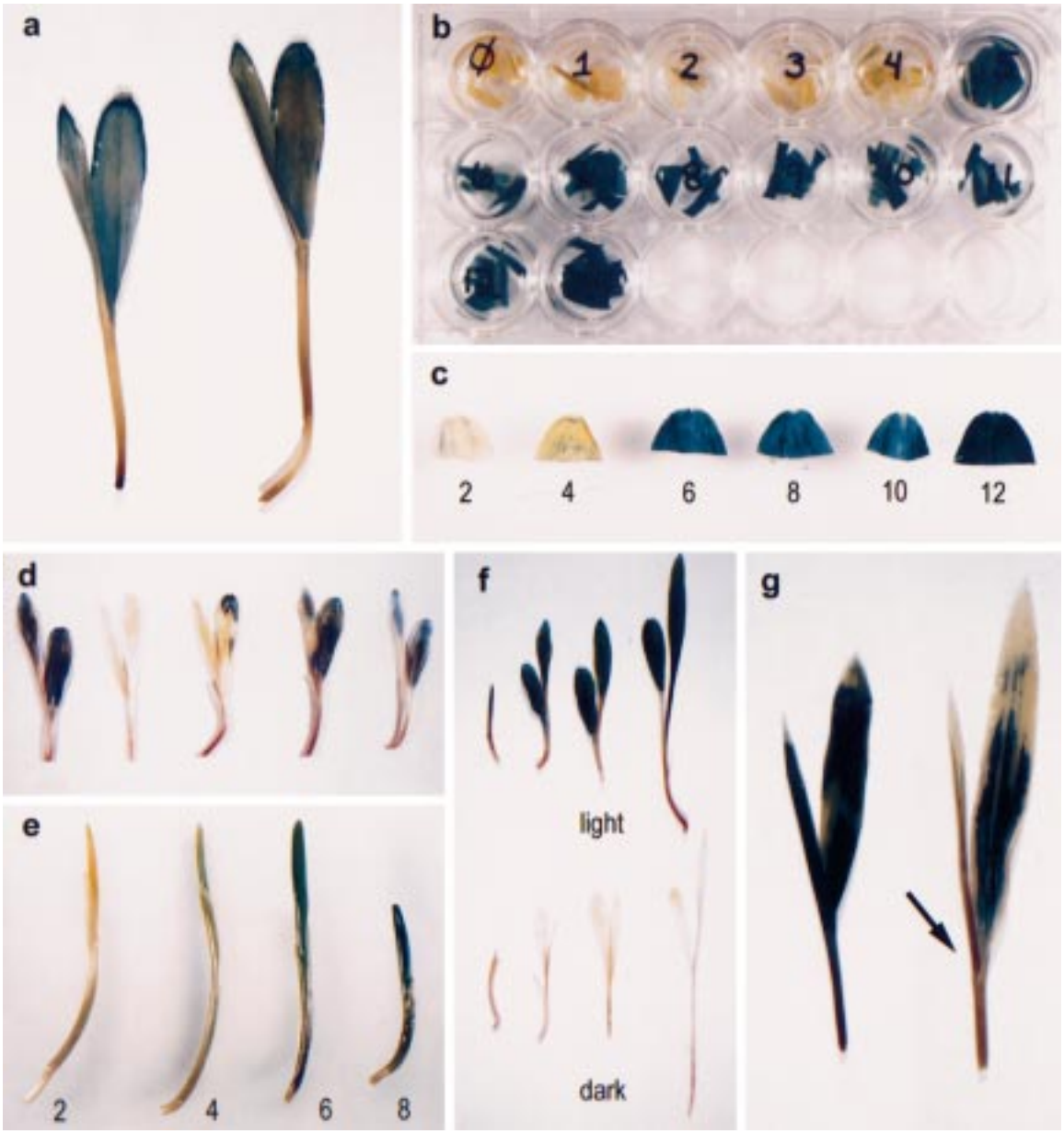

Figure 5. Light and metabolic induction of *PPCZm1-GUS* in transformed plants. a. *PPCZm1-GUS* expression in hydroponically grown seedlings of transformant 04 after 24 h light. b. Time course of light induction (transformant 04) for 0–12 and 24 h. c. Time course of light induction (h) in the first leaf (transformant 07). d. Inhibition of *PPCZm1-GUS* response to light by chloramphenicol (CAP), glucose and acetate. Light-grown control, dark-grown control, 200 mg/l CAP, 200 mM glucose, and 3 mM acetate (left to right). e. Time course of light induction (h) in etiolated seedlings (transformant 07). f. Developmental sequence of dark-grown plants (transformant 04) after 12 h light. g. Acetate repression of *PPCZm1-GUS* expression induced by light in the third leaf. Dark-grown plants watered without (left) and with (right) 3 mM acetate and illuminated for 24 h. Only the second (arrow) and third leaves are shown.

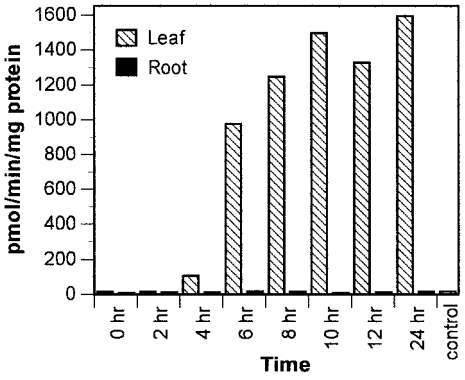

Figure 6. GUS activities detected in the second leaf and roots of dark-grown seedlings after exposure to light. GUS activities were measured from samples pooled from three transformed plants and are expressed in pmol of the reaction product 4-methylumbelliferone (MU) generated per minute per milligram protein.

day low light exposure were etiolated (Figure 5e) and did not stain for GUS activity, but showed light induction of GUS 4–6 h after exposure to light. Examination of a developmental sequence of dark-grown seedlings of transformant 04, exposed to 150 μE m^{-2} s^{-1} for 24 h during early growth showed that induction of GUS activity was not growth-stage-dependent (Figure 5f). These experiments were repeated several times with consistent results and GUS localization in these shoots was always correlated with the patterns of greening.

GUS activity was quantified relative to the duration of light exposure. Samples of the second leaf and root material were collected and pooled from three plants during light induction for quantitative GUS activity assays. Results for GUS activity during light induction showed detectable activity in the second leaf after 4 h light exposure (Figure 6) and ca. 1600 pmol/min per mg protein after 12 h. These results indicate that the level of GUS activity in the second leaf of transgenic seedlings is light-inducible. Light-inducible expression of the *PPCZm1-GUS* construct is several hundred times higher than in transformed dark-grown controls and untransformed light-grown leaf tissues after 6 h of light exposure. However, the levels of GUS activity in seedling leaves was ca. 1000-fold lower than in mature leaves of the same transformant. Root tissue exposed to the same levels and duration of light showed no activity above untransformed control leaf tissue.

The relationship of chloroplast development to light induction of GUS activity was further investigated by the use of an inhibitor of chloroplast protein synthesis, chloramphenicol (CAP), on dark-grown seedlings. After five days growth in darkness, 50–200 mg/l CAP was added 24 h prior to light induction (12 h at 150 μE m^{-2} s^{-1} light followed by GUS staining). Except for occasional sporadic patches, which may result from incomplete translocation of CAP in seedling tissues, the leaves of plants treated with CAP did not green in the light and did not stain positive for GUS activity (Figure 5d). The positive GUS activity in the first leaf of the seedling shown in Figure 5d was also green. Chloramphenicol will inhibit mitochondrial as well as plastid translation, and in sufficiently high levels can even reduce (directly or indirectly) cytoplasmic translation. This drug is not specific to plastid ribosomes, and a direct connection between plastid development and GUS activity cannot be established by this type of study alone.

Metabolite induction studies

Expression of photosynthetic genes is known to be modulated by various metabolites. Therefore, experiments were conducted to examine the effect of various metabolic signals on the response of *PPCZm1-GUS* transformants to light. We were also interested in examining the effects of nitrate and salt on dark-grown seedlings. After five days growth in darkness in hydroponics or Perlite, selected seedlings were transferred to half-strength Clark's medium containing either 200 mM glucose or 3 mM acetate for 24 h prior to light induction, or 200 mM NaCl or 16 mM KNO_3 and left in darkness. Light- and dark-grown controls were placed in fresh half-strength Clark's medium. Light induction was for 12 h at 150 μE m^{-2} s^{-1} followed by staining for GUS activity (Figure 5d). Plants treated with glucose or acetate appeared as green as control plants in the light (not shown), but they had reduced GUS activity (Figures 5d and 7). These results are especially apparent in comparisons between samples of the second leaves from treated seedlings. The differential repression by glucose and acetate in the first and second leaves could be due to uptake and age differences during the incubation. The effect of acetate on the emerging third leaf was also examined. Dark-grown seedlings of transformant 04 were transferred to Perlite and grown for 7 days by watering with half-strength Clark's medium. Before light induction, plants were watered and misted with half-strength Clark's medium, with and without 3 mM potassium acetate, and then exposed to 150 μE m^{-2} s^{-1} illumination for 12 h. All plants greened normally, and those

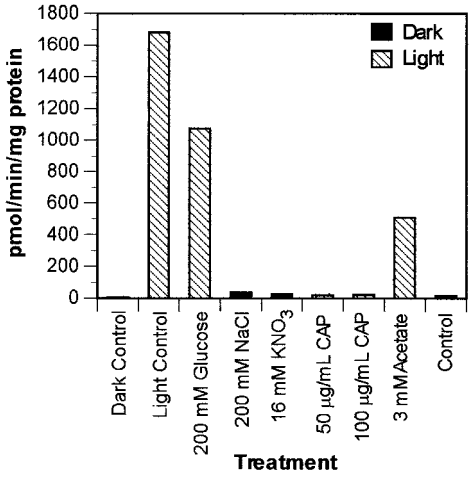

Figure 7. GUS activity detected in the second leaf seedlings after exposure to light and various metabolic stimuli. GUS activities were measured from samples pooled from three transformed plants and are expressed in pmol of the reaction product 4-methylumbelliferone (MU) generated per minute per milligram protein.

exposed to acetate showed a dramatic reduction in histological GUS staining in the third leaf (Figure 5g).

Pooled samples were made for quantitative GUS activity assays from the second emergent leaves taken from three individuals following the above induction studies. The results reflect those observed by histological staining (Figure 7) and show a two- to three-fold reduction in GUS activity in plants treated with either glucose or acetate. Consistent with previous observations on light induction, light was capable of inducing a more than 1500-fold increase in GUS activity compared with dark-grown controls. Consistent with observations of histological GUS staining, plants that had been pre-treated with chloramphenicol (50–200 μg/ml) prior to light induction do not show GUS activity levels above those of untransformed control plants. Salt (NaCl) stress has been shown to induce activity of PEPCase in the CAM halophyte *Mesembryanthemum* (Cushman *et al*., 1989), and exposure to neither NaCl nor KNO_3 induced GUS activity in transformed seedlings in the absence of light. These experiments were repeated five times for each treatment with similar results.

Discussion

The purpose of our study was to investigate the developmental, cell-specific, light and metabolic regulation of the homologous C_4 *PPCZm1* promoter in transgenic maize. We have characterized the pattern of homologous C_4 *PPCZm1* expression in maize, using a *PPCZm1-GUS* chimeric gene expressed in stable transformants. In addition, we have used the transformants to conduct physiological induction studies of the GUS reporter. Our results indicate that the expression of the C_4 *PPCZm1-GUS* gene is mesophyll-specific and influenced by development, light, glucose, acetate and, perhaps, chloroplast biogenesis in stable transgenic maize plants.

This report is the first to analyze the activity of a promoter of a C_4 photosynthetic gene in a transgenic C_4 monocot. Schäffner and Sheen (1991) suggested that *PPCZm1* was created after acquiring a new promoter from a pre-existing *PPC* gene and this may have been an essential event for the evolution of C_4 photosynthesis. From sequence data, it is likely that *PPCZm1* originated from a member of the *PPCZm3* subgroup, because of nearly identical sequences in the 5′-coding, untranslated, and proximal promoter regions. The C_4 gene, *PPCZm1*, gained a specific expression pattern through the acquisition of a unique upstream and a TATA-box-like TATTT sequence via genomic rearrangements at that point. The functional significance of such a hypothetical evolutionary event was demonstrated by transient expression assays, and is reiterated in the present analysis of transformed plants.

Our analysis of the maize C_4 *PPC* promoter *(PPCZm1)* in transgenic maize is consistent with previous investigations of transcriptional and developmental control of this gene in isolated maize protoplasts (Schäffner and Sheen, 1992). Moreover, our results are consistent with recent transgenic studies of *PPC* promoters in other plant species (Matsuoka *et al.*, 1994; Stockhaus *et al*., 1997). The C_4 *PPCZm1* promoter was shown to preferentially direct mesophyll-specific expression in transgenic C_3 rice plants (Matsuoka *et al.*, 1994). Recently, Stockhaus *et al.* (1997) reported a comparison of expression driven by the promoters for the C_4 and C_3 isoforms of *PPC* from *Flaveria* spp. in transgenic C_4 and C_3 dicotyledonous plants. They showed that the promoter region of the *F. trinervia* C_4 *PPCA1* gene directed mesophyll-specific leaf expression in stable transformed C_4 *F. bidentis*, and was restricted to the palisade parenchyma in the transformed C_3 tobacco plants. Furthermore, the promoter from the orthologous non-photosynthetic *PPC* gene in the C_3plant *F. pringle* was expressed in vascular tissue as well as in the mesophyll in transgenic C_4 *F. bidentis.* These results reconfirm that *cis*-acting

elements of the *PPC* gene have probably been altered during evolution to result in mesophyll-specific leaf expression. It is intriguing that the same *cis* elements in the C_4 *PPCZm1* promoter can direct light-inducible and leaf-specific expression in rice, a C_3 monocot plant (Matsuoka *et al.*, 1994). Thus, the evolution of the C_4 gene might simply involve combining the existing structural gene and regulatory sequences through unequal recombination in C_4 plants.

Our results show that the maize C_4 *PPC* promoter is developmentally regulated and influenced by light and metabolites. We have demonstrated that 1.7 kb of the 5′-flanking sequences of *PPCZm1* alone is sufficient to direct leaf-specific expression primarily in mesophyll cells of stable maize transformants. McCormac *et al.* (1997) examined expression of *PPC* genes in photosynthetic and non-photosynthetic leaf regions of the C_4 *Amaranthus tricolor*. They showed that alterations in either photosynthetic activity or development that result in a loss of activity can affect *PPC* gene expression at multiple regulatory levels. In plants grown under lower humidity in the greenhouse, hydroponics, or wetted Perlite, the *PPCZm1* promoter directs seedling leaf-specific and light-dependent expression causing GUS to accumulate to high levels in greening and fully green leaves. We observed expression during greening to be initiated in mesophyll and pre-mesophyll cells and that these constraints may become lessened during leaf maturation. The methylation site 3 kb upstream of the 5′-flanking region of the *PPCZm1* gene might not be essential for the C_4-specific expression patterns (Langdale *et al.*, 1991). Recently, Morishima (1998) reported the identification of a preferred binding site of a light-inducible DNA-binding factor (MNF1) within the 5′-flanking region (-1012 to -695) of the C4 PPC gene in maize. Quantitative measurements of GUS activity indicate that the *PPCZm1* promoter is expressed at a low level in expanding seedling leaves and at much higher levels in the mature leaf blade. We have also demonstrated expression in other leaf-like organs, such as flower parts, that have not been previously investigated in molecular or transient expression studies. However, expression in those organs was confined to mesophyll or mesophyll-like cells in the glumes and outer husk leaves; we did not observe expression in roots, stems or shoot apical meristems.

Our results are in strong agreement with previous studies which have shown that C_4 gene expression in maize is dependent on cell differentiation (Langdale *et al.*, 1988a, b, 1991). Based on *in situ* mRNA and protein localization studies, Ramsperger *et al.* (1996) found that individual C_4 genes, including *PPC*, in the dicot C_4 *Amaranthus hypochondriacus* are independently regulated as they become localized to pre-mesophyll cells. Similarly, Stockhaus *et al.* (1997) demonstrated that the C_4 *PPC* promoter of *Flaveria trinervia* becomes activated when a certain developmental stage has been achieved by mesophyll cells during differentiation. Our results indicate that the sequences required for proper developmental control of heterologous gene expression driven by the C_4 *PPCZm1* promoter are within this 1.7 kb fragment in a monocot C_4 maize; however, the molecular signals required during development are not known.

However, all of the selected transgenics examined in our analysis were high-copy-number transformants, raising the possibility that some effects observed are due to aberrant stoichiometry between the transgenic PPC promoters and endogenous regulatory factors. This may not pose a problem with results from leaf staining patterns where corroborative data are present in the literature, but further studies are necessary to substantiate the staining patterns observed in other organs for which less is known about the behavior of the endogenous PPC gene, and for which stoichiometry effects could skew results. Also, it is not known from our analysis how many of the copies are non-expressing as incomplete and/or truncated inserts. The transgenic plants grew normally with respect to untransformed controls and showed no sign of chlorosis; however, the effect between the high copy number of transgenic PPC promoters and endogenous regulatory factors is not known.

Light induction studies of the *PPCZm1* promoter showed visible and measurable GUS activity in illuminated dark-grown seedlings, regardless of germination conditions, after only 6 h of exposure. Several other maize photosynthetic gene promoters can respond to immediate and constant illumination for light induction, whereas *PPCZm1* is not subjected to such transcriptional control (Yanagisawa and Izui, 1990; Schäffner and Sheen, 1991, 1992; Sheen, 1991). The light induction of *PPCZm1* during the greening process might be solely dependent on light-mediated developmental changes (Yanagisawa and Izui, 1990; Schäffner and Sheen, 1992). The plants used for our studies were grown under similar conditions to those used in earlier transient expression assays, with electroporated protoplasts from the second expanding leaf of dark-grown seedlings (Schäffner and Sheen, 1991, 1992 Sheen, 1991). The results with this promoter

expressed in transgenic plants are consistent with results derived from transient expression assays with the protoplast system. Inhibition of both greening and expression of *PPCZm1-GUS* occurred in stable transgenics exposed to CAP and light. Chloramphenicol will inhibit mitochondrial as well as plastid translation, and in high enough concentrations can even reduce (directly or indirectly) cytoplasmic translation. While CAP is not specific to plastid ribosomes, and a direct connection between plastid development and GUS activity cannot be established by this type of study alone, our results may indicate that chloroplast biogenesis may be necessary for light-mediated developmental changes required for *PPCZm1-GUS* expression. Further experiments are necessary on the role of chloroplast development on photosynthetic gene expression.

Expression of photosynthetic genes is known to be modulated by various metabolites (Sheen, 1990; Jang and Sheen, 1994), and certainly production of these metabolites is developmentally regulated. Metabolic repression of photosynthetic gene expression by various hexose sugars (Jang and Sheen, 1994) and acetate (Sheen, 1990) has been previously demonstrated in protoplast transient expression assays. Our experiments with *PPCZm1-GUS* stable expression in the second emergent leaf of transgenic seedlings substantiate these results. We have shown a two- to three-fold decrease in GUS activity in greening transformants pre-treated with glucose and acetate. The repression in intact seedlings is not as strong as in isolated protoplasts probably due to limited accessibility of glucose and acetate and/or the lack of antagonist signals, such as nitrate and hormones, in cultured protoplasts (Jang and Sheen, 1997). Salt (NaCl) stress was shown to induce a 50-fold increase in activity of PEPCase in the CAM halophyte *Mesembryanthemum* (Cushman *et al*., 1989). Suzuki *et al.* (1994) reported on transcriptional and postrtranscriptional regulation of nitrogen-responding expression of *PPCZm1* in maize. The induction of *PPCZm1* in maize was reported to be nitrogen-dependent and was increased markedly by supply of a nitrogen source. Neither NaCl nor KNO_3 had a measurable effect on stimulation of the *PPCZm1* promoter in dark-grown plants.

The use of stable transgenic maize plants has allowed us to examine expression characteristics imposed by the 5′-flanking region of the C_4 *PPCZm1* gene. The generation of transgenic maize plants expressing the *PPCZm1-GUS* gene construct has enabled evaluation of the developmental expression of this promoter in a homologous system *in planta* and in a broad variety of cell types not possible in protoplast transient assays. This study is novel in that it extends measurements of the maize C4 PPC promoter activity to intact plants, permitting the testing of the effects of light, developmental stage, and application of metabolites. This study is also novel in that it relies on transgenic maize as a test system. There are still few reports in the literature that describe the characteristics (and caveats) of maize transgenics. The creation of a series of transgenic maize plants with various promoters would be useful in elucidation of interacting factors involved in developmental and cell-type-specific expression of C_4 photosynthetic genes in monocots.

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