A Rice cab Gene Promoter Contains Separate cis-Acting Elements That Regulate Expression in Dicot and Monocot Plants

Sheng Luan¹ and Lawrence Bogorad²
Department of Cellular and Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

The major light-harvesting chlorophyll a/b binding proteins of the photosynthetic apparatus are encoded by families of nuclear cab genes. The expression of most cab genes is tissue specific and photoregulated in angiosperms. In transgenic tobacco plants, expression of the reporter gene β-glucuronidase (GUS) is photoregulated and tissue specific from 5' upstream sequences of the rice cab1R gene; deletion of sequences upstream from position -170 with respect to the transcription start site eliminates the enhanced and photoregulated expression in the transgenic plants. Using an in situ transient expression assay, we have determined that the sequence OCT-R, an octamer repeat that lies within the -269 to -170 region of cab1R, is essential for photoregulated expression of the chimeric GUS gene in leaf cells of maize and rice but is not required for expression in illuminated tobacco leaves. Conversely, box III*- and G-box-like sequences found near OCT-R in cab1R are necessary for high-level transient expression of the reporter gene in tobacco leaf tissue but are not required for transient expression in maize or rice leaves.

INTRODUCTION

A number of plant nuclear genes are activated by light. The best studied examples are genes of the families encoding chlorophyll a/b binding proteins (cab) and the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS) (for reviews, see Tobin and Silverthorne, 1985; Dean et al., 1989). Because light regulation has been determined to be mainly at the transcriptional level (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984), studies in the past few years have been directed at identifying cis- and trans-acting elements that mediate light responsiveness to delineate the pathway of light signal transduction in plants.

In addition to "TATA" and "CAAT" boxes, a few other conserved sequences have been found in the 5' upstream regions of light-responsive genes (Kuhlemeier et al., 1987; Gilmartin et al., 1990). The GT-1 binding sites, which are present in most rbcS genes, are among the best characterized (Green et al., 1987; Lam and Chua, 1990). These sequence elements include box I, box II, box III*, and box III. They bind to the same protein factor and are functionally redundant in the rbcS-3A promoter. The tetramer of box II can confer light responsiveness and tissue specificity on the expression of a truncated cauliflower mosaic virus (CaMV) 35S promoter (~90 version) in transgenic tobacco (Lam and Chua, 1990). The G-box is another conserved sequence in rbcS genes that is required for high activity of the Arabidopsis rbcS-1A promoter in transgenic tobacco (Giuliano et al., 1988; Donald and Cashmore, 1990). GATA motifs conserved in both rbcS and cab genes (Castresana et al., 1987; Gidoni et al., 1989) are also involved in high-level expression by illuminated plants (Donald and Cashmore, 1990). Other sequence elements, such as AT-1 binding sites (Datta and Cashmore, 1989) and 3AF1 binding sites (Lam et al., 1990), may also play important roles in light-regulated expression of some genes.

The functional studies of dicot genes described above were conducted in transgenic dicot plants. Several light-responsive genes have been isolated from monocot plants (Lamppa et al., 1985a; Luan and Bogorad, 1989; Matsuoka and Sanada, 1991). Although some of these genes can be regulated properly in transgenic tobacco (Lamppa et al., 1985b; Matsuoka and Sanada, 1991), others cannot (Keith and Chua, 1986). Some short sequences in the promoter regions of monocot genes have limited homology to light-responsive elements of dicot genes. However, none of the sequence elements has been characterized functionally in monocot plants because of the difficulty of producing transgenic monocots.

We have analyzed the roles of sequences in the 5' portion of the photoregulated rice cab1R gene (Luan and Bogorad, 1989) by studying the expression of a reporter gene in transgenic tobacco and in situ transient expression assays. In
the course of this work, we have identified a 100-bp segment of the rice cab1R gene that is required for light-regulated expression in both transgenic tobacco and bombarded leaves of tobacco, maize, and rice. An octamer repeat within this 100-bp region has been characterized and shown to be required for photoresponsiveness of the cab1R promoter in maize and rice leaves. However, elimination of the octamer repeat had no effect on expression in tobacco leaves. Other sequences, including previously recognized dicot control regions, affect expression in leaves of this dicot plant.

RESULTS

Deletion Analysis of the cab1R Promoter in Transgenic Tobacco

To investigate the functional elements of the cab1R promoter sequence, chimeric gene constructs containing different 5'to 3'deletions of the promoter region fused to the β-glucuronidase (GUS) reporter, as shown in Figure 1, were introduced into the genome of tobacco SR1 plants by leaf disc transformation via *Agrobacterium tumefaciens* (Horsch et al., 1985). As a positive control, the *CaMV 35s*-GUS construct pB1121 (Jefferson et al., 1987) was introduced into SR1 plants.

As shown in Figure 2A, the average GUS activity in leaves of parenta1 transgenic plants with the 35s-GUS construct is about three times greater than in the plants with the cab1R "full promoter" (-750/+114-GUS), designated cab1R-GUS, construct shown in Figure 1B. A deletion mutant containing only 269 bp of 5' upstream sequence also exhibited near maximal GUS activity, but no GUS activity was observed from the -170 construct. Comparable results were obtained when GUS activities of F1 seedlings containing the various constructs were tested histochemically (Figure 2B). F1 seeds from transgenic plants with different constructs were surface sterilized and grown in the light for 10 days on MS (Murashige and Skoog, 1962) solidified medium. The tiny seedlings were dropped into a solution of the X-gluc substrate for GUS and kept in the dark for 16 hr. As shown in Figure 2B, the cotyledons of the light-grown seedlings with cab1R-, -584-, -346-, and -269-GUS as well as 35S-GUS turned blue showing GUS activity, but the -170-GUS-containing transformant did not.

To determine whether GUS activity in transgenic tobacco conferred by cab1R is light regulated and, if so, which part (or parts) of the promoter is required for the light responsiveness, 10-day-old etiolated seedlings with cab1R-GUS and
Figure 2. Expression of the GUS Gene in Tobacco Plants Transformed with 35S-GUS and cab1R Deletion Constructs.

(A) GUS activity of leaves from parental transgenic plants was measured by a fluorometric assay (Jefferson, 1987). GUS activity was assayed in leaves of eight to 12 plants (data from each is shown as a dot) with each construct. GUS activity is expressed as production of nanomoles of methylumbelliferone per milligram of protein per minute.

(B) Histochemical assay of 10-day-old, light-grown F₁ seedlings shown after incubation for 24 hr with GUS substrate. From left to right are seedlings with -750, -584, -346, -269, -170, and 35S-GUS constructs.

(C) From left to right are a 10-day-old, dark-grown 35S-GUS seedling and two cab1R-GUS seedlings, one illuminated for 24 hr and the next maintained in darkness before incubation with GUS substrate.
etiolated tobacco plants have very tiny cotyledons, light/dark transient expression experiments were not performed.

The parallels between the results of GUS expression in transgenic tobacco leaves and the spot-counting assay opened the way for using the latter to study in situ transient expression from cab1R gene promoter constructs in maize and rice leaves. As can be seen from the data in Figure 4A, GUS gene expression from various cab promoter constructs in tobacco leaves can be quantified using the spot-counting assay by performing a number of replicates, but we tested to see whether an internal control could further aid quantitation. A convenient, unique, visible marker established for maize tissues (Ludwig et al., 1990) proved to be an excellent internal control. The Rlc gene encodes a protein factor that induces cell anthocyanin autonomous pigmentation for most maize tissues (Ludwig et al., 1989). After delivering DNAs of cab1R-GUS and 35S-Rlc (Figure 1B) into living maize leaf tissue, the expression level of the Rlc protein can be assessed by counting the number of pigmented cells (Ludwig et al., 1990), and this result can be compared with the number of blue spots that develop after the leaf segments are incubated with X-gluc.

Although we were interested in ultimately testing cab1R promoter constructs in rice leaves, we chose maize leaves as the major target material for other reasons as well: (1) maize seedlings grow well in darkness providing large amounts of leaf material as target for bombardment in general and for studying the effects of illumination on expression in particular; and (2) maize is closely related to rice but is a C4 plant that contains two types of photosynthetic leaf cells, mesophyll and bundle sheath, thus providing the possibility of studying the behavior of a C3 plant gene (cab1R) in cells of a C4 plant.

To determine whether the cab1R promoter is light responsive in the transient assay in maize leaf tissues, we introduced cab1R-GUS or 35S-GUS into etiolated maize leaves together with 35S-Rlc by bombardment (see Methods). In Figure 4B, the relative GUS activity is given as the number of blue spots per 100 red pigmented cells. The cab1R-GUS gene was expressed about fourfold more actively in illuminated than in unilluminated leaves of dark-grown maize seedlings, whereas GUS activity was expressed from 35S-GUS equally well in either condition.

Because a flash is generated by the gun powder in the upper chamber of the PDS 1000 but some light can leak into the sample chamber through a small hole, a mock shot (no microprojectiles and no DNA) was made to monitor the effect of the flash on the expression of endogenous cab genes in etiolated maize leaves. The cab mRNA level was not detectably different in the bombarded and unbombarded tissues after 6 hr of incubation in the dark (data not shown). The single flash was apparently not sufficient to trigger a detectable increase in expression of cab genes in the leaf tissues. Accordingly, far-red light illumination (4 μmol m−2 sec−1 for 5 min) did not significantly change the level of GUS activity in the samples incubated in darkness after bombardment (data not shown).

To delineate the region of the promoter that contains elements involved in the photoregulation of cab1R, we delivered GUS constructs with different 5′ deletions of the cab1R promoter into etiolated maize and rice leaves. The deletion construct with only 269 bp upstream of the transcription start site of the cab1R gene can still express the GUS reporter gene in a light-responsive manner (Figure 4C). The −170 construct expressed GUS activity at about the same low level as the longer 5′ sequences did in unilluminated tissues but, unlike the longer promoter sequences, illumination did not result in any significant increase in expression. GUS was expressed from constructs with CAAT and TATA sequences (−80) or with only the TATA box (−40) but at a much lower level than from the −170 construct in both maize (Figure 4C) and rice (Figure 4D).

**Dissection of the cis-Acting Elements in the Promoter Region (−269 to −170)**

The deletion analyses described above showed that sequences required for light-responsive expression of cab1R in maize, rice, and tobacco leaves are located between positions −170 and −269. To assess the ability of DNA, including this sequence, to confer photoresponsive expression on a heterologous promoter, the cab1R sequence from positions −94 to −389 was ligated in both orientations to a GUS reporter gene (Jefferson et al., 1987) under the control of a truncated CaMV 35S RNA promoter carrying a 5′ deletion terminating 89 bp upstream from the transcription start site. This promoter, designated −90/35S, retains TATA and CAAT boxes but not the upstream enhancer sequences (Fang et al., 1989). The −90 version of the 35S promoter has been used frequently as a heterologous...
Figure 4. Transient Expression of cab1R-GUS in Maize, Rice, and Tobacco Leaves.

(A) Deletion analysis of the cab1R promoter in bombarded tobacco leaves.

(B) Light-regulated expression of cab1R-GUS in maize leaves.

(C) Light responsiveness of the deletion constructs in maize leaves.

(D) Light-regulated expression of the deletion constructs in rice leaves.

The bombardment procedure is described in Methods and in the text.

basic promoter for analyzing enhancer activity in plants (Chen et al., 1988; Thomas and Flavell, 1990). When delivered into etiolated maize leaf tissue by the particle gun, both gene fusions, designated 295A and 295B, as shown in Figure 5A, expressed GUS activity in a light-dependent manner, as shown in Figure 5B. The expression of the GUS gene was threefold higher in illuminated than in unilluminated leaves of dark-grown maize seedlings. On the other hand, the control construct, -90/35S-GUS, expressed GUS activity at a low basal level that was about the same in light and darkness.

In comparing the cab1R promoter region with sequences of the light-responsive elements identified earlier (Gilmartin et al., 1990), we found sequences, as shown in Figure 6A, that have significant homology to the box III*, G-box, and 3AF1-binding site, sequences previously discovered and analyzed in dicot genes (Green et al., 1987; Giuliano et al., 1988; Lam et al., 1990). In addition, we noticed a sequence containing an octamer repeat (OCT-R) (Figures 1A and 6A). Using polymerase chain reaction (see Methods), we generated specific mutations in each of these sequences (Figure 6A). The wild-type (295A) and mutant constructs were tested for their capacity to confer photoresponsive expression on the -90/35S-GUS gene in etiolated maize leaves.

As shown in Figure 6B, mutations in the sequences resembling box III*, G-box, and 3AF1 binding sites (Figure 6A) did not affect the activity or light responsiveness of the GUS
Figure 5. Analyses of the Light-Responsive Enhancer Element in Maize Leaves.

(A) Diagrams of constructs -90/35S-GUS and 295A/295B. (B) The expression of these constructs in illuminated or unilluminated leaf tissue of dark-grown maize seedlings.

Constructs in maize leaves, but deletion of the 15 bp containing most of the repeated octamer sequences (Figure 6A, D34) eliminated the capacity of the gene to exhibit light-dependent GUS expression. Similar results were obtained in rice leaves (data not shown).

To determine whether both repeats are required for the enhancer activity, two mutant plasmids were constructed, OCTm-1 and OCTm-2 (Figure 6A). In each of the mutant constructs, one of the octamer repeats was deleted from 295A. As shown in Figure 6B, deletion of either repeat reduced GUS expression to the dark level and eliminated the response to light. Deletion of one or both of the octamer repeats changed the spacing of sequences in the -170 to -269 region. The distance between two interacting cis-acting elements has been shown to be important for regulation of some genes (Gilmartin and Chua, 1990). To examine the possibility that the changes in spacing rather than elimination of the octamer sequences in the cab1R gene affects photoregulation, base replacements were generated in the octamer repeats. Two base changes (CC to GG) were made in each octamer sequence (Figure 6A, CG-1). These alterations had about the same effect as deleting the repeated sequences, as shown in Figure 6B (CG-1).

To study the function of the octamer repeat in the context of the original cab1R promoter instead of with the -90/35S sequence, we deleted the 15 bp from the "full" promoter of cab1R (-750/cab1R). A BglII-HincII fragment containing 750

Figure 6. Mutagenesis of Several cis-Acting Elements.

(A) Various sequences and mutations discussed in the text. Section I shows a comparison of cab1R sequences with consensus sequences (Gilmartin et al., 1990). Mutations were made to the underlined bases. Section II shows the sequence containing the octamer repeat and various mutants of it, D34, OCTm-1, OCTm-2, and CG-1. Deletions are represented as spaces; base changes are underlined. Section III shows alignment of part of the pea rbcS-3A promoter region (-261 to -240; Gilmartin et al., 1990) with a segment of cab1R promoter in the -170 to -269 region (-254 to -234 in Figure 1). (B) The effect of mutations on GUS expression in maize leaves.
bp upstream of the transcription start site and part of the coding region (−750 to +114; Figure 1A) of cab1R was cloned into BamHI/Smal sites of pBluescript SK+. After deleting the same 15-bp sequence as in D34 (Figure 6A), a GUS reporter gene from pBI101 (Jefferson et al., 1987) was excised by PstI/EcoRI digestion and fused in frame with either the wild-type or mutated cab1R sequence (−750 to +114) in pBluescript SK+ to form cab1R-GUS and CD34, as shown in Figure 7A. Both wild-type cab1R-GUS and CD34 DNAs were delivered into etiolated maize leaves. The light-dependent expression of cab1R-GUS was completely eliminated by the 15-bp deletion (Figure 7B).

The Repeated Sequence Is Not Sufficient to Confer Light Responsiveness

To determine whether OCT-R might be sufficient for the cab1R promoter to respond to light, we tested the capacity of a tetramer of the octamer repeat to confer photoresponsive expression on a heterologous basal promoter. In Figure 8A, a tetramer of the 22-bp element containing both octamer sequences was made and fused to −90/35S-GUS to generate the construct TET-90/35S-GUS. Using −90/35S-GUS as a negative control and 295A as a positive control, we tested the ability of TET-90/35S-GUS to drive photoresponsive expression in maize leaves. Whereas the sequence that included the wild-type cab1R enhancer (−389 to −94) consistently increased GUS expression at least threefold in a photoregulated manner, TET-90/35S-GUS was not capable of responding to light and expressed GUS at a low level like −90/35S-GUS (Figure 8B). This suggests that other sequence elements within the −389 to −94 region are also required for light responsiveness.

The Octamer Repeat Does Not, but Other Sequences Do, Affect Expression of cab1R in Tobacco Leaf Tissue

The 5′ deletion experiments showed that sequences between −170 and −269 are required for photoregulated expression of cab1R-GUS in both transgenic tobacco and bombarded leaves of rice and maize. OCT-R within this region is required
which of the other bases in this 22-bp sequence are also critical for the cabR promoter to respond to light. The effect of mutation of CC to GG shows that these bases are important for its function. In addition, there are other nearly identical tamer repeats, AGCTCACCCCAAGCTCACCAAG, is required for light-regulated expression of the GUS reporter in maize or rice leaves. This suggests that the sequence containing the octamer repeat in the -269 to -170 region to drive the expression of the GUS gene in tobacco leaves. Conversely, mutations in G-box-like and box III*-like sequences (Figure 6A) each reduced expression in tobacco by about 50% (Figure 9) but had no effect on expression of the GUS reporter in maize leaves (Figure 6B). Because etiolated tobacco cotyledons are so small, we did not study the effects of illumination on expression from mutated promoter regions.

**DISCUSSION**

In searching for sequences required for photoresponsiveness of the cabR promoter, we found a sequence element containing an octamer repeat in the -269 to -170 region. Deletion of either or both of the repeated sequences or replacement of bases (CACCC to CAGG) in both octamers eliminated the light-dependent expression of the GUS reporter in maize or rice leaves. This suggests that the sequence containing the octamer repeats, AGCTCACCCCAAGCTCACCAAG, is required for the cabR promoter to respond to light. The effect of mutation of CC to GG shows that these bases are important for presumptive trans-acting factor(s) to bind and function. However, the data presented in this report do not reveal exactly which of the other bases in this 22-bp sequence are also critical for its function. In addition, there are other nearly identical symmetrical sequences in the sequence, such as CCAAGCTCACCCCAAGCTCACCAAG; additional mutations will be required to fully define this sequence element. Because deletion of either half of the element abolished light regulation, there could be an interaction between the putative trans-acting factors that may bind to the two octamers or the two octamers could act as a single protein binding element. A similar repeated sequence is present in the promoter region of another rice cab gene (cab2R) that is not expressed as strongly as cabR (Luan and Bogorad, 1986; Luan, 1991). However, in cab2R the repeats are separated by a sequence of 40 bp in length. It would be interesting to determine whether this repeat is important in light-regulated expression from the cab2R promoter.

A number of cases have been reported in which a promoter-containing DNA segment of a monocot gene fused to a reporter sequence can be expressed and regulated properly in a widely heterologous system, such as a transgenic dicot (for example, Lamppa et al., 1985b). We have found this to be true of the cabR promoter segment: cabR-GUS is expressed in a light-responsive and organ-specific manner in transgenic tobacco. Because the sequence between -269 and -170 is required for expression in tobacco as well as in maize or rice, it seemed possible that common cis-acting sequences might exist for light regulation in these plants. However, more detailed analyses have shown that the cabR promoter contains separate sequences for expression of cabR-GUS in tobacco and photoregulated expression in the monocot plants maize and rice. Although deletion of a sequence containing an octamer repeat completely eliminated light-dependent expression in maize or rice leaves, it had no effect on expression in illuminated tobacco leaves.

Box III* and G-box sequences have been implicated in light-regulated expression in dicot plants (reviewed in Gilmartin et al., 1990). In comparing the cabR promoter segment (-269 to -170) with the promoter sequence of pea rbcS-3A, we found that box III*-like and G-box-like sequences are next to each other in both promoters (Figure 1A and Figure 6A, section iii). Replacement of bases in either box III*-like or G-box-like sequences reduced expression of the GUS gene in tobacco leaves by about 50%, but such changes did not alter expression in maize or rice leaves. Box III*-like and G-box-like sequences may be required for proper regulation of the cabR promoter in transgenic tobacco, whereas regulation in a more closely related plant, maize, as well as in rice, requires another set of sequence elements including OCT-R. It is possible that the box III*- and G-box-like sequences function in some maize or rice tissues under some circumstances. The fact that the rice cabR gene has regulatory sequences for expression in diverse species implies both genetic conservation (retaining sequences from dicot plant genes) and genetic diversity (new elements for specific control) during evolution.

These experiments have also shown that a histochemical in situ spot-counting transient assay in which plant tissues are bombarded with DNA-coated microprojectiles can be used to study the functions of a promoter of a monocot gene in homologous or heterologous plants in cases where the production...
of transgenic plants is difficult or very lengthy. However, we cannot exclude the possibility that some differences may exist between the in situ transient assay and transgenic plant systems. In our case, the -170 construct gave hardly detectable GUS activity in transgenic tobacco but was expressed at a higher level in bombarded tissues. This might be attributed to differences in the fluorometric and histochemical assays for GUS activity (Luan, 1991).

In conclusion, we have found that there are separate cis-acting elements in the rice cab1R gene 5' upstream region that are required for expression in tobacco versus in maize and rice. Curiously, this gene also contains separate sequences, resembling some previously described elements in dicot genes, that regulate expression in tobacco. More detailed analysis of the monocot-specific sequence elements and identification of protein factors binding to them should yield further insights into the mechanisms for light-regulated gene expression in diverse species of plants. These would be important steps toward delineating the differences and similarities in light signal transduction pathways among these monocot and dicot plant species.

METHODS

Plant Materials

Maize (W23 r-g, AbC, PI) kernels (kindly provided by Dr. E. Coe) and rice (Labelle) seeds (kindly provided by Dr. Charles M. Bollich) were surface sterilized and imbibed in water for 24 hr at room temperature. Both maize and rice seedlings were grown in the dark at about 28°C for 9 days before their leaves were used for bombardment. Expanded tobacco leaves from plants grown sterilely on MS medium (Murashige and Skoog, 1962) were taken for bombardment.

Constructs and Deletions

35S-GUS was made by cloning the 3.0-kb Hindlll-EcoRI fragment containing the CaMV 35S promoter-controlled GUS gene from pBl121 (Jefferson et al., 1987) into pUC19. To generate cab1R-GUS, a BglII-Hindlll fragment from cab1R extending from positions -750 to +114 (in relation to the transcription start site; see sequence in Figure 1) was cloned into BamHI/Smal sites of pBluescript SK+ and then fused in frame with a GUS cassette from pBI101 (Jefferson et al., 1987). To construct 35S-Rlc, the GUS cassette was removed from 35S-GUS by BamHI and SacI, and an XbaI fragment from an Rlc cDNA clone (Ludwig et al., 1989) was inserted in place of GUS. The 5' end deletions of the cab1R-GUS were generated by Exonllmung bean nucleases from Stratagene following the manufacturer's protocol. For Ti plasmid-mediated transformation, a cassette containing promoter deletions and the GUS gene was cloned into a binary vector pBIN19 (Bevan, 1984) (see Figure 1B).

Transgenic Tobacco Production and Analyses

The leaf disc infection procedure (Horsch et al., 1985) was employed to produce transgenic tobacco plants. Briefly, discs were made from leaves of 6- to 8-week-old explants of tobacco SR1. After incubation for 36 hr with Agrobacterium tumefaciens harboring both the disarmed Ti plasmid (pGV2260) (Deblaere et al., 1985) and the binary vector pBIN19 (Bevan, 1984) containing the transgene constructs, leaf discs were thoroughly washed in liquid B5 medium and placed on regenerating medium (0.1 μg/mL indoleacetic acid, 0.15 μg/mL kinetin, and 250 μg/mL cefotaxime in B5 medium with 3% sucrose and 0.8% agar) for 4 days before transfer to selection medium (50 μg/mL kanamycin in regenerating medium). Plants were transferred to rooting medium (selection medium without hormones) until they grew into plants with four to five leaves. Leaves at the same position on 10 to 15 transgenic plants generated from each construct were analyzed. The level of GUS activity varied up to 10-fold among different plants with the same construct. DNA gel blot analyses indicated that most plants contained only one copy of the transgene. GUS activity was not related to the apparent number of copies of the integrated genes (data not shown). The plants were then transferred into pots and raised to flowering in a growth chamber (14 hr of light/10 hr of dark, 26°C). Some of the flowers were self-pollinated to produce F1 seeds.

Fluorometric GUS Assay

A 1-cm-wide segment from leaves at the same position of each of the transgenic tobacco plants was pulverized in a microcentrifuge tube in the presence of liquid nitrogen and lysed in GUS assay buffer (Jefferson, 1987). After centrifugation at 12,000 rpm in a microcentrifuge (Fisher) for 10 min, aliquots of the supernatant were taken for enzyme assay, as described by Jefferson (1987).

Microprojectile Bombardment and Histochemical GUS Assay

A biolistic system (PDS 1000; Du Pont) was used for all the microprojectile bombardments. Three-centimeter-long middle sections from the second leaves of etiolated 9-day-old maize seedlings were placed on MS agar (0.6%) plates (three pieces per 4.5-cm-diameter plate). Each plate was bombarded with 2 μL of a 10-μL mixture containing 2 μg of DNA precipitated onto 1.5 mg of tungsten particles with an average diameter of 1.2 μm (for details, see Klein et al., 1987, 1989). For bombardment of rice leaves, 35S-Rlc DNA was mixed with GUS constructs in a molar ratio of 1:2. In light/dark experiments, tissues were bombarded under a green safelight in a darkroom. After incubation at 25°C for 48 hr under white fluorescent lamps (250 μmol m⁻² sec⁻¹) or in darkness, red dots of anthocyanin generated by Rlc-induced pigmentation were visible in maize leaf tissue. The red dots were counted under the microscope. The tissues were then cut into about 4-mm-wide segments and put into a microcentrifuge tube containing 0.8 mL of GUS substrate solution (Jefferson, 1987) and incubated at 37°C for 24 hr. The number of blue spots was then counted under the microscope, and the ratio of blue spots to red dots was calculated. This was the basis for the quantitative GUS assay. For bombardment of rice leaves, 3-cm-long leaf segments were arranged on an agar plate and bombarded as described above. Tobacco leaves (about 4 cm wide and 8 cm long) from sterile culture were split into halves along the midvein and cut into quarters; two quarters served as the target for one shot.

Site-Directed Mutagenesis

A region of the cab1R promoter (−389 to −94) was obtained by restriction digestion of −750cab1R DNA using ClaI and BalI (see...
Figure 1). After filling in the ends by T4 polymerase, this DNA fragment was ligated in two orientations to −9035s-GUS in the pBluescript vector to form the constructs 295A and 295B (Figure 5A). The mutagenesis procedure was basically as described by Sarkar and Sommer (1990), except for some minor changes. Briefly, the DNA fragment being mutagenized was inserted into the pBluescript SK+ vector. In the mutagenesis reactions, three primers were used in the two-round polymerase chain reactions (PCRs). One primer contained the desired mutations (mutant primer), and the other two were generated from vector sequences (vector primers)—in this case, the M13 −20 primer sequence and the reverse primer sequence. The first PCR round was performed using the mutant primer and one of the two vector primers under the reaction conditions of Sarkar and Sommer (1990), except that the temperature and time for the annealing process were changed depending upon the mutations. To generate the 15-bp deletion, for instance, we annealed at 32°C for 2 min per cycle. The product of the first PCR was then used as a primer together with the other vector primer in the second PCR round to produce the whole insert sequence containing the desired mutations. The mutations were verified by sequencing, and the mutated insert sequence was cloned back into the constructs containing the −9035s-GUS cassette. The 295-bp fragment was mutagenized at various places using essentially the procedure described above while cloned in pBluescript SK+. The mutated −389 to −94 sequence was then excised and cloned back upstream of −9035s-GUS to generate the various mutants: D34, OCTm-1, OCTm-2, BIIIm-m, GBm-m, and 3AF1-m. The cab1R promoter in pBluescript SK+ was used as a template for producing the CD34 mutant.

ACKNOWLEDGMENTS

We thank Dr. Susan Wessler for the ic cDNA clone. We are grateful to Dr. James DeCamp for critical reading of the manuscript. This work was supported in part by a research grant from the National Institute of General Medical Sciences. Early portions of this work were supported in part by the Rockefeller Foundation.

Received March 10, 1992; accepted June 19, 1992.

REFERENCES


Keith, B., and Chua, N.-H. (1986). Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. EMBO J. 5, 2419–2425.


A rice cab gene promoter contains separate cis-acting elements that regulate expression in dicot and monocot plants.

S Luan and L Bogorad

*Plant Cell* 1992;4:971-981

DOI 10.1105/tpc.4.8.971

This information is current as of July 8, 2017