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Transient expression from *cab-m1* and *rbcS-m3* promoter sequences is different in mesophyll and bundle sheath cells in maize leaves

(gene regulation/light induction/*in situ* transient expression assay/mesophyll-specific sequence/35S cauliflower mosaic virus promoter)

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ABSTRACT **Cell-specific and light-regulated expression of the β-glucuronidase (GUS) reporter gene from maize *cab-m1* and *rbcS-m3* promoter sequences was studied in maize leaf segments by using an *in situ* transient expression microprojectile bombardment assay. The *cab-m1* gene is known to be strongly photoregulated and to be expressed almost exclusively in mesophyll cells (MC) but not in bundle sheath cells (BSC). Expression of GUS from a 1026-base-pair 5′ promoter fragment of *cab-m1* is very low in dark-grown leaves; GUS expression is increased about 10-fold upon illumination of dark-grown leaves. In illuminated leaves, the ratio of GUS expression in MC vs. BSC is about 10:1. The *cab-m1* region between 868 and 1026 base pairs 5′ to the translation start confers strong MC-preferred expression on the remainder of the chimeric gene in illuminated leaves, but a region between −39 and −359 from the translation start is required for photoregulated expression. Transcripts of *rbcS-m3* are found in BSC but not in MC and are about double in BSC of greening dark-grown seedlings. In contrast to the behavior of the *cab-m1*–GUS construct, GUS expression driven by 2.1 kilobase pairs of the *rbcS-m3* 5′ region was about twice as high in MC as in BSC of unilluminated dark-grown maize leaves. The number of BSC, but not MC, expressing GUS nearly doubled upon greening of bombarded etiolated leaves. These data suggest that the 5′ region of *rbcS-m3* used here could be responsible for most of the light-dependent increase in *rbcS-m3* transcripts observed in BSC of greening leaves and that transcriptional or posttranscriptional mechanisms are responsible for the lack of *rbcS-m3* transcripts in MC.**

Two types of photosynthetic cells—mesophyll cells (MC) and bundle sheath cells (BSC)—are involved in carbon dioxide fixation via the C4 pathway in maize leaves (1). BSC are in a single layer around each vascular bundle; MC form a concentric sheath around the cylinder of BSC. The two cell types are differentiated from sister cells (2). MC have 3–4 times more of the light-harvesting chlorophyll a/b binding protein associated with photosystem II (LHCPII) than do BSC (3). LHCPIIs are encoded by nuclear genes of the *cab* family, and *cab* transcripts are more abundant in MC than in BSC (4); this is the result of differences in preferential expression of individual *cab* genes in the two cell types (4). The gene *cab-m1* is preferentially expressed in MC and is strongly positively photoregulated; its transcript is the most abundant of all the maize *cab* genes and accounts for 30% of total LHCPII mRNA in leaves greened for 24 hr (4).

Ribulose bisphosphate carboxylase/oxygenase, in contrast to LHCPII, is abundant in maize BSC but is absent from MC (5, 6). The small subunit of ribulose bisphosphate carboxylase/oxygenase is encoded by nuclear genes of the *rbcS* family. Transcripts of *rbcS* and of the chloroplast gene *rbcL* encoding the large subunit of the enzyme are found solely in BSC (6–10). Transcripts of *rbcS-m3* gene are barely detectable in MC of illuminated or unilluminated dark-grown maize leaves but are found in BSC of unilluminated leaves, where they double in amount during greening (5, 7). Transcripts of *rbcS-m3* constitute about 35% of the total leaf *rbcS* mRNA in 24-hr illuminated dark-grown maize (5).

Mechanisms for regulating the cell specificity of *cab-m1* and *rbcS-m3* genes are not known; how illumination affects their expression is poorly understood. Until now it has not been possible to analyze *in vivo* the promoter regions of maize genes that are expressed differently in MC and BSC. Although the recent generation of transgenic maize (11) carrying β-glucuronidase (GUS) reporter gene constructs makes this possible in principle, maize transformation and regeneration procedures are lengthy and thus are not convenient for the analysis of numerous modified forms of genes.

We have found that DNA precipitated on tungsten microprojectiles can be delivered into MC and BSC *in situ* in maize leaf segments and that MC-specific and light-induced GUS expression from the *cab-m1* promoter[§] requires two widely separated sequences 5′ to the translation start site. In contrast, GUS expression from the *rbcS-m3* promoter is about the same in MC and BSC of 24-hr greened leaves, but expression of the *rbcS* chimeric gene is promoted by light in BSC.

MATERIALS AND METHODS

Plant Material. Seeds of maize (*Zea mays*; $FR9^{cms}$ × FR37; Illinois Foundation Seeds, Champaign, IL) were sown in damp Vermiculite and grown at 30°C in darkness. Ten-day-old seedlings were harvested under a dim-green safelight, and their second leaves were used in the transient *in situ* expression assay described below.

Transient *in Situ* Expression Assay. Four 3.5-cm-long segments of the upper halves of the second leaves of 10-day-old dark-grown maize seedlings, a total area of about 12 cm^2, were flattened on 0.8% agar Murashige and Skoog medium (GIBCO) in a 5-cm Petri plate with the lower epidermis facing upward. DNA was precipitated on tungsten particles (0.5 mg of 1.1-μm size particles coated with 1 μg of supercoiled plasmid DNA for each shot). The leaf segments were bombarded by using the Biolistics Particle Delivery System-1000

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Abbreviations: MC, mesophyll cell(s); BSC, bundle sheath cell(s); GUS, β-glucuronidase; CaMV, cauliflower mosaic virus; LHCPII, light-harvesting chlorophyll a/b binding protein associated with photosystem II.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M87020).

(DuPont), as described by Klein *et al.* (12). The leaf segments were bombarded under ambient light and were then allowed to green for 24 hr under white fluorescent lamps (≈300 microeinsteins·m^{-2}·s^{-1}). After this, the segments were incubated with the GUS enzyme substrate 5-bromo-4-chloro-3-indolyl glucuronide (Biosynthag, Skokie, IL) in the dark at 37°C (13, 14) for 48 hr, at which time the number of blue spots, indicating where the GUS enzyme was being expressed, had reached a plateau (data not shown). To study gene expression in unilluminated leaves of dark-grown seedlings, segments of etiolated leaves were harvested and bombarded with chimeric gene constructs under a dim-green safelight and were then maintained in darkness for 24 hr. Northern analysis has shown that the light flash produced by the firing of the gun powder charge in the Biolistics apparatus is insufficient to cause *cab* mRNA to accumulate in etiolated maize leaves after incubation in darkness (15).

Localization of GUS Activity *in Situ* in Various Leaf Cell Types. Leaf segments with blue spots were infiltrated with 0.1 M sodium phosphate-buffered 4% (wt/vol) paraformaldehyde at pH 6.8 (16), embedded in 4% (wt/vol) agarose, and sliced transversely with a Vibrocutter (World Precision Instruments, Sarasota, FL) into 50-μm-thick serial sections. The leaf cell type in which the indigo dye was found was recorded. The MC/BSC ratio approached a constant value after 40 or more spots had been mapped from a single experiment.

RESULTS

GUS Expression from the Cauliflower Mosaic Virus (CaMV) 35S Promoter in Different Leaf Cell Types. To develop a convenient method for analyzing expression of *cab* and *rbcS* promoters in the transient expression assay *in situ*, we first sought to determine if conditions could be found for delivering DNA to equal numbers of MC and BSC of maize leaf segments. As reporters for gauging the distribution of microprojectiles among various leaf cell types, we used the 35S promoter of CaMV (17) linked to the *uidA* sequence that encodes GUS in both pBI221 (14) and pDPG208. pDPG208 is expressed in both MC and BSC of transgenic maize leaves (11).

We found a large number of blue spots in leaves of dark-grown maize seedlings bombarded with pBI221 and then illuminated for 24 hr (see Fig. 3*A*). Also, the mean number of spots per shot was not much different at the two levels of shooting tested: 9 cm (level 3) and 12 cm (level 4) from stopping plate to target (Table 1). Under both shooting conditions, expression was much more frequent in MC and BSC than in guard cells or epidermal cells (Table 1), and no GUS expression was detected in vascular bundles. However, the ratio of MC to BSC expressing GUS was 1.1 at shooting level 3, but at level 4 the MC/BSC ratio was 2.6 (Table 1). The plasmid pDPG208 performed essentially the same as pBI221 when shot at level 3 (Table 1); its expression in all types of cells in transgenic maize leaves indicates that this 35S promoter–GUS construct is most probably neutral with respect to MC vs. BSC expression (11). The parallel transient expression of pDPG208 and pBI221 *in situ* shows that the latter is also a suitable control for MC vs. BSC transient expression in maize leaves. These results indicated (*i*) that the 35S promoter in pBI221 can drive GUS expression in almost all maize leaf cell types including MC and BSC [as expected from the behavior of this promoter in transgenic maize (11)] and (*ii*) that MC and BSC are transformed with equal frequency at the shorter bombardment distance tested—i.e., at level 3. Consequently, we chose to shoot leaf segments at level 3 in most of our subsequent experiments; deviations from the MC/BSC ratio of about 1:1 could be interpreted without further corrections as resulting from cell-specific regulatory expression. The terms "cell-specific" and "cell-preferred" expression are used interchangeably here. Although the former term is generally used to describe the phenomena we are examining, the latter is a more accurate description of the behavior of *cab-m1* and *rbcS-m3* genes (4, 5) in MC and BSC and of the chimeric genes derived from them.

Table 1. Summary of histochemical localization of GUS expression in different cell types of greening and etiolated maize leaves bombarded with pBI221, pDPG208, pM1CAB1.1, or pM3TSSU2.1 plasmids

Construct	Shooting level	Spots per shot (*n*)*	Distribution of cells expressing GUS†				MC/BSC ratio
			MC	BSC	GC	EC	
		Light					
pBI221	3	133 ± 15 (8)	54	50	9	1	1.1
	4	107 ± 13 (10)	69	27	6	5	2.6
pDPG208	3	113 ± 4 (4)	62	47	11	3	1.3
pM1CAB1.1	3	40 ± 6 (8)	82	8	7	1	10.2
	4	48 ± 7 (10)	83	6	11	2	13.8
pM3TSSU2.1	3	51 ± 10 (6)	53	48	1	0	1.1
	4	54 ± 10 (4)	61	39	0	0	1.6
		Dark					
pBI221	3	75 ± 5 (8)	24	45	1	0	0.5
pM1CAB1.1	3	4 ± 1 (6)	ND	ND	ND	ND	ND
pM3TSSU2.1	3	36 ± 4 (8)	54	22	1	0	2.5

ND, not determined; GC, guard cells; EC, epidermal cells.
*The values given are the means ± SEM. The numbers in parentheses represent the number of independent assays.
†Leaf segments used for sectioning to map GUS-expressing cells were picked randomly from several independent experiments. For example, 1064 spots were counted in eight experiments (column 3) when pBI221 was tested in illuminated leaves, and 114 of these spots were sectioned.

In some cases a blue spot in a leaf could be attributed to a single blue cell, but in most cases several adjacent cells were blue. However, among the almost 1300 blue spots we sectioned, not a single case was found of concomitant GUS expression in adjacent MC and BSC. In the latter cases, we do not know whether the bombarded DNA entered each cell separately or whether the DNA or the GUS enzyme diffused from cell to cell. But, irrespective of the number of blue cells it contained, an individual spot was treated as a single expression event for scoring purposes (Table 1). Transient GUS expression from various deletion constructs of the rice *cab-1R* promoter, determined as the number of blue spots per shot in tobacco leaves in the microprojectile bombardment assay, is directly correlated with the GUS activity of the same set of constructs in fluorogenic assays of transgenic tobacco leaves (15). These results show that the microprojectile bombardment assay can be used effectively to study MC- vs. BSC-specific expression of maize genes.

Isolation and Characterization of the *cab-m1* Gene and the Construction of pM1CAB1.1. A maize genomic library constructed in λDash II phage (Stratagene) from a partial *Sau*3A digest of maize DNA was screened for sequences homologous to the unique 3′ sequence of the maize cDNA clone Δ 8–11 derived from the *cab-m1* gene (4). The sequence of this probe (data not shown) differs from other known maize *cab* sequences (18–20). One hybridizing recombinant phage was isolated, and the appropriate DNA sequences were determined by the dideoxynucleotide method. The sequence of the 5′ upstream region is shown in Fig. 1. pM1CAB1.1 was constructed as a translational fusion by inserting 1026 bp of DNA upstream of the *cab* transit peptide sequence plus the initial six codons of the transit peptide sequence into the *Sal* I/*Nco* I site of the pRAJ275 plasmid (Clontech). The nopaline synthase terminator of *Agrobacterium tumefaciens* (25) was placed at the 3′ end of the GUS reporter gene (Fig. 2).

Expression of GUS in MC and BSC from the *cab-m1* Promoter in pM1CAB1.1. In greening leaves, using

```
   *(-1026)
 1 TTGATTTTAG AAAAATAACG AAATCAGTTT CATAATTTTC TAAGTTAAGA TGAATTTACA

                (AT-1)AAT ATTTTTATT
61 AAGATTAGTT TAGATTTAAT ATTTTTtcTG AAAAAATACC GATTTCGGAA ACGGGCAAAA
                                                    *(-867)
121 GAGATCCAAA CTATTTCTGT TTTTTTTTTA CCGATTTCAT TTCCGTATTT TCGGTAACGG
                                                                *(-789)
                                                 (AT-1)AATA TTTTTATT
181 TTTCCGGTTT CGTATGACCC TAAATTTTGG TAAAGTTTCG AAAAAAAATA TTTTaAgaAC

241 TGAAAATTAA CGTTCCTGTT TTCATCCATA CTAATGGCTC TTTACCGCTA AAATGTTGCC

301 CACAATCATT GAGTAGGTTT AGACGTGAGA GCAAACAGTA CAACATTACG ATTCGCCTTG
              *(-656)
361 CCAAATTTAC ATGCCTTTTC CCTACGGAAA CAACATAGAA TCAAGTTGAC TGGGTTACTT

421 ACATTGAAGT GGCCAAACTG ATGGTAGCTG TAGATTTGGA TGTATGTTTT CTATAAATTA
     *(-544)                                  *(-511)
481 GTCAAAATTG AGACAAAATA AACTGCAATT TAAAACTGAG GAAATAGTAA AAAAAGGTG

541 AAGAAGGGAG GAAGAGGAAA TCAGAAGCAA AAAATGGGCA ACTTTAGGCC CATTATCTCG

                                                (PPD-1 Binding Site)TCGTGC
601 ATGGTCTCGT CGGAGTCCAG ATATGTGATT GACGGATTGG ATTGGGCCGT ACATCtCTGCA

         *(-359)                                                (I BOX)TCTTA
661 TGAGAGTTCG CCAAGATTTC ATTGTTTAAC AAGAAGCGCG TGACAACAAA ACCAAgCcTA

    TCTCATCC                          (ABRE)CACGTG GC
721 TCTCATCCAC TCTTTTTTTC CGTTCCCACA ATGGCAaGTG GCAGCTCGTG ATTCGGTCTG

      (ABRE)CA CGTGGC
781 GCCATTCCtA CGTGGCAGAC ACCAGGATTC TTGTGTGATA GGCCACTGGG TCCCACCCAC

841 CAGGTGCCAC ATCAGACGCC AAAGCCATCC CGGCAGACCA ATCCCAGCCC AGCAACAGAT

901 GGTCTGCTAT CCAGTTCCAA CTGTATAAAA GCAGCTGCTG TGTTCTGTTA ATGCACAGCC
                                  *(-39)
961 ATCACACGGA CGCATACACA GCACAGAGTG AGGTAAGCAT CCGAAAAAAG CTGTGATCTG
      *(-1)
1021 ATCGACATGG CCGCCGCCAC CATGGCTCTC ACCTCCC
                          NcoI
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FIG. 1. The DNA sequence 5′ to the coding region of *cab-m1*. The CAAT box, TATA box, translation start site, and the *Nco* I site used for the *cab-m1*–GUS gene fusion in pM1CAB1.1 are overlined. The deletion endpoints of the constructs for *cab-m1* promoter analysis are indicated by asterisks. The 158-base-pair (bp)-long distal promoter region required for MC-preferred expression is underlined, and the proximal promoter region conferring light-regulated expression is indicated in boldface letters. Sequences resembling the consensus sequences of the AT-1 motif (21), PPD-1 binding site (22), I box (23), and ABA response element (ABRE) (24) found in various other genes are shown above the corresponding homologous sequences, and mismatches are revealed by lowercase letters in the maize *cab-m1* sequence.

pM1CAB1.1 DNA, the mean numbers of blue spots per shot were 40 ± 6 and 48 ± 7, at shooting levels 3 and 4 (Table 1), respectively. However, GUS expression was much lower in etiolated leaves: on average, only 4 ± 1 spots per shot were counted in six trials at level 3 (Table 1).

Plasmids containing 5′ to 3′ deletions of the *cab-m1* promoter region were created by linearizing pM1CAB1.1 by *Sph* I and *Sal* I, treating with exonuclease III and mung bean nuclease, and religating with T4 DNA ligase. Deletions with 5′ ends at −867, −789, −656, −544, −511, −359, and −39 bp from the translation initiation site of *cab-m1* (Fig. 1) were made. The endpoint of each deletion construct was determined by dideoxynucleotide sequencing. The total number of spots per shot, in greening and etiolated leaves, were about the same with the −789, −544, and −359 deletion plasmids as with pM1CAB1.1, but the −39 deletion was completely inactive (Table 2). Therefore, the region responsible for high-level and light-regulated *cab-m1* expression lies in the 320 bp between the −359 and −39 endpoints (Fig. 1).

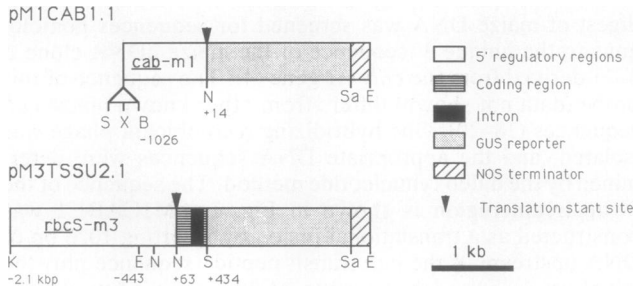

FIG. 2. Diagrams of the chimeric gene constructs used in bombardment of maize leaves. Distances are indicated from the translation start for pM1CAB1.1 and from the transcription start for pM3TSSU2.1. B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; N, *Nco* I; S, *Sal* I; Sa, *Sac* I; X, *Xba* I; NOS, nopaline synthase.

In the case of pM1CAB1.1, there were 10 times more spots in MC than in BSC of greening leaves shot at level 3 (Table 1). Examples of expression from this construct in MC are shown in Fig. 3 *B* and *C*. At level 4, the MC/BSC expression ratio increased slightly to 14 (Table 1). At both shooting levels, these results are in striking contrast to the MC/BSC expression ratios of about 1:1 obtained with bombardment with 35S–GUS gene constructs. Thus we conclude that the 1026 bp of the *cab-m1* promoter sequence drives the expression of the GUS gene in a strongly MC-preferred manner. However, GUS expression from the promoter construct containing only 867 bp upstream of the *cab-m1* translation start site was not MC specific; in this case the MC/BSC ratio was close to 1:1, rather than 10:1, at level 3 (Table 2). The MC/BSC ratios from the constructs with 5′ end deletions terminating between nucleotides −867 and −359 were also about 1:1. Therefore, we conclude that the promoter region of *cab-m1* that lies between nucleotides −1026 and −868 is required for strong MC-preferred expression of the *cab-m1*–GUS gene tested here.

Expression of GUS in MC and BSC from the *rbcS-m3* Promoter in pM3TSSU2.1. Transcripts of *rbcS-m3* are found exclusively in BSC (5). The 3′ noncoding region of a previously described *rbcS* genomic clone (26) hybridized strongly to the *rbcS-m3*-specific cDNA probe SS7 (5) and did not hybridize to two other *rbcS* gene-specific cDNA probes (data not shown). A 2.5-kilobase-pair (kbp) *Kpn* I/*Sal* I fragment of the *rbcS-m3* genomic clone, containing 2.1 kbp of the promoter region and 0.4 kbp of the transcribed region, was inserted in-frame with and upstream of the GUS reporter gene coding sequence (see Fig. 2) in a Bluescript II KS(−) vector (Stratagene). The GUS reporter gene, composed of the GUS coding region followed by the nopaline synthase terminator, was isolated from pBI101.2 (Clontech).

DNA of pM3TSSU2.1 (Fig. 2) was bombarded into leaves; at level 3 the mean number of spots obtained was 36 ± 4 in etiolated leaves and 51 ± 10 in greening leaves (Table 1), and GUS expression occurred in both BSC and MC (Fig. 3 *D*, *E*,

Table 2. Summary of histochemical localization of GUS expression in different cell types of greening maize leaves bombarded with different deletion constructs of the *cab-m1* promoter

Construct	Spots per shot*		Distribution of cells expressing GUS				MC/BSC ratio
	Light	Dark	MC	BSC	GC	EC	
−1026†	40 ± 6 (8)	4 ± 1 (6)	82	8	7	1	10.2
−867	33 ± 2 (6)	ND	32	27	0	0	1.2
−789	30 ± 3 (6)	1 ± 0.4 (6)	21	17	0	1	1.2
−656	45 ± 5 (8)	ND	27	36	2	1	0.8
−544	42 ± 7 (8)	2 ± 0.4 (6)	48	25	0	2	1.9
−511	33 ± 7 (8)	ND	32	40	3	1	0.8
−359	34 ± 5 (6)	2 ± 0.4 (6)	37	34	1	0	1.1
−39	0 (4)	ND	ND	ND	ND	ND	ND

ND, not determined; GC, guard cells; EC, epidermal cells.
*Leaves were bombarded at level 3. The values given are the means ± SEM. The numbers in parentheses represent the number of independent assays.
†Data given for construct −1026, which is pM1CAB1.1, are repeated from Table 1.

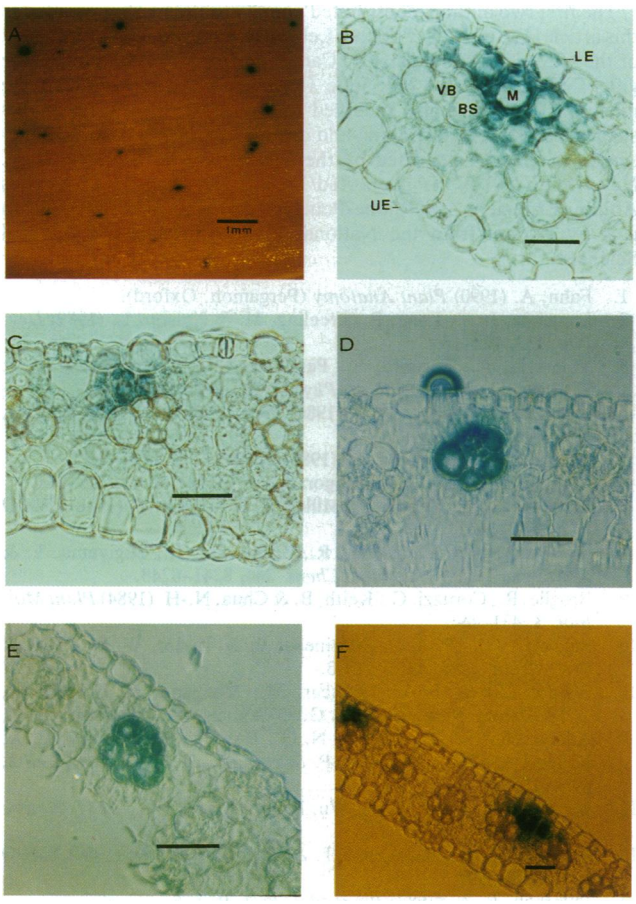

FIG. 3. *In situ* GUS activity in MC and BSC of maize leaves bombarded with pBI221, pM1CAB1.1, or pM3TSSU2.1. (*A*) A leaf segment bombarded with pBI221 shows blue spots resulting from GUS activity after incubation with the 5-bromo-4-chloro-3-indolyl glucuronide substrate for GUS. (*B–F*) Transverse sections of bombarded leaves showing sites of *in situ* GUS activity in MC and BSC. (*B* and *C*) pM1CAB1.1 expression in MC. (*D* and *E*) pM3TSSU2.1 expression in BSC. (*F*) pM3TSSU2.1 expression in MC. CaMV 35S promoter expression was very similar to the expression of pM1CAB1.1 in MC (*B*) and of pM3TSSU2.1 in BSC (*D* and *E*). pM1CAB1.1 expression in BSC was barely detectable. Leaf segments were bombarded through the lower epidermis. LE, lower epidermis; M, mesophyll cell; BS, bundle sheath cell; VB, vascular bundle; UE, upper epidermis. (*B–F*, bars = 50 μm.)

and *F*). The number of BSC expressing GUS was about twice as great in illuminated as in unilluminated leaves of dark-grown maize seedlings, whereas MC expression was relatively high but was unaffected by illumination (Table 1). As a result of the light-induced increase in expression in BSC, the MC/BSC expression ratio of about 2.5:1 in unilluminated leaves dropped to about 1:1 in greening leaves (Table 1). Thus, the principal effect of illumination on the expression of pM3TSSU2.1 is to promote expression in BSC by about 2.5-fold. We conclude that the 2.5-kbp *Kpn* I/*Sal* I region of *rbcS-m3* tested here is responsible for part or all of light-regulated accumulation of *rbcS-m3* mRNA in BSC.

Expression of GUS in MC and BSC from the CaMV 35S Promoter in pB1221. It was surprising that the expression of GUS from the 35S CaMV promoter in pB1221 in MC was substantially greater in illuminated than in unilluminated leaves of dark-grown maize seedlings, whereas expression in BSC was not affected by illumination (Table 1).

DISCUSSION

The particle bombardment method has been previously used to study light-regulated gene expression of a reporter gene (27) and to generate transgenic plants (11, 28). An adaptation of this method based on counting blue spots produced as a result of GUS expression from deletions of the rice *cab-1R* promoter has been worked out in leaves of tobacco, rice, and maize (15). We have found that this method can be further adapted for studying regulation of gene expression *in situ* in MC vs. BSC of maize leaves. When the distance between the stopping plate of the Biolistic apparatus and the target is 9 cm, the MC/BSC expression ratio of a GUS reporter gene driven by the CaMV 35S promoter in pBI221 is 1:1. We also found that pDPG208, which is known to express strongly in both leaf cell types in transgenic maize (11), behaved about the same as pBI221; the MC/BSC expression ratio was 1.3:1 when the leaves were shot at 9 cm. These experiments indicated that CaMV 35S promoter–GUS constructs can be used as controls to determine conditions for introducing DNA into equal numbers of MC and BSC of maize leaves.

When expression was under the control of 1026 bp of *cab-m1* 5′ flanking sequence, >80% of the GUS-expressing cells in greening leaves were MC, whereas only 6–8% were BSC (Table 1). This pattern of expression of the *cab-m1* promoter parallels the steady-state levels of *cab* mRNA in MC vs. BSC of greening maize leaves (4). Under the same bombardment conditions, the number of cells expressing GUS in etiolated leaves was 1/10th the number obtained with greening leaves. This is also in accord with the very low level of *cab-m1* mRNA in MC of dark-grown seedling leaves (4). We therefore conclude that both cell-specific and light-regulated expression of *cab-m1* are controlled primarily at transcription.

Deletion analyses of *cab-m1* 5′ flanking sequences showed that the 158-bp region between −1026 and −868 is required for MC-specific expression of this gene. However, this region does not appear to solely repress expression in BSC or to solely promote expression in MC. If it only repressed expression in BSC, expression in MC would be unaffected by deletion of the −868 to −1026 sequence, and expression in BSC would rise to the same level as in MC; this does not occur. If this region only had a role in promoting MC expression, its elimination would be expected to lead to a drop in MC expression to the very low BSC level; this does not occur. Removal of the −868 to −1026 segment results in decreased expression in MC and increased expression in BSC. The simplest conclusion to be drawn from these results is that some sequences included in the 158-bp segment between −868 and −1026 suppress expression in BSC and enhance expression in MC; a single sequence or two separate sequences could be involved.

The 5′ 1026-bp flanking sequence of *cab-m1* contains the sequence (TCTTGC) that resembles the PPD-1 binding site (TCGTGC), which is required for pyruvate orthophosphate dikinase (PPDK) gene expression in maize mesophyll cells (22). As shown in Fig. 1, the sequence similar to the PPD-1 binding site of the PPDK gene is not located in either the MC-specifying area or in the region controlling photoregulation of the *cab-m1* gene. We did not detect a sequence in *cab-m1* resembling a 14-bp directly repeated sequence found in the phospho*enol*pyruvate carboxylase (PEPC) promoter region (29) or a G+C-rich sequence (22, 30) found in the 5′ regions of both the PPDK and PEPC genes. An AT-1 motif present in the negative regulatory element of the *Nicotiana plumbaginifolia cab-E* gene (21, 31) and light-regulated promoters of other dicots (23) occurs in the MC-specifying 5′ region of the *cab-m1* gene. It remains to be determined whether this A+T-rich element between −949 and −937 is involved in negative regulation of *cab-m1* expression in BSC.

The region between −359 and −39, which is required for enhanced and light-regulated expression of *cab-m1*, contains DNA sequences homologous to the I box of the tomato *rbcS-3A* gene (23). Mutation in an I box of the *Arabidopsis*

thaliana rbcS-1A promoter causes reduced light-regulated expression of an *adh* reporter gene (32). Two sequences that are very similar to the ABA response element of wheat Em gene (24) are also found in the −359 to −39 region. ABA is reported to regulate *cab* and *rbcS* gene transcription in tomato (33). Although *cab-m1* is normally expressed at extremely low levels in BSC, once the distal MC-specifying region is deleted, it is strongly positively photoregulated in BSC. This indicates that trans-acting factors for photoregulating *cab-m1* are present in BSC as well as MC.

The number of BSC expressing GUS from pM3TSSU2.1 in leaves of unilluminated dark-grown seedlings was less than half that in greening leaves. This 2-fold increase in GUS expression in BSC upon greening is comparable in magnitude to the increase in *rbcS-m3* transcripts in BSC at 24 hr into greening (5). Therefore, we conclude that the 5′ 2.5 kbp of *rbcS-m3* in pM3TSSU2.1 is responsible for its photoregulated expression in BSC and that the increase in *rbcS-m3* transcripts in BSC upon illumination can probably be accounted for largely by a change in the rate of transcription. Transcripts of *rbcS* are absent from MC; however, expression of GUS from pM3TSSU2.1 is strong and appears not to be photoregulated in MC. The segment of *rbcS-m3* present in pM3TSSU2.1 could lack some normally present essential MC-specific repressor sequence(s). Control of transcription from sequences downstream of the translation start site has been suggested for petunia *rbcS* genes (34). Alternatively and/or additionally, *rbcS-m3* transcripts could be degraded rapidly in MC; RNA sequences recognized by such a nuclease could be absent from the chimeric *rbcS-m3*–GUS transcript produced from pM3TSSU2.1. It has been reported that expression of chloramphenicol acetyltransferase from the 5′ region extending from −229 to +64 of *rbcS-m3* is about 7-fold lower in mesophyll protoplasts from etiolated leaves maintained in the dark than in those incubated in light after electroporation (35). The discrepancy between these results and ours could be due to differences in stabilities of the chloramphenicol acetyltransferase and GUS mRNAs and proteins or to differences in the states of the cells in the electroporation and *in situ* transient expression assays.

Surprisingly, GUS expression from the 35S promoter in pBI221 was found to be photoregulated in MC (Table 1). It is not known what 35S promoter sequences may be involved. Overall GUS expression from the 35S promoter was lower in the dark than in the light. The frequency of GUS expression in MC of etiolated leaves is lower than in MC of greening leaves, but illumination has no comparable effect on expression in BSC. This expression pattern from the 35S promoter in the dark is thus opposite to differences in expression in MC and BSC from the *rbcS-m3* promoter in the dark. In a way, these results confirm that the photostimulation of GUS expression from the *rbcS-m3* promoter in BSC of greening maize leaves is specific and not a result of a general increase in transcription, for example.

It should be emphasized that in the experiments reported here similar segments of the second leaves of 10-day-old dark-grown maize seedlings were bombarded. Segments were maintained in darkness or in the light only after bombardment. That is, the internal structures of all the leaf segments were the same at the time of bombardment, and thus the distribution of the DNA-coated microprojectiles would be expected to be the same regardless of whether or not leaves were subsequently illuminated. The relative expression of −867 through −359 *cab-m1*–GUS constructs in MC and BSC vs. that of the −1026 construct validates the use of GUS expression from the 35S CaMV promoter in pBI221 and pDPG208 in illuminated leaves of dark-grown maize seedlings as empirical penetration references.

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