Plant Molecular Biology **44:** 543–557, 2000.

Binding of cell type-specific nuclear proteins to the 5′-flanking region of maize C_4 phospho*enol*pyruvate carboxylase gene confers its differential transcription in mesophyll cells

Mitsutaka Taniguchi[1,*], Katsura Izawa[1], Maurice S.B. Ku[2], Jenq-Horng Lin[3], Hideaki Saito[4], Yuji Ishida[4], Shozo Ohta[4], Toshihiko Komari[4], Makoto Matsuoka[5] and Tatsuo Sugiyama[1]
[1]*Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan (*author for correspondence; e-mail: G44485A@nucc.cc.nagoya-u.ac.jp);* [2]*School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA;* [3] *Department of Botany, National Chung-Hsing University, 250 Kuo-Kuang Road, Taichung 40227, Taiwan;* [4]*Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438, Japan;* [5]*BioScience Center, Nagoya University, Nagoya 464-8601, Japan*

Received 4 January 2000; accepted in revised form 30 June 2000

Key words: C_4 photosynthesis, maize, phospho*enol*pyruvate carboxylase, transgenic plant, transcription, gene expression

Abstract

C_4-type phosph*enol*pyruvate carboxylase (C_4PEPC) acts as a primary carbon assimilatory enzyme in the C_4 photosynthetic pathway. The maize C_4PEPC gene (*C4Ppc1*) is specifically expressed in mesophyll cells (MC) of light-grown leaves, but the molecular mechanism responsible for its cell type-specific expression has not been characterized. In this study, we introduced a chimeric maize *C4Ppc1* 5′-flanking region/β-glucuronidase (GUS) gene into maize plants by *Agrobacterium*-mediated transformation. Activity assay and histochemical staining showed that GUS is almost exclusively localized in leaf MC of transgenic maize plants. This observation suggests that the introduced 5′ region of maize *C4Ppc1* contains the necessary *cis* element(s) for its specific expression in MC. Next, we investigated whether the 5′ region of the maize gene interacts with nuclear proteins in a cell type-specific manner. By gel shift assays with nuclear extracts prepared from MC or bundle sheath cells (BSC), cell type-specific DNA-protein interactions were detected: nuclear factors PEP_{Ib} and PEP_{Ic} are specific to MC whereas PEP_{Ia} and PEP_{IIa} are specific to BSC. Light alters the binding activity of these factors. These interactions were not detected in the assay with nuclear extract prepared from root, or competed out by oligonucleotides corresponding to the binding sites for the maize nuclear protein, PEP-I, which is known to bind specifically to the promoter region of *C4Ppc1*. The results suggest that novel cell type-specific positive and negative nuclear factors bind to the maize *C4Ppc1* 5′-flanking region and regulate its differential transcription in MC in a light-dependent manner.

Abbreviations: BAR, phosphinothricin acetyltransferase; BSC, bundle sheath cell(s); CaMV, cauliflower mosaic virus; DTT, dithiothreitol; GUS, β-glucuronidase; MC, mesophyll cell(s); ME, malic enzyme; 4-MU, 4-methylumbelliferone; NOS, nopaline synthase; PEPC, phospho*enol*pyruvate carboxylase; PMSF, phenylmethylsulfonyl fluoride; pro, promoter; ter, terminator.

Introduction

C_4 plants possess a specialized Kranz-type leaf anatomy, which contains two distinct photosynthetic cell types, mesophyll (MC) and bundle sheath cells (BSC). The C_4 dicarboxylate cycle of photosynthetic carbon assimilation occurs between the two cell types and acts as a 'CO_2 pump' to concentrate CO_2 in BSC chloroplasts (for review, see Hatch, 1988; Leegood,

1997; Kanai and Edwards, 1999). Photosynthetic enzymes involved in the C_4 pathway are not unique to C_4 plants; their counterparts are also found in C_3 plants at low levels. Therefore, it has been suggested that most of the genes encoding the C_4 enzymes had evolved from the corresponding ancestral genes in C_3 plants and that mechanisms responsible for the C_4-specific expression patterns of these genes (high-level expression, induction by light, and organ- and cell type-specific expression) had been acquired during the evolution of C_4 plants (Furbank and Taylor, 1995; Ku *et al.*, 1996).

At least three isozymes of phospho*enol*pyruvate carboxylase (PEPC) have been recognized in C_4 plants (C_4-type, C_3-type and root-type) (for reviews, see Chollet *et al.*, 1996; Ku *et al.*, 1996). The C_4-type PEPC (C_4PEPC) is a primary carbon assimilatory enzyme specifically confined in MC. Only the C_4PEPC gene among the PEPC gene family shows light inducibility and MC-specific expression in leaf. Several molecular studies have attempted to address the underling regulatory mechanisms of cell type- and tissue-specific expression of the C_4PEPC gene (*C4Ppc1*). First, nuclear run-off experiments with nuclei isolated from maize MC and BSC directly demonstrated that *C4Ppc1* is transcribed in MC but not in BSC (Schäffner and Sheen, 1991). This observation leads to the supposition that the cell type-specific expression of *C4Ppc1* is regulated at the transcriptional level. Second, by gel shift assay, at least two binding sites for leaf-specific nuclear proteins (MNF1 and PEP-I) have been identified in the promoter region of *C4Ppc1* (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991; also see Figure 2). However, MNF1 also interacts with the cauliflower mosaic virus (CaMV) 35S promoter (Yanagisawa and Izui, 1992), and its specific role in C_4 gene expression remains to be established. Third, homologous transient expression assay has indicated that two long direct repeated sequences including the PEP-I binding site enhance the transcription of a reporter gene in isolated maize mesophyll protoplasts (Schäffner and Sheen, 1992). Schäffner and Sheen (1992) also reported that leaf-specific expression of *C4Ppc1* is mainly controlled by its 5′-upstream region and the induction by light relies on light-mediated developmental changes of MC instead of an immediate activation of transcription found for other maize genes, such as the C_4 pyruvate, orthophosphate dikinase (PPDK) gene, the chlorophyll *a/b*-binding protein gene, and the Rubisco small subunit (*rbcS*) gene. Fourth, Yanagisawa and colleagues have earlier cloned two maize cDNAs from the Dof gene family (Yanagisawa and Izui, 1993; Yanagisawa, 1995). Recently, Dof1 and Dof2 have been shown to bind to the AAAG motif found in the 5′-upstream region of *C4Ppc1* (Yanagisawa and Sheen, 1998). Dof1 is a transcriptional activator while Dof2 acts as a repressor of Dof1-mediated transcriptional activation. Based on these observations, they proposed a model for tissue-specific and light-regulated expression of *C4Ppc1* mediated by Dof proteins. Fifth, analyses with transgenic rice plants with an introduced reporter gene driven by the maize *C4Ppc1* promoter (Matsuoka *et al.*, 1994) or an intact maize *C4Ppc1* gene (Ku *et al.*, 1999) have revealed that the necessary *trans*-acting factors required for the MC-specific and light-dependent expression of *C4Ppc1* are also present in rice, a C_3 plant.

These observations suggest that the maize *C4Ppc1* possesses *cis*-acting elements necessary for its organ- and cell type-specific expression, but so far no specific element has yet been recognized. One of the possible reasons for this slow progress is the lack of an efficient stable transformation system for C_4 plants, especially C_4 monocots. Recently, an *Agrobacterium*-mediated transformation system for maize has been developed (Ishida *et al.*, 1996). We have applied this system to introduce chimeric genes consisting of the β-glucuronidase (GUS) gene directed by the 5′-flanking region of the maize *C4Ppc1* into maize. GUS expression analysis of the transgenic maize plants revealed that GUS gene is expressed in a similar manner to endogenous *C4Ppc1* (Sheen and Bogorad, 1987; Hudspeth and Grula, 1989). This observation leads us to conclude that the introduced 5′-flanking region of the maize *C4Ppc1* contains *cis*-acting element(s) necessary for conferring its C_4-specific patterns of gene expression.

It is possible that the expression of *C4Ppc1* is regulated by binding of cell type-specific *trans*-acting factors on the *C4Ppc1* promoter, caused by changes in abundance, localization or affinity of the factors during development. To test this hypothesis, we examined the cell type-specific distribution of nuclear proteins which bind to the *C4Ppc1* 5′-flanking region. In previous gel-shift assays for detection of nuclear proteins binding to the *C4Ppc1* promoter, nuclear extracts were prepared by weakly blending whole maize leaves (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991). Consequently, it is likely that the nuclear extracts thus obtained might have preferentially come from MC and only the interaction between MC nu-

clear proteins and the *C4Ppc1* promoter was detected. Although *C4Ppc1* is expressed only in MC, it is important to compare the binding activities of nuclear proteins isolated from MC with those isolated from BSC in order to gain a complete understanding of cell type-specific expression mechanisms. In this study, we have separated mesophyll protoplasts and bundle sheath strands from maize leaves at different developmental stages for extraction of nuclear proteins. By gel shift assay, we found that MC- or BSC-specific nuclear factors bind the 5′-flanking region of *C4Ppc1* which may account for its specific expression in MC.

Materials and methods

Plant material and growth conditions

Wild-type or transformed maize (*Zea mays* L. inbred line A188) was grown in a growth chamber with 14 h of illumination (500 μE m^{-2} s^{-1}) at 28 °C and 10 h of darkness at 20 °C per day. Plants were fertilized regularly during growth.

For gel shift analysis, another maize strain (cv. Golden Cross Bantam T51) was used. Etiolated plants were grown in vermiculite in the dark at 23 °C for 12 days. For greening plants, the etiolated seedlings were exposed to continuous white light at an intensity of about 120 μE m^{-2} s^{-1} at 26 °C for 17 h. For green plants, seeds were grown in the growth chamber for 14 days. For isolation of roots, the seeds were grown in hydroponic culture with Arnon and Hoagland nutrient solution (Arnon and Hoagland, 1940) for 12 days in a growth chamber at 28 °C with 16 h of illumination (white light, 120 μE m^{-2} s^{-1}) and 8 h of darkness per day.

Construction of chimeric fusion genes and production of transgenic maize plants

For the PEPC(0.6)-GUS fusion gene construct, the 5′-upstream region (−571 to +131 relative to the transcription initiation site) of *C4Ppc1* (Hudspeth and Grula, 1989) was amplified from maize (B73 inbred line) genomic DNA by PCR with two synthetic oligonucleotides (sense primer 5′-AGACGACTCTTAGCCACAGC-3′, antisense primer 5′-TCGATGGAGTGGTGCTTCTC-3′. The PCR product was subcloned into pCR1000 vector, cut with *Nco*I (position +80) and treated with mung bean nuclease and Klenow fragment of DNA polymerase I to change the *Nco*I site to a blunt end. The clone was recut with *Hin*dIII, and the *Hin*dIII-*Nco*I fragment was inserted into the *Hin*dIII-*Sma*I site of pBI121 after removal of the CaMV 35S promoter. We confirmed the junction between *C4Ppc1* and the GUS gene by sequence analysis. The construct contained the *C4pc1* 5′-upstream region from −571 to +81.

The 3′ border of the 5′-upstream DNA fragment is located just 5′ of the translation initiation site in *C4Ppc1*. The insert (*C4Ppc1 pro-GUS-NOS ter*) of the modified plasmid was cut out and inserted between the left border and *CaMV 35S pro-BAR-NOS ter*) in the T-DNA region of intermediate vector pSB25 (Ishida *et al.*, 1996) (Figure 1). The PEPC(1.3)-GUS fusion gene which contains the 5′-upstream region (−1212 to +83) of *C4Ppc1* from maize Golden Cross Bantam strain was constructed as described previously (Matsuoka and Sanada, 1991) and inserted into pSB25. The 3′ border of the promoter fragment used for the PEPC(1.3)-GUS gene is located two nucleotides upstream to the translation initiation site in *C4Pc1*. There are several deletions and substitutions of nucleotides between the *C4Ppc1* 5′-upstream regions from the two maize genotypes (B73 and Golden Cross Bantam) and the major transcription initiation sites were different to each other (Hudspeth and Grula, 1989; Matsuoka and Minami, 1989). These derivatives of pSB25 were introduced to *Agrobacterium tumefaciens* strain LBA4404 (pSB1) that carried a helper plasmid and an acceptor vector, pSB1 (Komari *et al.*, 1996). Homologous recombination occurred between the acceptor vector and the modified intermediate vector.

An *Agrobacterium* strain that carried the hybrid vector was obtained by screening with spectinomycin and used to infect immature embryos of maize inbred line A188 (Hiei *et al.*, 1994; Ishida *et al.*, 1996). The inoculated embryos were cultured on a phosphinothricin-containing medium, and resistant calluses were obtained. Plants were regenerated from the calluses, transferred to soil in pots and grown to maturity in a greenhouse. Primary transformants were self-pollinated, and the resulting seeds (T_1 plants) were collected and used for further analyses. Construction of transgenic maize which contains the construct *CaMV 35S pro-GUS-NOS ter* was previously described (Ishida *et al.*, 1996).

GUS assay

Wild-type and T_1 transgenic plants were grown to maturity in the growth chamber. For fluorometric assay of GUS, the plant tissues harvested were ground in the

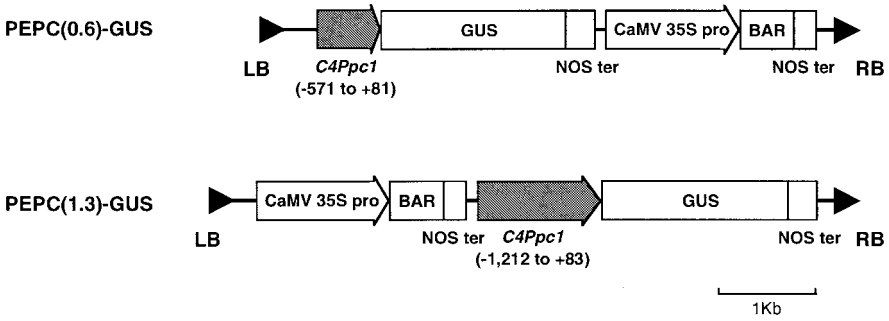

Figure 1. Construct of T-DNA region containing *C4Ppc1 pro-GUS-NOS ter* used for maize transformation. The 5′-upstream region (−571 to +81) of *C_4Ppc1* from maize B73 strain and the 5′-upstream region (−1212 to +83) of *C_4Ppc1* from Golden Cross Bantam strain were used for PEPC(0.6)-GUS and PEPC(1.3)-GUS constructs, respectively. GUS, coding region of the β-glucuronidase gene; NOS ter, 3′ signal of the nopaline synthase gene; CaMV 35S pro, cauliflower mosaic virus 35S promoter; BAR, coding region of the phosphinothricin acetyltransferase gene; LB, left border; RB, right border.

soluble protein extraction medium (50 mM HEPES-KOH pH 7.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EDTA, 5 mM DTT, 5% v/v glycerol) with a mortar and a pestle at 4 °C. The homogenate was centrifuged at 12 000 × *g* for 10 min. The supernatant of the homogenate was used for fluorometric assay of GUS activity by the method of Jefferson (1987). Protein concentration in the supernatant was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

For measurement of GUS activity in MC and BSC, mesophyll protoplasts and bundle sheath strands were isolated by digestion of green leaves from transgenic plants according to a previously reported method (Ohnishi and Kanai, 1983). The MC and BSC preparations were macerated in the soluble protein extraction medium at 4 °C, centrifuged, and the supernatant used for assay of GUS activity. The cross-contamination in the preparations was estimated by measuring the activities of PEPC (Hatch and Oliver, 1978) and NADP-malic enzyme (ME) (Kanai and Edwards, 1973), the respective marker enzymes for MC and BSC. The purity of the MC preparation was estimated by calculating the ratio of NADP-ME activity in the MC and BSC preparations (Edwards *et al.*, 1979) and that of the BSC preparation was calculated with values of PEPC activity in both the preparations.

For histochemical analysis of GUS activity, plant tissues were harvested and cut into small pieces of ca. 1 cm. The tissues were embedded in 5% agar and sectioned with a micro-slicer (DTK-100, Dosaka EM, Kyoto, Japan). The sections were soaked in histochemical staining solution (50 mM sodium phosphate buffer pH 7.0, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 20% v/v methanol) and subjected to a brief vacuum. After incubation for several hours at 37 °C, 70% ethanol was used to wash the tissues. The stained sections were mounted on microscope slides, and examined and photographed using an Olympus BH-2 microscope. For histochemical detection of GUS activity in epidermal cells, small pieces of leaf blades were directly immersed in the histochemical staining solution and incubated. After an ethanol wash, the leaf segments were observed with a light microscope.

Isolation of mesophyll protoplasts and bundle sheath strands and preparation of nuclear extracts

Mesophyll protoplasts were isolated by digestion of well differentiated leaves from etiolated, greening and green plants according to a previously reported method (Ohnishi and Kanai, 1983) except that 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM 2-mercaptoethanol were included in the digestion medium. Bundle sheath strands were mechanically isolated using a Polytron homogenizer (Agostino *et al.*, 1989). In brief, leaves were sliced with a razor blade and blended twice in a precooled blending medium (0.35 M sorbitol, 25 mM Hepes-KOH pH 7.5, 2 mM $MgCl_2$, 2 mM potassium phosphate, 2 mM sodium ascorbate) with a Polytron homogenizer for 30 s at speed 10. The homogenate was then filtrated through two layers of Miracloth (Calbiochem, La Jolla, CA) and washed with an excess volume of the blending medium to remove cellular extracts from broken cells. The same blending procedure was repeated twice, and the residual bundle sheath strands on Miracloth were recovered. Both cell preparations were immediately frozen in liquid nitrogen and stored

at −80 °C until use. The degree of contamination by MC in the preparations was examined first under a light microscope (Sheen and Bogorad, 1985). The contamination was also estimated by measuring the activities of the marker enzymes PEPC and NADP-ME. Chlorophyll concentration and *a/b* ratio were determined according to Arnon (1949).

Crude nuclei were isolated from the frozen mesophyll protoplasts, bundle sheath strands, roots and whole leaf blades powdered in liquid nitrogen as previously described (Suzuki *et al.*, 1994). The frozen powder was immersed in NIB (1 M hexylene glycol, 10 mM Pipes-KOH pH 7.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.5% v/v Triton X-100) and blended with a Polytron homogenizer. For extraction of nuclei from root, 20 mM 2-mercaptoethanol and 5 mM sodium ascorbate were added to NIB. The condition for blending the powdered samples with a Polytron homogenizer was modified according to the samples. In the cases of MC and roots, the blending procedure was omitted while the samples of BSC and whole leaf blades were blended for 30 s at speed 8. The blending procedure for BSC samples was repeated once. The homogenates were filtered through a 130 μm nylon mesh and then through a 30 μm nylon mesh. Crude nuclear pellets were harvested by centrifugation (2000 × *g*, 10 min) and washed twice with NIB and once with NIB without Triton X-100. The nuclear pellets were suspended in sterile distilled water, and one fourth volume of 5× nuclear extract buffer (1× NEB is 15 mM Hepes-KOH pH 7.9, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% v/v glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml antipain) was added to the suspension. Nuclei were lysed by adding 5 M NaCl to a final concentration of 0.4 M, and the mixture was gently stirred at 4 °C for 30 min.

After centrifugation at 117 000 × *g* for 1 h at 4 °C, the supernatant was dialyzed against a dialysis buffer (15 mM Hepes-KOH pH 7.9, 1 mM EDTA, 100 mM NaCl, 15% v/v glycerol, 10 mM 2-mercaptoethanol, 0.5 mM PMSF). Insoluble material was removed by centrifugation at 12 000 × *g* for 10 min, and the extracts were divided into small aliquots, frozen in liquid nitrogen and stored at −80 °C.

Probe and competitor DNA preparation

DNA fragments I and II containing the 5′-upstream region of *C4Ppc1* from maize Golden Cross Bantam strain were described previously (Kano-Murakami *et al.*, 1991). These fragments were labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I, purified by Sephadex G-50 spun columns (ProbeQuant G-50 Micro Columns, Amersham Pharmacia Biotech) and used as probe DNAs in gel shift assays. A DNA fragment containing the PEP-I binding region was produced by subcloning of synthetic oligonucleotides. In short, oligonucleotides that spanned both strands of PEP-I binding region (5′-GACGCCCTCTCCACATCCTGC-3′) were chemically synthesized with a 5′-CCGG-3′ sequence attached to the 5′ end. The synthetic oligonucleotides were phosphorylated at the 5′ end, annealed together and cloned into the *Xma*I site of pBluescript II KS+ plasmid. DNA fragments containing the monomer of the oligonucleotides were excised with *Bam*HI and *Pst*I from the recombinants and used as competitor DNA and gel-shift probes.

Gel shift assays

The standard binding reaction was carried out in a volume of 12.5 μl containing 0.25 fmol of ^{32}P-labeled DNA fragment, 0.5 μg nuclear protein extract, 1 μg poly(dI-dC), 15 mM Hepes-KOH pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% v/v glycerol and 10 mM 2-mercaptoethanol at 25 °C for 20 min. Of the reactions 10 μl was loaded on 4.9% or 5% polyacrylamide gel in 50 mM Tris, 380 mM glycine, 2.1 mM EDTA and 2.7% v/v glycerol pH 8.5. After electrophoresis (at 8.8 V/cm), the gel was dried and radioactive signals were detected by a Bio Imaging Analyzer (BAS 2000, Fujix, Tokyo, Japan).

Results

Chimeric C4Ppc1-GUS *fusion genes and maize transformation*

To test the transcriptional activity of the maize *C4Ppc1* promoter in transgenic maize plants, two different lengths of the 5′-flanking regions of the maize gene (−571 to +81 or −1212 to +83 relative to the transcription initiation site) were fused to the coding region of the GUS gene linked to the terminator of the nopaline synthase gene (Figure 1). These two chimeric genes were designated as *PEPC(0.6)-GUS* and *PEPC(1.3)-GUS*, respectively. The two PEP-I binding sites are located between −470 and −375 while the MNF1 binding sites are located between −879 and −804 of the 5′ region of the maize *C4Ppc1*

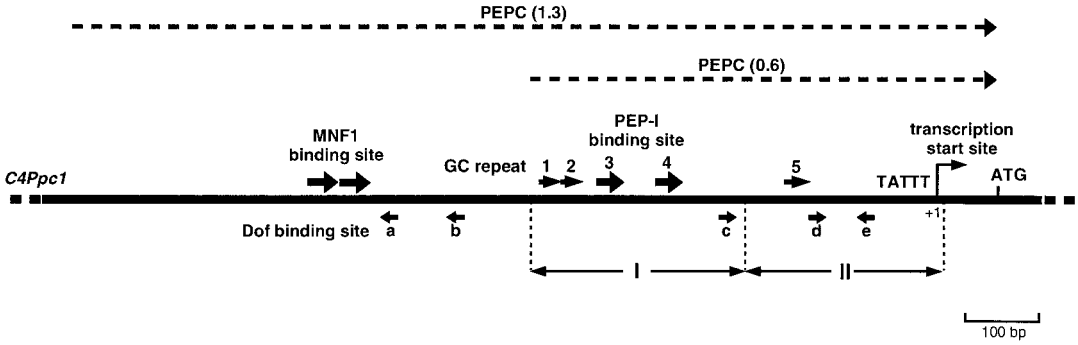

Figure 2. Structure of the 5′-upstream region of maize *C4Ppc1*. The heavy line represents the maize *C4Ppc1*. Large arrows indicate the binding sites for the nuclear proteins MNF1 and PEP-I which have been identified in maize (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991). The 5′-upstream region of *C4Ppc1* also contains GC repeats 1–5 (small arrows; conserved core sequence: 5′-CCCTCTCCACATCC-3′, and the PEP-I binding sites coincides with GC repeats 3 and 4. Small arrows a to e below the *C4Ppc1* line indicate the AAAG motifs which the Dof proteins can bind (Yanagisawa and Sheen, 1998). Dotted arrows above the *C4Ppc1* line indicate the 5′ regions used for the PEPC-GUS constructs. DNA fragments I and II used in gel shift assays are shown below the *C4Ppc1* line.

from Golden Cross Bantam strain (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991; Figure 2).

A total of 22 *PEPC(0.6)-GUS* transformants, with GUS activity ranging from 329 to 28 872 pmol 4-MU formed per minute per mg protein, were obtained and ten of them were selected for further analysis of tissue specificity of GUS expression (Table 1) and two of the transformants were selected for a cell specificity study (Table 2). In addition, two *PEPC(1.3)-GUS* transformants were obtained and used for tissue specificity analysis (Table 1). The chimeric genes, along with a *35S(0.8)-GUS* fusion gene, were introduced to maize inbred line A188 with an *Agrobacterium*-mediated transformation system, as described previously (Ishida *et al.*, 1996). All of the primary transformants were grown in a greenhouse. After self-pollination of the primary transformants, the resulting T_1 progeny were grown to maturity and used for experiments. All of the transgenic plants exhibited a normal phenotype.

Chimeric C4Ppc1-GUS *genes are expressed mainly in MC of leaf blades*

The distribution of GUS activity in different organs of the transformants were measured in ten PEPC(0.6)-GUS, two PEPC(1.3)-GUS and two 35S(0.8)-GUS plants (Table 1). For PEPC-GUS transgenic plants, among the tissues tested, leaf blade had the highest GUS activity with sheath exhibiting less than 3% of the activity in the leaf blade. Husk leaf, stem, tassel and root had no or very little GUS activity. Although the activity of GUS varied considerably among the transgenic plants, presumably due to differences in site of insertion, copy number and segregation of the inserted gene, the expression pattern of GUS among the organs remains the same. Therefore, it is concluded that transcription from the *C4Ppc1* 5′-upstream region, either 0.6 or 1.3 kb in length, occurs mainly in leaf blade. In contrast, the 35S promoter from CaMV was transcribed constitutively in all tissues examined, while the untransformed maize plants exhibited little GUS activity.

The cell type specificity of GUS expression in PEPC-GUS transgenic plants was determined by histochemical analysis of GUS activity using one of the plants with high-level expression (Figure 3). In leaf blade, heavy blue staining of GUS was observed only in MC but no or very low staining was observed in BSC, epidermal cells or vascular bundles (Figure 3a). Similar staining patterns were observed in the cross-sections of leaf sheath (Figure 3b). However, in leaf sheath, only MC adjacent to BSC exhibited heavy staining whereas MC located away from BSC showed weak staining. In great contrast, heavy staining was observed in both MC and BSC of the 35S-GUS plants (Figure 3c and d), indicating a constitutive expression pattern. The PEPC-GUS transgene was also expressed in stomatal guard cells, with staining heavily concentrated in the adjacent sides of the two guard cells (Figure 3a and e).

To apply quantitative analysis to intercellular distribution of GUS activity, we assayed the marker enzymes in separated MC and BSC from maize plants transformed with PEPC-GUS or 35S-GUS independently (Table 2). PEPC and NADP-ME, known to be specifically located in MC and BSC of maize leaves, respectively (Kanai and Edwards, 1973), were used

Table 1. Distribution of GUS activities in various organs of untransformed and PEPC-GUS and 35S-GUS transgenic maize plants.

Plant	GUS activity (pmol 4-MU formed per minute per mg protein)				
	leaf blade	leaf sheath	husk leaf	stem	root
PEPC(0.6)-GUS 3 (1)	9151	212	ND[a]	ND	31
PEPC(0.6)-GUS 3 (2)	11506	165	ND	ND	29
PEPC(0.6)-GUS 8-2 (1)	6945	ND	12	5	ND
PEPC(0.6)-GUS 8-2 (4)	17284	568	13	17	37
PEPC(0.6)-GUS 8-2 (7)	11144	219	ND	ND	30
PEPC(0.6)-GUS 8-2 (8)	10364	ND	8	17	ND
PEPC(0.6)-GUS 28-1-2	968	37	30	26	68
PEPC(0.6)-GUS 33-2 (4)	22794	179	9	4	29
PEPC(0.6)-GUS 33-2 (8)	28872	278	26	3	29
PEPC(0.6)-GUS 35-1	395	90	31	13	41
PEPC(1.3)-GUS 110 (3)	629	28	15	4	50
PEPC(1.3)-GUS 110 (5)	651	21	14	9	53
35S(0.8)-GUS (1)	211	81	80	343	179
35S(0.8)-GUS (3)	3595	4560	3330	23642	1167
Non-transformant 1	10	41	2	16	20
Non-transformant 2	12	26	2	11	29
Non-transformant 3	11	47	30	19	18

[a]ND, not determined.

to estimate the purity of the isolated cells. Since the PEPC activities in the BSC preparations were very low (less than 2% of those in the MC preparations) and the NADP-ME activities in the MC preparations were not detectable as compared with their counterpart cell preparations, it is clear that the degree of cross-contamination of both cell preparations was very low. The GUS activities in the BSC preparations from PEPC-GUS transgenic maize plants were less than 2% of those in the MC preparations. The results suggest that GUS is expressed only in MC and that the 652 bp 5′-upstream region of the maize *C4Ppc1* used in this study is sufficient to direct MC-specific expression of an introduced reporter gene. In contrast, the GUS activities in the BSC preparations from 35S-GUS transgenic maize plants were about 30–40% of those found in the MC preparations. This result shows that the constitutive 35S CaMV promoter functions in both photosynthetic cells in maize leaves, consistent with previous observations on the transcriptional activity of this promoter in transgenic maize plants (Gordon-Kamm *et al.*, 1990; Ishida *et al.*, 1996). Our results obtained from both histochemical staining (Figure 3) and enzyme assay with isolated cells (Table 2) are in accordance and clearly demonstrate that the maize *C4Ppc1* is transcribed in a MC-specific manner.

Preparation of MC and BSC nuclear extracts and estimation of purity

To investigate whether the cell type-specific expression of maize *C4Ppc1* is regulated by binding of cell type-specific *trans*-acting factors on the 652 bp 5′-upstream region, we examined the interaction between the nuclear proteins isolated from both cell types and the 5′-upstream region of *C4Ppc1* by gel shift assay. Mesophyll protoplasts were isolated enzymatically and bundle sheath strands were isolated mechanically from etiolated, greening and green leaves of non-transgenic plants. Etiolated seedlings have already showed distinct Kranz anatomy but lack the C_4 enzymes (Langdale *et al.*, 1988). Differential cell wall toughness of MC and BSC is the same in etiolated and greening leaves as in well-developed green leaves (Sheen and Bogorad, 1985). Although enzymatically separated mesophyll protoplasts and bundle sheath strands gave a high degree of purity for both cell types (Table 2), mechanical blending of whole

Table 2. Distribution of PEPC, GUS and NADP-ME activities between MC and BSC preparations from the PEPC-GUS and 35S-GUS transgenic maize plants.

Plant	Cell type	PEPC[a] (μmol/min per mg protein)	GUS[a] (pmol-4MU formed per minute per mg protein)	NADP-ME (μmol/min per mg protein)
PEPC(0.6)-GUS 8-2	MC	4.35 (100)	22403 (100)	ND[b]
	BSC	0.068 (1.6)	377 (1.7)	2.57
PEPC(0.6)-GUS 33-2	MC	3.98 (100)	13001 (100)	ND
	BSC	0.024 (0.60)	187 (1.4)	1.11
35S(0.8)-GUS 102	MC	10.66 (100)	20215 (100)	ND
	BSC	0.096 (0.90)	8011 (40)	2.11
35S(0.8)-GUS 131	MC	3.79 (100)	42660 (100)	ND
	BSC	0.013 (0.34)	13322 (31)	1.01

[a]The values in parenthesis are the relative values between MC and BSC for each enzyme activity.
[b]NADP-ME activities in MC were too low to permit accurate determination of purity.

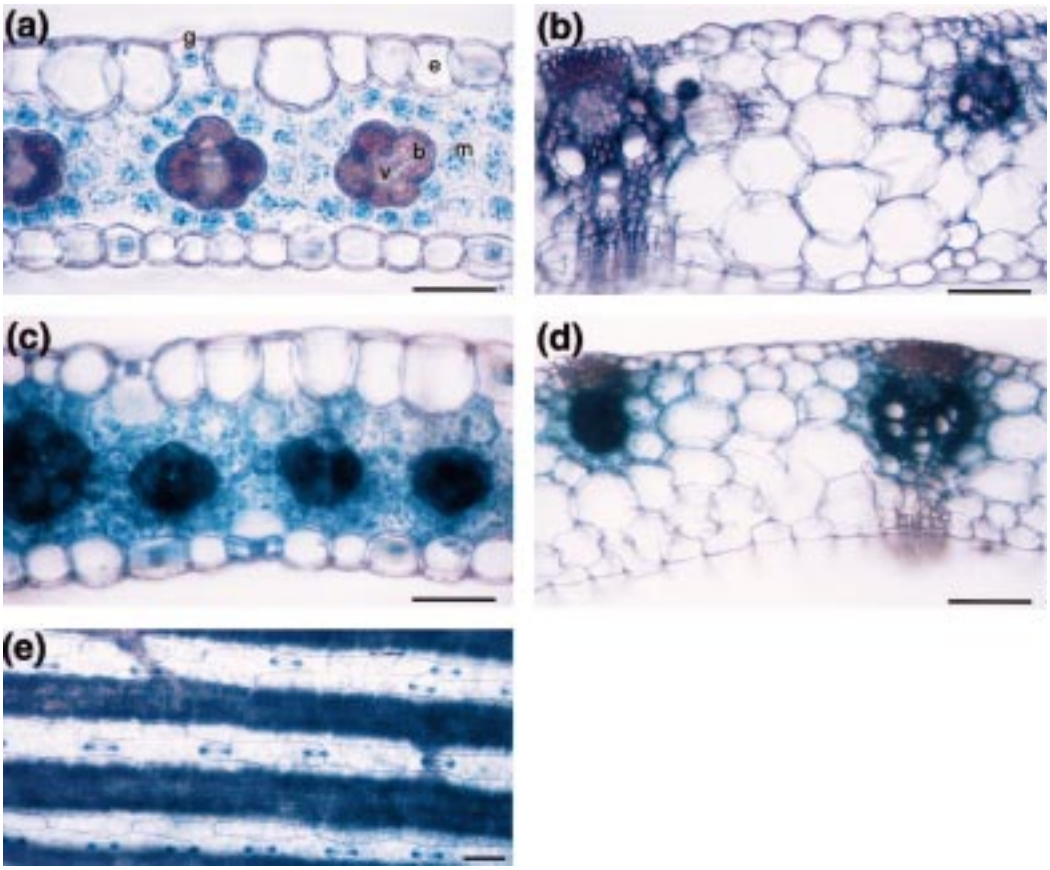

Figure 3. Histochemical localization of GUS activity in leaves of transgenic maize plants. (a) to (d) Cross sections of leaf blade (a, c) and leaf sheath (b, d) from PEPC(0.6)-GUS (a, b) or 35S-GUS (c, d) plants were stained for GUS activity. (e) Epidermis containing stomatal guard cells from a PEPC(0.6)-GUS plant was stained for GUS activity. b, bundle sheath cell; e, epidermal cell; g, guard cell; m, mesophyll cell; v, vascular bundle. Bars in (a) to (e) = 50 μm.

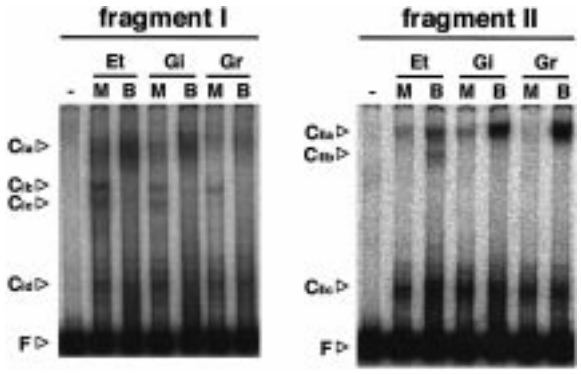

Figure 4. Changes of intercellular distribution of the nuclear factors binding to the *C4Ppc1* promoter regions during greening. Gel shift assays were performed with individual ^{32}P-labeled DNA fragments I and II from the 5′-upstream region of *C4Ppc1* denoted in Figure 2 in the absence (−) or the presence of nuclear extracts (each 1 μg of protein) prepared from MC (M) and BSC (B) of etiolated (Et), 17 h greening (Gi) and green (Gr) leaves. Electrophoresis was carried out in a 4.9% gel. F, free probe; C_{Ia}, C_{Ib}, C_{Ic}, C_{Id}, C_{IIa}, C_{IIb} and C_{IIc}, bound DNA-protein complexes.

leaves was necessary for isolating large quantities of bundle sheath strands and sufficient nuclei.

The degrees of cross-contamination of the MC and BSC preparations isolated from green leaves were determined to be less than 2% and 19%, respectively, as estimated by the activities of PEPC and NADP-ME, the respective marker enzymes for MC and BSC. The preparations of mesophyll protoplasts and bundle sheath strands from green leaves had chlorophyll *a/b* ratios of 3.4 and 5.0, respectively, and the preparations of these two cell types from greening leaves had chlorophyll *a/b* ratios of 3.7 and 4.6, respectively. The chlorophyll *a/b* ratios between the two cell-type preparations are close to the expected values for the enzymatically purified cell types (3.2 vs 6.4, respectively) (Kanai and Edwards, 1973). Light microscopic examination showed that the preparations of bundle sheath strands had few MC attached (data not shown). Nuclear extracts were prepared from the MC and BSC preparations. The protein profiles of the SDS-polyacrylamide gel, as revealed by staining with Coomassie brilliant blue, were similar among the six preparations of nuclear extracts (data not shown). Therefore, we conclude that the purities of the nuclear preparations are nearly equal.

MC- and BSC-specific nuclear factors differentially bind to the C4Ppc1 promoter

To reveal the interactions between leaf cell-specific nuclear binding factors and the 5′-upstream region of *C4Ppc1*, gel shift assays were performed using two DNA fragments of the maize Golden Cross Bantam strain *C4Ppc1* (I, −570 to −273; II, −272 to +7) which encompass the 5′-upstream region used for the transgenic analysis (Figure 2). As shown in Figure 4, more than two DNA-protein complexes were detected with each DNA fragment. The retarded complexes (C_{Ia}, C_{Ib}, C_{Ic}, C_{Id}, C_{IIa}, C_{IIb} and C_{IIc}) were competed out by excess amounts of the same unlabeled DNA fragments (50- or 100-fold molar excess relative to the labeled probes) and not by DNA fragments with unrelated sequences (Figure 6 and data not shown). Therefore, it is concluded that these complexes were formed specifically with each probe. Bands of the retarded complex C_{Ia} was relatively broad. The amount of the complex C_{Ia} was higher in the nuclear extract from BSC than from MC and decreased progressively with the extract prepared from etiolated to greening and green leaves. The retarded complexes, C_{Ib} and C_{Ic}, were exclusively associated with the MC extracts but not with the BSC extracts, and the intensities of these two complexes gradually decreased during greening. The formation of complex C_{IIa} was mainly associated with the BSC extracts from etiolated and greening leaves and its intensities increased during greening. In green leaves, complex C_{IIa} was exclusively associated with the BSC extracts. Complex C_{IIb} was weak and only observed in the BSC extract from etiolated leaves. In contrast, complexes C_{Id} and C_{IIc} were observed with all the extracts (from different cell types and different greening stages), showing no cell type specificity and light dependence. It is likely that they are formed with constitutive factors expressed in both MC and BSC. Thus, complexes C_{Ia} and C_{IIa} are specifically formed with BSC nuclear proteins and their formation with MC nuclear proteins is inhibited by light during greening of leaves. On the other hand, complexes C_{Ib} and C_{Ic} are specifically formed with MC nuclear proteins and their formation is attenuated by light during greening of leaves.

The MC- or BSC-specific nuclear factors do not exist in roots

Earlier studies with maize *C4Ppc1* promoter showed that the DNA-protein complexes formed with the nuclear proteins, MNF1 and PEP-I, are detected only in the extracts from leaves but not from roots (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991). In order to examine the tissue distribution of the MC- or BSC-specific nuclear factors, gel shift assays were

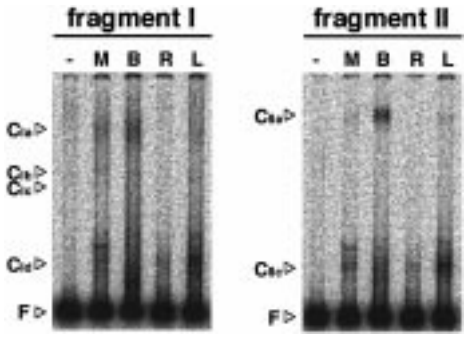

Figure 5. Distribution of the nuclear factors binding to the *C4Ppc1* promoter regions among different tissues. Gel shift assays with individual ^{32}P-labeled DNA fragments I and II from the 5′-upstream region of *C4Ppc1* were performed in the absence (−) or the presence of nuclear extracts (each 0.5 μg of protein) prepared from MC (M) and BSC (B) of 17 h greening seedlings, roots (R) and whole green leaf blades (L).

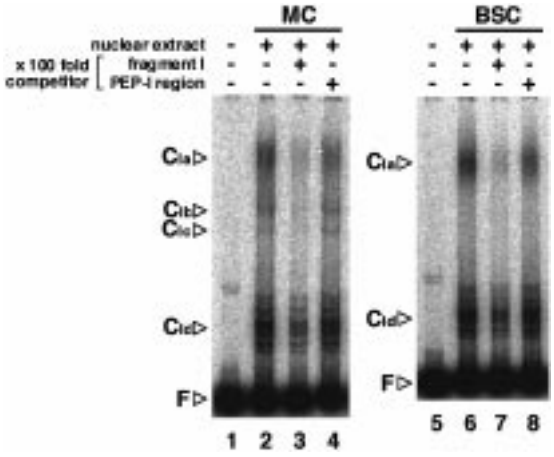

Figure 6. Gel shift competition assay for the nuclear proteins binding to the DNA fragment I from *C4Ppc1*. The gel shift assay was performed using ^{32}P-labeled fragment I containing the PEP-I binding site in the absence (lanes 1 and 5) or the presence of nuclear extracts from MC (lanes 2 to 4) or from BSC (lanes 6 to 8) of greening leaves. The reaction mixture was incubated in the absence (lanes 1, 2, 5 and 6) or the presence of cold DNA fragment I (lanes 3 and 7) or a single PEP-I binding region (lanes 4 and 8) as a competitor (100-fold molar excess).

performed under similar conditions with nuclear extracts prepared from roots (Figure 5, lane R). The slowly migrating complexes (C_{Ia}, C_{Ib}, C_{Ic} and C_{IIa}) were not detected in the extracts from roots. These slowly migrating complexes could not be detected even when a large amount of nuclear extracts (3.5 μg) from roots was used (data not shown). In contrast, there was no significant difference in formation of the fast-migrating complexes (C_{Id} and C_{IIc}) among the extracts from MC, BSC and roots. These findings suggest that the nuclear factors showing differential distribution between MC and BSC are localized specifically in leaf tissues but not in roots, consistent with the tissue specificity of expression of the maize *C4Ppc1* gene.

We also conducted a gel shift assay with nuclear proteins extracted from whole leaves. Whole green leaf blades were frozen in liquid nitrogen immediately after harvesting and a nuclear extract was prepared by weak blending with a Polytron homogenizer in the same manner as previously reported (Yanagisawa and Izui, 1990; Kano-Murakami *et al.*, 1991). The nuclear extract prepared from whole leaf blades showed similar gel-shift patterns with the nuclear extract from MC but not from BSC (Figure 5, lane L). This result clearly shows that, as suspected, the binding assays with the *C4Ppc1* promoter in the previous studies were performed with nuclear extracts predominantly released from MC. Another conclusion that can be drawn from this experiment is that the MC-specific retarded complexes that were detected with the nuclear proteins extracted from mesophyll protoplasts are not false bands due to the cell digestion procedure.

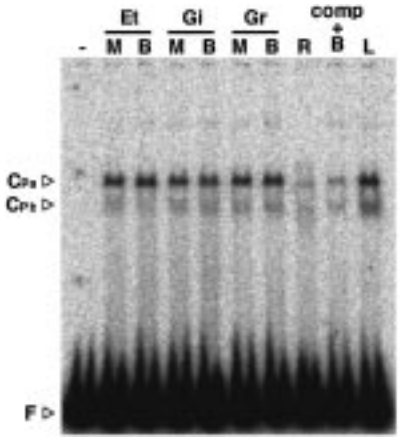

Figure 7. Intercellular distribution of the nuclear factors binding to the PEP-I binding site. Gel shift assays were performed with ^{32}P-labeled DNA fragment corresponding to the single PEP-I binding region in the absence (−) or the presence of nuclear extracts (each 0.5 μg of protein) prepared from MC (M) and BSC (B) of etiolated (Et), 17 h greening (Gi) and green (Gr) leaves, roots (R) and whole green leaf blades (L). In the lane 'comp + B', unlabeled DNA corresponding to the probe was added to the reaction mixture with BSC nuclear extracts from the greening leaves. Electrophoresis was carried out in a 5% polyacrylamide gel. F, free probe; C_{Pa} and C_{Pb}, bound DNA-protein complexes.

The cell-specific nuclear factors identified do not interact with the known PEP-I binding sites

Since previous reports showed that a monomer of the oligonucleotide corresponding to the PEP-1 binding region of *C4Ppc1* can interact with maize leaf nuclear proteins (Kano-Murakami *et al.*, 1991; Matsuoka *et al.*, 1994), we examined whether the MC- or BSC-specific nuclear factors identified in this study would interact with the PEP-I binding sites. A gel shift assay was performed with a monomeric PEP-I binding region as a competitor. As shown in Figure 6, the BSC-preferred complex C_{Ia} and MC-specific complexes C_{Ib} and C_{Ic} were not competed out by the oligonucleotide corresponding to the PEP-I binding site. Next, we performed gel shift assays using the oligonucleotides containing the PEP-I binding site as a probe (Figure 7). At least two shifted complexes (C_{Pa}, C_{Pb}), which interacted with leaf nuclear extracts (lane L), were detected with the probe, whereas the signal intensities of these complexes with the root nuclear extract were low (lane R). The shifted complexes were markedly reduced when excess of unlabeled DNA of the PEP-I binding site was added to the binding reaction (lane comp + B). This result, consistent with previous reports (Kano-Murakami *et al.*, 1991; Matsuoka *et al.*, 1994), suggests that some leaf-specific proteins interact with the DNA probe which contains the PEP-I binding site. The amount of the complex C_{Pa} or C_{Pb} was not significantly changed between nuclear extracts from MC and BSC or during greening. From the results of Figures 6 and 7, we conclude that the MC- or BSC-specific nuclear factors identified in the experiments of Figure 4 are distinct proteins from the nuclear factor PEP-I and do not interact with the PEP-I binding site.

Discussion

The 5′-flanking region of maize C4Ppc1 *is sufficient to drive MC-specific expression*

Previous studies with maize showed that transcripts for the C_4-type PEPC gene are accumulated only in photosynthetic tissues (Hudspeth *et al.*, 1986; Kawamura *et al.*, 1990; Schäffner and Sheen, 1992), and the accumulation of PEPC mRNA in leaves induced by light is MC-specific (Sheen and Bogorad, 1987). Homologous transient expression assays also indicated that transcription from the *C4Ppc1* promoter occurs only in mesophyll protoplasts but not in stem and root protoplasts (Schäffner and Sheen, 1992). These observations are in agreement with our data on promoter analysis using homologous transgenic maize plants: the activity of the reporter GUS was highest in leaf blade in the transgenic maize plants harboring two different chimeric *C4Ppc1* 5′ region/β-glucuronidase genes (Table 1). Histochemical analysis and a GUS activity assay of enzymatically separated MC and BSC revealed that the GUS activity is almost exclusively localized in MC (Figure 3 and Table 2). Furthermore, the expression pattern of the GUS gene directed by the maize *C4Ppc1* 5′ region coincides with that of the endogenous C_4-type PEPC gene. Our results demonstrate that the 652 bp 5′-upstream region (-571 to $+81$) is sufficient to direct MC-specific expression and that the 5′-upstream region contains *cis*-acting element(s) necessary for conferring MC-specific expression. An earlier study by Langdale *et al.* (1991) reported that differential methylation in a far upstream (-3.5 kb) site of maize *C4Ppc1* is correlated with the cell type-specific expression. However, our experiments suggest that the methylation site in the further upstream sequences is not required for MC-specific expression.

One of the interesting questions relative to evolution of C_4-specific genes is whether a similar molecular regulatory mechanism is being employed by C_4 plants for cell- and organ-specific expression. In a similar study with transgenic maize plants, we also showed that the 900 bp maize *C4Pdk* (encoding mesophyll chloroplast-located pyruvate, orthophosphate dikinase) promoter sequences contain the necessary *cis*-acting elements for directing its MC-specific and leaf-preferred expression of the GUS reporter gene (Taniguchi *et al.*, 2000). Therefore, the 5′-flanking sequences for both maize *C4Ppc1* and *C4Pdk* are sufficient to direct cell type- and organ-specific expression. However, for the maize *rbcS*-m3 gene, which is specifically expressed in BSC chloroplasts, not only the 5′-untranscribed region but also the 3′-transcribed region of the gene are necessary for photoregulated suppression of its expression in MC (Viret *et al.*, 1994). In the case of *Flaveria bidentis Me1*, which encodes the C_4 form of NADP-ME located in BSC chloroplasts, the 3′ region of the gene acts as an enhancer (Marshall *et al.*, 1997). Thus, different molecular regulatory mechanisms may be employed by C_4 plants to express the different photosynthetic genes between the two cell types. Recently, Stockhaus *et al.* (1997) reported that 2 kb of the 5′-flanking region of the *Flaveria trinervia C4 PpcA1* gene is sufficient

to direct MC-specific expression of the GUS reporter gene in transgenic *F. bidentis* plants. Further work is needed to clarify whether similar *cis*-acting elements are responsible for the C_4-specific expression function in both monocotyledonous and dicotyledonous plants.

Previous *in situ* hybridization and immunolocalization studies of PEPC mRNA and protein by Langdale *et al.* (1987, 1988) demonstrated that C4PEPC gene is expressed only in MC adjacent to BSC but not in MC located far from BSC. They hypothesized that a light-induced signal is transported from provascular tissues, passing into surrounding BSC and MC where it induces the C_4 pattern of enzyme expression. It is proposed that the signal does not reach more distant MC and C_3-like expression pattern occurs in the distant MC. In our sections of leaf sheath from PEPC-GUS transgenic maize, GUS staining was observed not only in MC adjacent to the vacular tissues but also in MC not in direct contact with BSC although the staining was weaker (Figure 3b). These MC have a relatively larger cell volume, but contain fewer organelles (e.g. chloroplasts) than MC directly adjacent to BSC. Therefore, it is likely that the expression of *C4Ppc1* is coupled to the extent of development of photosynthetic cells rather than to the distance from BSC. Stockhaus *et al.* (1997) also pointed out that in the C_4 dicot plant *Flaveria trinervia*, MC located more than two cell layers apart from BSC contain very few chloroplasts. In the transgenic *F. trinervia*, these mesophyll cells show much lower activity of GUS, driven by its own *Ppc* promoter, then do MC surrounding BSC.

Another interesting observation made in this study is the expression of PEPC-GUS transgene in stomatal guard cells (Figure 3). In contrast, the neighboring subsidiary cells and epidermal cells did not express the transgene. The activity of GUS and, thus, the transcriptional activity of the *C4Ppc1* in the guard cells was not determined in this study. Further work is needed to determine whether C_4PEPC protein has a physiological function in the guard cells and whether the regulatory mechanism of its expression in guard cells is similar to that in MC. In guard cells, PEPC may play an important role in synthesis of the organic anion malate in the cytosol, which is subsequently stored in the vacuole to balance the positively charged inorganic ions (e.g. K^+) during stomatal opening (Taiz and Zeiger, 1998). Langdale *et al.* (1987) also detected not only PEPC but also pyruvate, orthophosphate dikinase, NADP-malate dehydrogenase, NADP-ME and Rubisco proteins in guard cells of maize leaf blade and sheath by using immunolocalization assays.

Cell-specific nuclear factor binding sites and the known MNF and PEP-I binding sites

The 5′-upstream region of maize *C4Ppc1* is known to contain GC-rich sequences which are repeated five times between positions −550 and –200 (GC repeats 1 to 5 Figure 2) (Matsuoka and Minami, 1989). Two laboratories have studied the maize nuclear proteins that bind to the repeat sequences, but discrepancies between the findings are noticeable. Kano-Murakami *et al.* (1991) have shown that maize nuclear protein PEP-I binds specifically to GC repeats 3 and 4 by gel shift assays and this activity was detected only in the nuclear extracts of green leaves but not in the extracts of roots or etiolated leaves. Moreover, by a gel shift competition assay PEP-I also was found to bind to the GC-repeat 5 in DNA fragment II (Figure 2). It is speculated that PEP-I is a positive factor for *C4Ppc1* expression. In contrast, Yanagisawa and Izui (1990) detected the binding of nuclear protein MNF2a to GC repeats 3 and 4 and MNF2b to GC repeat 5 (Yanagisawa and Izui, 1990). They suggested that both of these nuclear factors are distinct proteins judged from the differences in chromatographic behavior. Furthermore, the binding activity of the factor MNF2a was higher in etiolated leaves than in green leaves. Thus, MNF2a is considered a negative regulator with respect to light-dependent expression of *C4Ppc1* in leaves. One cause of the inconsistency among the observations might be the differences in the methods of extraction of nuclear factors and in the conditions for the gel shift assays.

In our gel shift experiment with DNA fragment 1 containing GC repeats 3 and 4, at least four retarded complexes were revealed (Figure 4). The signal intensity of complex C_{Ia} was higher in the extracts from BSC than from MC; however, complexes C_{Ib} and C_{Ic} were detected exclusively in the MC extracts. All of these three complexes were detected in etiolated leaves at the highest level and the signal intensities decreased during greening. The signal intensities of these complexes were not reduced when oligonucleotide containing the PEP-I binding region was used as a competitor (Figure 6). In contrast, complex C_{IIa} was detected preferentially in BSC extracts, and its signal intensity increased during greening (Figure 4). Also, complex C_{IIa} was not diminished by competitor DNA containing the PEP-I binding site (data not shown).

Our gel shift analysis using the PEP-I binding region as a probe showed that there is no difference in binding activity to the PEP-I binding site between MC and BSC (Figure 7). These results indicate that there are no cell type-specific nuclear factors interacting with the PEP-I binding site. These retarded complexes were not competed out by excess amount of DNA fragment corresponding to the MNF1 binding site and nuclear proteins did not interact with the MNF1 binding site in a cell type-specific manner (data not shown). Therefore, the abundance or DNA-binding affinity of the nuclear factors for complexes C_{Ia}, C_{Ib}, C_{Ic} and C_{IIa} (designated PEP_{Ia}, PEP_{Ib}, PEP_{Ic}, and PEP_{IIa}), but not for nuclear factors PEP-I and MNF, appears to be regulated by light in different directions between the two photosynthetic cell types. Taken together, the results suggest that these novel cell type-specific nuclear factors bind to the maize *C4Ppc1* 5′-flanking region and regulate its differential transcription in MC and BSC in a light-dependent manner. Thus, maize *C4Ppc1* may have multiple regulatory sequences which are required for cell type-specific expression. Similarly, the 5′-upstream region of the maize *cab-m1* gene, which is preferentially expressed in MC, also contains multiple sequences functioning in active stimulation of transcription in MC or in suppression of transcription in BSC (Bansal *et al.*, 1992; Bansal and Bogorad, 1993).

Recently, the properties of maize nuclear zinc finger proteins, Dof1 and Dof2, have been reported (Yanagisawa and Sheen, 1998). Both proteins have been shown to bind to the AAAG motifs in the 5′-upstream region of *C4Ppc1* and the binding motifs are different from the PEP-I and MNF1 binding sites (Figure 2). Earlier, the direct repeats including the motifs have been demonstrated to have high enhancer activity in MC protoplasts (Schäffner and Sheen, 1992). In maize, Dof1 is constitutively expressed in leaves, stems and roots, whereas Dof2 is expressed mainly in stems and roots but not in leaves. Based on homologous transient expression analysis with MC protoplasts, it is proposed that Dof1 is a transcriptional activator while Dof2 is a transcriptional repressor, for tissue-specific and light-regulated expression of *C4Ppc1* respectively (Yanagisawa and Sheen, 1998). In accordance with their hypothesis, Dof1 should be active in MC of green leaf and Dof2 should be active in BSC. Whether these Dof proteins are capable of binding to the *C4Ppc1* promoter in a cell type-specific manner and functioning in cell type-specific gene expression requires further investigation. In a preliminary gel shift experiment, we could not detect any retarded complexes between the putative Dof binding sites on *C4Ppc1* promoter and the crude nuclear extracts from maize leaves.

Effect of light on cell-specific DNA-protein interaction

Light plays two important roles for photosynthetic gene expression in C_4 plants. First, light stimulates overall levels of photosynthetic gene transcripts and proteins. Second, light has a role in positional regulation that represses incorrect gene expression. For example, Rubisco is expressed in both MC and BSC of etiolated maize leaves, whereas the transcript level of *rbcL* and *rbcS* decreases only in MC upon illumination (Sheen and Bogorad, 1985, 1986). Unlike Rubisco, *C4Ppc1* is not expressed in MC or BSC in the dark; light triggers induction of *C4Ppc1* expression in MC. However, the light-induced expression of *C4Ppc1* is controlled by a light-mediated developmental change of MC rather than by an immediate activation of transcription (Schäffner and Sheen, 1992). *In situ* hybridization analysis for photosynthetic gene transcripts in maize shows that the expression of *C4Ppc1* is very low in the leaf primordium when Kranz anatomy is not yet evident (Langdale *et al.*, 1988). The mesophyll protoplasts and bundle sheath strands used in our experiments were prepared from well differentiated leaves, although they were under different stages of greening. It is quite possible that MC- or BSC-specific nuclear factors which function in cell type-specific expression have already existed in etiolated leaves and, therefore, we could detect cell type-preferred interaction in etiolated leaves by gel shift assay (Figure 4). Thus, light may effect the positional regulation of maize *C4Ppc1* expression by altering the abundance or binding affinity of cell type-specific nuclear factors.

The DNA binding activity as revealed by gel shift assay does not always reflect the change in abundance or binding affinity of the nuclear factor *in vivo*. It is also likely that *in vitro* interaction of protein with naked DNA fragment does not accurately reflect *in vivo* interaction of the protein with chromatin. Several plant DNA-binding proteins which bind to the *cis* elements of light-regulated gene promoters have been characterized, but most of these proteins show constitutive DNA-protein interactions in both light- and dark-grown tissues (Terzaghi and Cashmore, 1995). However, the wheat nuclear factor, WF-1, has been shown to bind to the upstream sequences of fructose-1,6-bisphosphatase and

sedoheptulose-1,7-bisphosphatase genes in light- and development-regulated manners (Miles *et al.*, 1993). Also, interactions of several animal nuclear proteins with cell-specific regulatory sequences have been confirmed by gel-shift analysis (Mori *et al.*, 1995; Kamachi and Kondoh, 1993). Nuclear factors, such as muscle-specific factor MyoD (Edmondson and Olson, 1993) and lens-specific factor Maf (Ogino and Yasuda, 1998), which function in cell type-specific expression mechanisms have been cloned and characterized. In contrast, information on cell-specific nuclear factors from plant cells is limited. Neighboring photosynthetic MC and BSC in leaves of C_4 plants express different genes in a cooperative manner whereas both cell types are functionally differentiated. The cell type-specific expression of C_4 photosynthetic genes will, therefore, serve as a good model system to dissect molecular mechanism of plant cell differentiation. In this report, we have revealed the interactions between both positive and negative cell type-specific nuclear factors and the maize *C4Ppc1* 5′-upstream region, which are necessary for its specific transcription in MC. Further refined study is needed to identify the binding sites and binding proteins which are involved in the DNA-protein interactions in order to illustrate how these factors are specifically involved in the cell type-specific expression mechanism of *C4Ppc1*.

Acknowledgements

This work was supported by grants from the Ministry of Educations, Science, and Culture of Japan (Scientific Research Grants 10740368 and 12740434, and Scientific Research on Priority Areas Grants 09274101 and 09274102).

References

Agostino, A., Furbank, R.T. and Hatch, M.D. 1989. Maximising photosynthetic activity and cell integrity in isolated bundle sheath cell strands from C_4 species. Aust. J. Plant Physiol. 16: 279–290.

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.

Arnon, D.I. and Hoagland, D.R. 1940. Crop production in artificial solutions and soils with special reference to factors influencing yield and absorption of inorganic nutrients. Soil Sci. 50: 463–471.

Bansal, K.C. and Bogorad, L. 1993. Cell type-preferred expression of maize cab-m1: repression in bundle sheath cells and enhancement in mesophyll cells. Proc. Natl. Acad. Sci. USA 90: 4057–4061.

Bansal, K.C., Viret, J.-F., Haley, J., Khan, B.M., Schantz, R. and Bogorad L. 1992. Transient expression from cab-m1 and rbcS-m3 promoter sequences is different in mesophyll and bundle sheath cells in maize leaves. Proc. Natl. Acad. Sci. USA 89: 3654–3658.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Chollet, R., Vidal, J. and O'Leary, M.H. 1996. Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 273–298.

Edmondson, D.G. and Olson E.N. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. 268: 755–758.

Edwards, G.E., Lilley, R.McC., Craig, S. and Hatch, M.D. 1979. Isolation of intact and functional chloroplasts from mesophyll and bundle sheath protoplasts of the C_4 plant *Panicum miliaceum*. Plant Physiol. 63: 821–827.

Furbank, R.T. and Taylor, W.C. 1995. Regulation of photosynthesis in C_3 and C_4 plants: a molecular approach. Plant Cell 7: 797–807.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R. Jr., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P. and Lemaux, P.G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2: 603–618.

Hatch, M.D. 1988. C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim. Biophys. Acta 895: 81–106.

Hatch, M.D. and Oliver, I.R. 1978. Activation and inactivation of phospho*enol*pyruvate carboxylase in leaf extracts from C_4 species. Aust. J. Plant Physiol. 5: 571–580.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271–282.

Hudspeth, R.L. and Grula J.W. 1989. Structure and expression of the maize gene encoding the phospho*enol*pyruvate carboxylase isozyme involved in C_4 photosynthesis. Plant Mol. Biol. 12: 579–589.

Hudspeth, R.L., Glackin, C.A., Bonner, J. and Grula J.W. 1986. Genomic and cDNA clones for maize phospho*enol*pyruvate carboxylase and pyruvate, orthophosphate dikinase: expression of different gene-family members in leaves and roots. Proc. Natl. Acad. Sci. USA 83: 2884–2888.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14: 745–750.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387–405.

Kamachi, Y. and Kondoh H. 1993. Overlapping positive and negative regulatory elements determine lens-specific activity of the delta 1-crystallin enhancer. Mol. Cell. Biol. 13: 5206–5215.

Kanai, R. and Edwards, G.E. 1973. Separation of mesophyll protoplasts and bundle sheath cells from maize leaves for photosynthetic studies. Plant Physiol. 51: 1133–1137.

Kanai, R. and Edwards, G.E. 1999. The biochemistry of C_4 photosynthesis. In: Sage, R.F. and Monson, R.K. (Eds.) C_4 Plant Biology, Academic Press, San Diego, CA, pp. 49–87.

Kano-Murakami, Y., Suzuki, I., Sugiyama, T. and Matsuoka, M. 1991. Sequence-specific interaction of a maize factor with a GC-

rich repeat in the phospho*enol*pyruvate carboxylase gene. Mol. Gen. Genet. 225: 203–208.

Kawamura, T., Shigesada, K., Yanagisawa, S. and Izui K. 1990. Phospho*enol*pyruvate carboxylase prevalent in maize roots: isolation of a cDNA clone and its use for analyses of the gene and gene expression. J. Biochem. 107: 165–168.

Komari, T., Hiei, Y., Saito, Y., Murai, N. and Kumashiro, T. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. 10: 165–174.

Ku, M.S.B., Kano-Murakami, Y. and Matsuoka, M. 1996. Evolution and expression of C_4 photosynthesis genes. Plant Physiol. 111: 949–957.

Ku, M.S.B., Agarie, S., Nomura, M., Fukayama, H., Tsuchida, H., Ono, K., Hirose, S., Toki, S., Miyao, M. and Matsuoka, M. 1999. High-level expression of maize phospho*enol*pyruvate carboxylase in transgenic rice plants. Nature Biotechnol. 17: 76–80.

Langdale, J.A., Metzler, M.C. and Nelson, T. 1987. The *argentia* mutation delays normal development of photosynthetic cell-types in *Zea mays*. Dev. Biol. 122: 243–255.

Langdale, J.A., Rothermel, B.A. and Nelson, T. 1988. Cellular pattern of photosynthetic gene expression in developing maize leaves. Genes Dev. 2: 106–115.

Langdale, J.A., Zelitch, I., Miller, E. and Nelson, T. 1988. Cell position and light influence C_4 versus C_3 patterns of photosynthetic gene expression in maize. EMBO J. 7: 3643–3651.

Langdale, J.A., Taylor, W.C. and Nelson T. 1991. Cell-specific accumulation of maize phospho*enol*pyruvate carboxylase is correlated with demethylation at a specific site >3 kb upstream of the gene. Mol. Gen. Genet. 225: 49–55.

Leegood, R.C. 1997. The regulation of C_4 photosynthesis. Adv. Bot. Res. 26: 251–316.

Marshall, J.S., Stubbs, J.D., Chitty, J.A., Surin, B. and Taylor, W.C. 1997. Expression of the C_4 *Me1* gene from *Flaveria bidentis* requires an interaction between 5′ and 3′ sequences. Plant Cell 9: 1515–1525.

Matsuoka, M. 1990. Structure, genetic mapping, and expression of the gene for pyruvate, orthophosphate dikinase from maize. J. Biol. Chem. 265: 16772–16777.

Matsuoka, M. and Minami, E. 1989. Complete structure of the gene for phospho*enol*pyruvate carboxylase from maize. Eur. J. Biochem. 181: 593–598.

Matsuoka, M. and Sanada, Y. 1991. Expression of photosynthetic genes from the C_4 plant, maize, in tobacco. Mol. Gen. Genet. 225: 411–419.

Matsuoka, M., Kyozuka, J., Shimamoto, K. and Kano-Murakami, Y. 1994. The promoters of two carboxylases in a C_4 plant (maize) direct cell-specific, light-regulated expression in a C_3 plant (rice). Plant J. 6: 311–319.

Miles, A.J., Potts, S.C., Willingham, N.M., Raines, C.A. and Lloyd, J.C. 1993. A light- and developmentally-regulated DNA-binding interaction is common to the upstream sequences of the wheat Calvin cycle bisphosphatase genes. Plant Mol. Biol. 22: 507–516.

Mori, Y., Folco, E. and Koren, G. 1995. GH3 cell-specific expression of Kv1.5 gene. J. Biol. Chem. 270: 27788–27796.

Ogino, H. and Yasuda, K. 1998. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science 280: 115–118.

Ohnishi, J. and Kanai, R. 1983. Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C_4 plants. I. Glycine oxidation by mitochondria. Plant Cell Physiol. 24: 1411–1420.

Schäffner, A.R. and Sheen, J. 1991. Maize *rbcS* promoter activity depends on sequence elements not found in dicot *rbcS* promoters. Plant Cell 3: 997–1012.

Schäffner, A.R. and Sheen, J. 1992. Maize C_4 photosynthesis involves differential regulation of phospho*enol*pyruvate carboxylase genes. Plant J. 2: 221–232.

Sheen, J.-Y. and Bogorad, L. 1985. Differential expression of the ribulose bisphosphate carboxylase large subunit gene in bundle sheath and mesophyll cells of developing maize leaves is influenced by light. Plant Physiol. 79: 1072–1076.

Sheen, J.-Y. and Bogorad, L. 1986. Expression of the ribulose-1,5-bisphosphate carboxylase large subunit gene and three small subunit genes in two cell types of maize leaves. EMBO J. 5: 3417–3422.

Sheen, J.-Y. and Bogorad, L. 1987. Differential expression of C_4 pathway genes in mesophyll and bundle sheath cells of greening maize leaves. J. Biol. Chem. 262: 11726–11730.

Stockhaus, J., Schlue, U., Koczor, M., Chitty, J.A., Taylor, W.C. and Westhoff, P. 1997. The promoter of the gene encoding the C_4 form of phospho*enol*pyruvate carboxylase directs mesophyll-specific expression in transgenic C_4 *Flaveria* spp. Plant Cell 9: 479–489.

Suzuki, I., Crétin, C., Omata, T. and Sugiyama, T. 1994. Transcriptional and posttranscriptional regulation of nitrogen-responding expression of phospho*enol*pyruvate carboxylase gene in maize. Plant Physiol. 105: 1223–1229.

Taiz, L. and Zeiger, E. 1998. Plant Physiology, Sinauer Associates, Massachusetts.

Taniguchi M., Izawa, K., Ku, M.S.B., Lin, J.-H., Saito, H., Ishida, Y., Ohta, S., Komari, T., Matsuoka, M. and Sugiyama, T. 2000. The promoter for the maize C_4 pyruvate,orthophosphate dikinase gene directs cell- and tissue-specific transcription in transgenic maize plants. Plant Cell Physiol. 41: 42–48.

Terzaghi, W.B. and Cashmore, A.R. 1995. Light-regulated transcription. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46: 445–474.

Viret, J.-F., Mabrouk, Y. and Bogorad, L. 1994. Transcriptional photoregulation of cell-type-preferred expression of maize *rbcS*-m3: 3′ and 5′ sequences are involved. Proc. Natl. Acad. Sci. USA 91: 8577-8581.

Yanagisawa, S. 1995. A novel DNA-binding domain that may form a single zinc finger motif. Nucl. Acids Res. 23: 3403–3410.

Yanagisawa, S. and Izui, K. 1990. Multiple interactions between tissue-specific nuclear proteins and the promoter of the phospho*enol*pyruvate carboxylase gene for C_4 photosynthesis in *Zea mays*. Mol. Gen. Genet. 224: 325–332.

Yanagisawa, S. and Izui, K. 1992. MNF1, a leaf tissue-specific DNA-binding protein of maize, interacts with the cauliflower mosaic virus 35S promoter as well as the C_4 photosynthetic phospho*enol*pyruvate carboxylase gene promoter. Plant Mol. Biol. 19: 545–553.

Yanagisawa, S. and Izui, K. 1993. Molecular cloning of two DNA-binding proteins of maize that are structurally different but interact with the same sequence motif. J. Biol. Chem. 268: 16028–16036.

Yanagisawa, S. and Sheen, J. 1998. Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. Plant Cell 10: 75–89.