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Localization and Interaction of the *cis*-Acting Elements for Abscisic Acid, VIVIPAROUS1, and Light Activation of the *C1* Gene of Maize

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The *C1* regulatory gene of the maize anthocyanin pathway is regulated by a combination of developmental and environmental signals that include the *Viviparous1* (*Vp1*) gene, abscisic acid (ABA), and light. Using protoplast electroporation and particle bombardment assays, we have defined *cis*-acting elements that are necessary and sufficient for the activation of *C1* by ABA, VP1, and light, respectively. The sequence from positions −142 to −132 (CGTCCATGCAT) is essential for VP1 activation, whereas a larger overlapping element from −147 to −132 (CGTGTCGTCCATGCAT) is necessary and sufficient for activation by ABA. A separate light (blue and red)-responsive *cis* element is located between positions −116 and −59. Light interacts synergistically with the ABA and VP1 responses in transient expression assays, suggesting that combinatorial interaction between modules plays a role in integrating these signals in the developing seed.

INTRODUCTION

Anthocyanins accumulate in certain tissues of the maize seed (the scutellum of the embryo and the aleurone layer of the endosperm) during the maturation phase of seed formation. The synthesis of anthocyanin in the maize seed involves an interaction between structural genes (*A1*, *A2*, *C2*, *Bronze1* [*Bz1*], and *Bz2*) and a cascade of regulatory genes (*C1*, *R1*, and *Viviparous1* [*Vp1*]; Coe and Neuffer 1977; McCarty 1995). The *C1* gene encodes a Myb-like DNA binding protein (Paz-Ares et al., 1987) with an acidic transcriptional activation domain (Goff et al., 1991). *C1* and *R1* interact to activate the structural genes in the anthocyanin pathway (Goff et al., 1991).

McCarty et al. (1989b) showed that the colorless phenotype of the *vp1* mutant is associated with failure to express the *C1* gene in the kernel, indicating that *Vp1* and *C1* are part of a regulatory gene hierarchy. In transient expression assays, the *C1* promoter is activated by VP1 and abscisic acid (ABA; Hattori et al., 1992). Light may also play a role in regulation of *C1* and anthocyanin synthesis in maize seeds. Wild-type maize ears that develop in total darkness produce colorless seeds (Cone et al., 1993; Dooner, 1994). In addition, if the colorless *vp1* seeds are removed from the ear before desiccation and allowed to continue to germinate in light, the seeds become pigmented (McCarty and Carson, 1990). An allele of C1, called *c1-p*, has an analogous light response. When exposed to light on the developing ears, *c1-p* kernels remain colorless. However, when subsequently germinated in the dark, these light-exposed seeds become pigmented, indicating that the light stimulus is somehow stored in the *c1-p* tissues (Chen and Coe, 1978). During seed germination, mRNA for a number of *C1* alleles increases significantly in the presence of light (Scheffler et al., 1994). These observations indicate that in addition to developmental regulation by VP1 and hormone regulation by ABA, *C1* is environmentally activated by light.

In a previous study, we showed that VP1 overexpression and ABA independently cause transcriptional activation of a reporter gene driven by the *C1* promoter in maize protoplasts (Hattori et al., 1992). Here, we define a 16-bp element that is sufficient for ABA and VP1 activation. We also find that light can activate transient expression from the *C1* promoter in various tissues, including protoplasts, developing and germinating embryos, and root and leaf tissues. Red and blue light are effective; far-red light inhibits *C1* expression, suggesting a possible involvement of phytochrome. The minimal light-responsive *cis* element is separate from the VP1/ABA response element and has features that resemble previously identified light response sequences.

RESULTS

Fine Structure of the VP1 and ABA Response Element

Previous studies have shown that ABA and overexpression of VP1 in maize protoplast activate a *C1* promoter–

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β-glucuronidase (*GUS*) reporter construct, C1-*Shrunken* (Sh)-GUS (C609), and that sequences necessary for the VP1/ABA response are located between positions −157 and −130 in the *C1* promoter (Hattori et al., 1992). To resolve the fine structure of the *cis* element, a series of site-directed mutants through this region (−157 to −130) was made (Figure 1A), and their responses to VP1 and ABA were tested in a protoplast electroporation assay (Figures 1B and 1C). The sequence from positions −142 to −132 (5′-CGTCCATGCAT-3′), which is basically the Sph element, is critical for VP1 activation, whereas sequences essential for ABA regulation overlap the critical VP1 region and extend 5 bp farther in the 5′ direction to include the base at position −147. In total, the region required for both ABA and VP1 activation is 16 bp long from positions −147 to −132 (5′-CGTGTCGTCCATGCAT-3′).

We placed a 16-bp synthetic oligomer of this sequence upstream of the cauliflower mosaic virus (CaMV) 35S core promoter (−46) and assayed this construct by protoplast electroporation. As shown in Figure 2, a dimer of this sequence is sufficient for both ABA and VP1 activation. No activation of a monomer construct was detected (data not shown). In contrast to the additive response of the native *C1* promoter to VP1 and ABA (Hattori et al., 1992), the Sph dimer construct exhibited a synergistic response to VP1 and ABA combined.

C1-Sh-GUS Is Light Activated in Protoplast and Particle Bombardment Systems

We found that light activation of the *C1* promoter occurs in protoplast electroporation and particle bombardment systems. Figures 3A and 3B show the time course of the light activation of C1-Sh-GUS in protoplast and leaf particle bombardment experiments. In protoplast electroporation and microprojectile experiments, gene activation was detected after 9 and 6 hr of continuous illumination, respectively. Light activation reached a maximum at ~24 hr after bombardment of leaf tissues (Figure 3B). Figure 3C shows that this light effect is specific to the *C1* promoter and does not affect various other promoters, some of which also include the *Sh1* first intron (ubiquitin [Ubi]-Sh-GUS and 35S-Sh-GUS). By using different wavelength cutoff filters, we determined that red light (>600 nm) and blue light (400 to 500 nm) activated C1-Sh-GUS, whereas far-red light (>700 nm) was inhibitory (Figure 4). This suggests that phytochrome may be one of the photoreceptors for light activation of C1-Sh-GUS.

In addition, Figure 5A shows that light activation of C1-Sh-GUS was observed in other tissues, including those of developing and germinating embryos, leaves, and roots (data not shown). Because the endogenous *C1* is not normally expressed in roots and leaves, we conclude that not all aspects of proper developmental control of light regulation are recovered in the transient expression assays. In contrast, as shown in Figures 5A and 5B, VP1 transactivation of C1-Sh-GUS occurs specifically in seed and not in leaf tissues. This pattern is in contrast

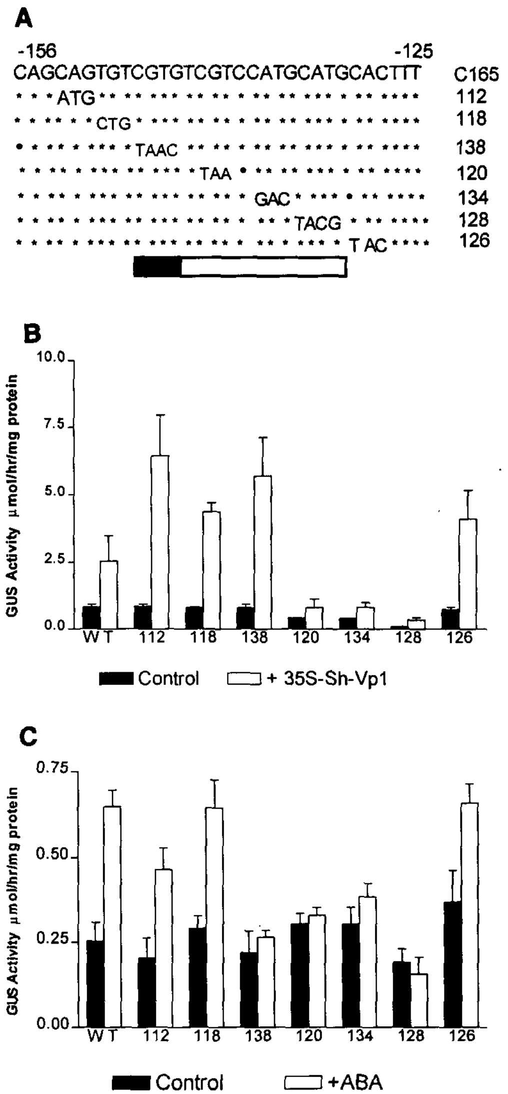

Figure 1. Localization of *C1* Promoter Sequences Critical for ABA Regulation and VP1 Transactivation in the Protoplast Electroporation Assay.

(A) A series of site-directed mutants was made between positions −156 and −125 in the promoter of C1-Sh-GUS (C165). Asterisks represent the wild-type sequences as shown in C165.

(B) Responses of the site-directed mutants to 35S-Sh-VP1 in transformed protoplasts.

(C) Responses of the mutants to ABA (10^{-5} M) in transformed protoplasts.

In **(A)**, the open rectangle represents the sequences critical for both ABA and VP1, and the dark rectangle represents the sequences specifically required for ABA regulation. In **(B)** and **(C)**, WT indicates the wild-type *C1* promoter reporter construct.

with the regulation of the Em-GUS reporter gene, which is transactivated by VP1 in leaves (Figure 5B) as well as in seed tissues (McCarty et al., 1991; Hoecker et al., 1995). This result suggests that in addition to the VP1 protein, there may be other seed-specific factors needed for *C1* activation by the VP1 protein.

The interactions of light, VP1, and ABA and their effects on C1-Sh-GUS (C609) in protoplast electroporation experiments are shown in Figure 5C. The light plus VP1 and light plus ABA activation are roughly multiplicative with respect to individual treatments, suggesting a positive synergism between light, VP1, and ABA activation.

Localization of Light-Responsive Sequences in the *C1* Promoter

To define the minimal light-responsive *C1* promoter, a series of 5′ deletions of the promoter region was tested (Figure 6A). As shown in Figure 6B, mutants deleted up to position −130 relative to the transcription start site still remained light responsive, whereas C48 lost the response. This result indicates that an essential light-responsive *cis* element is localized between positions −130 and −48. The light-responsive −130 promoter,

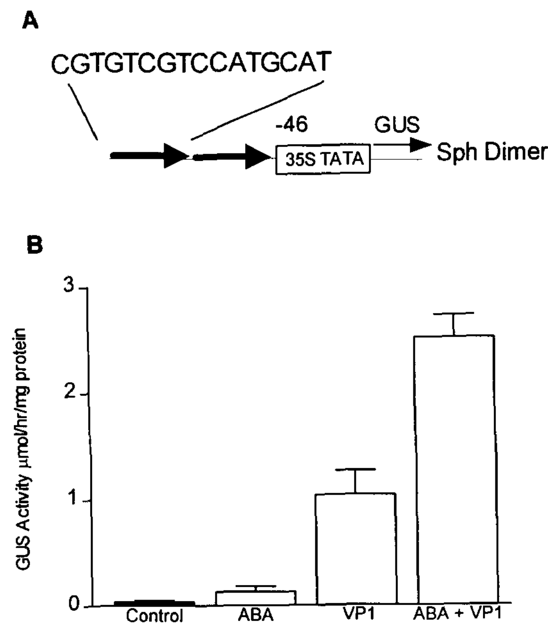

Figure 2. A 16-bp Sequence Dimer Is Sufficient for Both ABA and VP1 Regulation.

(A) Dimer of the sequence important for ABA/VP1 regulation in the minimal 35S (−46) promoter reporter construct. Arrows represent the tandem copies of the sequence.

(B) Responses of the dimer reporter construct to ABA and VP1 in the protoplast electroporation assay.

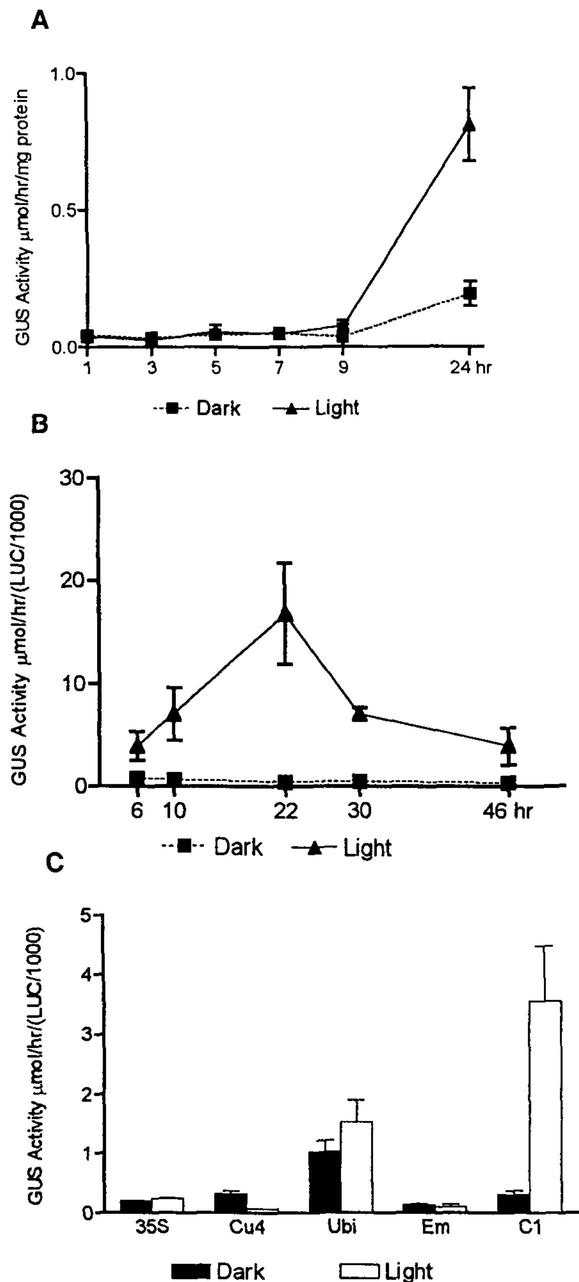

Figure 3. Light Activation and Interaction with VP1 and ABA in Transient Assays.

(A) and **(B)** Time course responses of light regulation in protoplast electroporation and leaf particle bombardment, respectively.

(C) Specificity of C1-Sh-GUS light regulation relative to four different promoter constructs in particle bombardment of leaves. 35S, CaMV 35S-GUS; Cu4, alcohol dehydrogenase (Adh1)-Adh1 intron 1-GUS; Ubi, ubiquitin (Ubi)-Sh-GUS; Em, Em-GUS; and C1, C1-Sh-GUS. Sh1-Sh-GUS and Sh2-Sh-GUS reporter constructs, data not shown. LUC, luciferase.

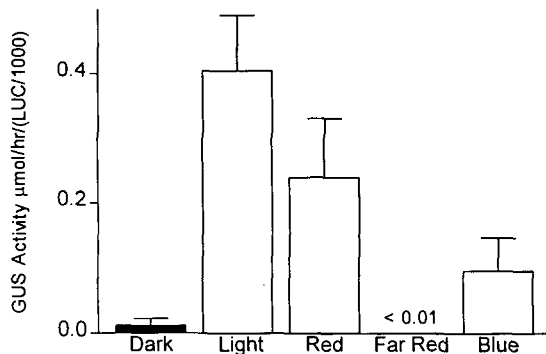

Figure 4. Effects of Different Light Wavelengths on C1-Sh-GUS Implicate Phytochrome as One of the Photoreceptors for *C1* Light Activation.

After bombardment, leaf tissue was incubated in different light wavelengths for 24 hr. Light, white light from a slide projector; Red, red light (>600 nm); Far Red, far-red light (>700 nm); Blue, blue light (400 to 500 nm); LUC, luciferase.

however, has no VP1 and ABA response (Hattori et al., 1992), indicating that light regulation can be separated from VP1/ABA activation.

The *C1* promoter contains at least three G-box–like sequences that are similar to light regulatory elements defined in the chalcone synthase promoter in parsley cells (Block et al., 1990). A copy found at position −96 was within the minimally responsive promoter defined by the terminal deletions. Figure 6C shows that an eight-base deletion (−104 to −96; DG96) and a two-base site-directed mutation (ACGT → AACT; MG96) of the −96 element have a critical effect on light activation. An eight-base deletion in the upstream ACGT element (−189 to −181; DG181) has no effect on the light response, indicating that −96 and −181 elements are not functionally redundant. These results, together with tests of deletions (C235ld3 and C130ld3; see below) that removed a third ACGT element located proximal to TATA at position −47, indicate that only the −96 G-box motif has an essential function in light regulation.

Structure of the Light Response Element

To further delimit sequences in the −130 promoter that are required for light regulation, a series of site-directed and internal deletion mutants was tested. As shown in Figure 7, the 5′ boundary of the critical light response region is defined by the 2-bp change in the 7677 mutant, which abolishes light activation. This result, together with the effects of the C235ld2 and C235ld3 internal deletion constructs, indicates that the essential light-regulatory sequences are located between positions −116 and −59. To define sequences that are sufficient for light regulation, we cloned oligonucleotide sequences from

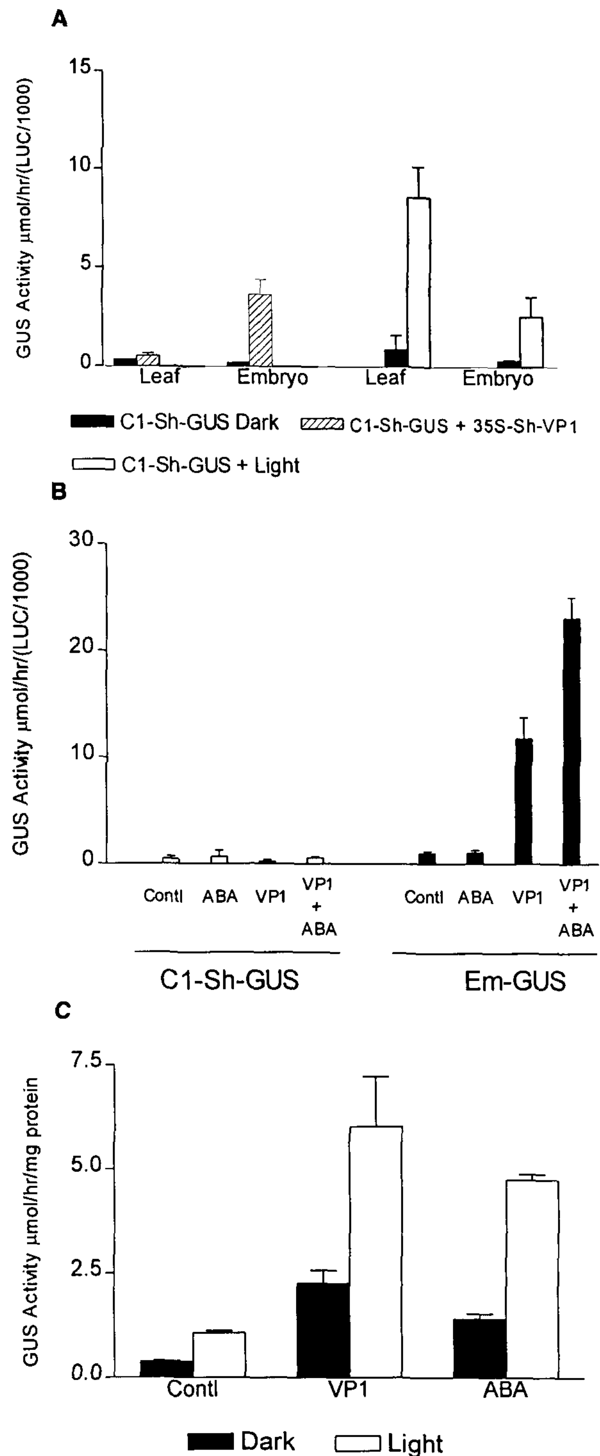

Figure 5. Tissue Specificity of Transactivation by the VP1 Protein and the Synergistic Interactions of Light Plus VP1 and Light Plus ABA Activation.

(A) Tissue specificity of transactivation by the VP1 protein in comparison with light activation of C1-Sh-GUS. Leaf, the third or fourth leaf

the minimal light-responsive region upstream of the 35S (−46) and *C1* (−48) core promoters and the *GUS* gene. Consistent with the results in Figure 7, a minimal promoter consisting of the 76 bp (−58 to −130) fused to the *C1* TATA element was light activated (C130ld3). Unexpectedly, however, this 76-bp sequence failed to confer light regulation to the 35S (−46) core promoter. To address the possibility that essential sequences may be located near TATA in the core promoter region of *C1*, we replaced the core TATA of C130 with the 35S promoter TATA and transcription start sites, as shown in Figure 8A. That we observed light activation of this construct (CLE35S) rules out special properties of the *C1* TATA and core promoter region (Figures 8B and 8C). Furthermore, the activities of the C130ld3 and C235ld3 constructs show that the −58 to −45 sequence per se and altered spacing between TATA and the upstream elements are not critical. One remaining feature that is common to all of the light-activated minimal constructs we tested is the presence of the *Sh1* first intron in the 5′ untranslated region of the *GUS* transcript.

Although the mechanism by which the *Sh1* intron enhances transient gene expression remains poorly understood, it is unlikely that the intron has a specific role in light activation (Vasil et al., 1989). The *Sh1* gene itself is not directly light regulated. A variety of unrelated promoter constructs that include the *Sh1* intron in similar contexts shows no light activation in our system (Figure 3C; C.-Y. Kao, I.K. Vasil, and D.R. McCarty, unpublished results). The existence of specific *C1* promoter mutants, which block light activation (i.e., DG96, MG96, and C235ld3) without affecting VP1 and/or ABA regulation, as well as the nonresponsive C48 construct clearly demonstrate that the intron is not sufficient for light activation in the context of the *C1* promoter. A possibly similar qualitative interaction between intron sequences and upstream regulatory elements has been demonstrated in transgenic potato (Fu et al., 1995a, 1995b).

DISCUSSION

Our results show that the region −147 bp upstream of the transcription start site in the *C1* promoter includes at least two independent regulatory modules, one for ABA/VP1 and another for light activation. Thus, like other light-regulated

Figure 5. (continued).

of wild-type (W22) seedlings; Embryo, developing embryos of *vp1* kernels (20 days after pollination).

(B) Comparison of tissue-specific VP1 transactivation between C1-Sh-GUS and Em-GUS in leaf particle bombardment.

(C) Light activation of C1-Sh-GUS (C609) has a synergistic effect on VP1 and ABA treatment in protoplasts.

LUC, luciferase; Contl, control.

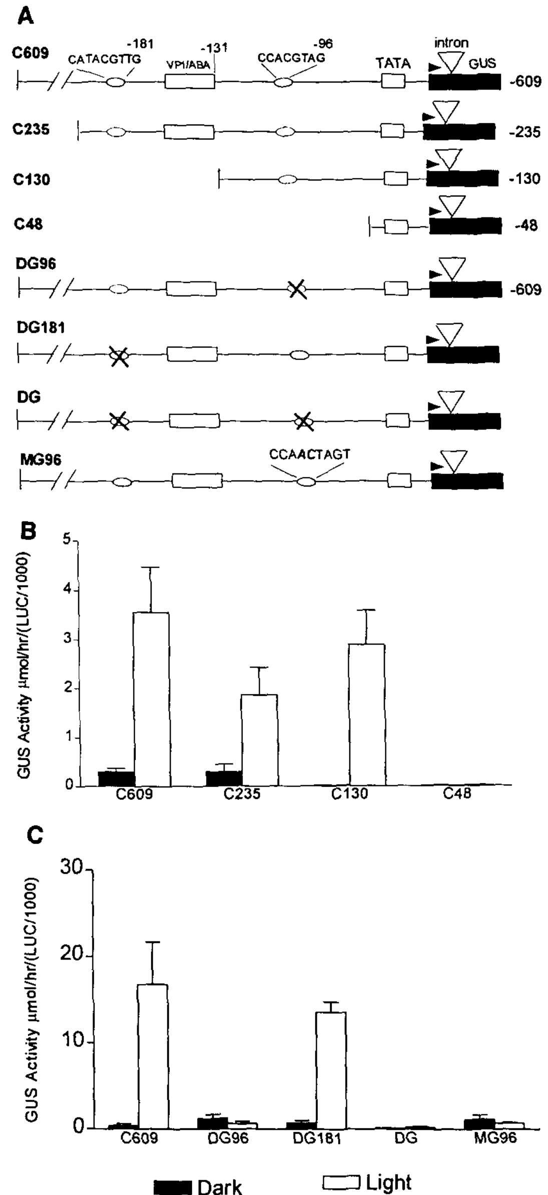

Figure 6. Localization of *C1* Promoter Sequences Required for Light Regulation.

(A) A series of 5′ deletion and G-box internal deletion mutants (indicated by X's) in the promoter of C1-Sh-GUS was tested for light regulation in leaves. Open ovals indicate the ACGT motifs; double slashes represent the upstream regions that are not drawn to scale.

(B) Responses of the 5′ deletion mutants to light in leaf bombardment. C130 is a deletion up to position −130 relative to the transcription initiation site that still remained light responsive. The C130 construct is not ABA/VP1 responsive (Hattori et al., 1992).

(C) Responses of the G-box internal deletion (DG96, DG181, and DG) and base change mutants (MG96) implicate involvement of the G-box sequence at position −96; a two-base change within the ACGT motif abolished the light response of the *C1* promoter.

LUC, luciferase.

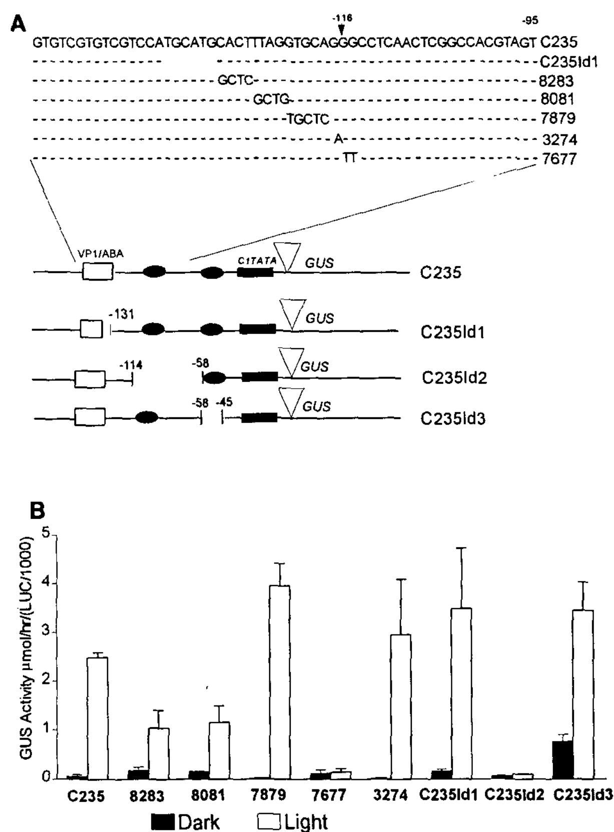

Figure 7. Resolution of the Light-Responsive Domain in the *C1* Promoter.

(A) A series of site-directed mutants 5′ to the G-box (GGCCACGTAGTTA) at position −96 and internal deletion mutants (C235ld1, C235ld2, and C235ld3) were tested for light regulation by leaf particle bombardment. Solid ovals represent ACGT motifs.

(B) Light response of these mutants defined the 5′ boundary of the light element (7677) at position −116, and light response of the C235ld3 defined the 3′ end of the light *cis* element. LUC, luciferase.

promoters that have been studied in detail (Kuhlemeier et al., 1988; Bruce et al., 1991), the *C1* promoter has a modular structure. The *cis*-acting element for ABA/VP1 is further resolved into the Sph element (CGTCCATGCAT), which is required for the ABA and VP1 responses, and an upstream element (CGTGTC) that is specifically required for ABA regulation in the absence of VP1 overexpression. This result is consistent with our previous finding that the GTGTCGTGTC repeat, which distinguishes wild-type *C1* alleles from the *c1-p* alleles, is necessary for ABA regulation. Our results indicate that the C residue (underlined) contributed by the upstream copy of the repeat is the critical difference between these alleles. The Sph element overlaps an extended RY motif (CATGCATGCAC) (Dickinson et al., 1988; Baumlein et al., 1992) and also resembles one component of the gibberellic

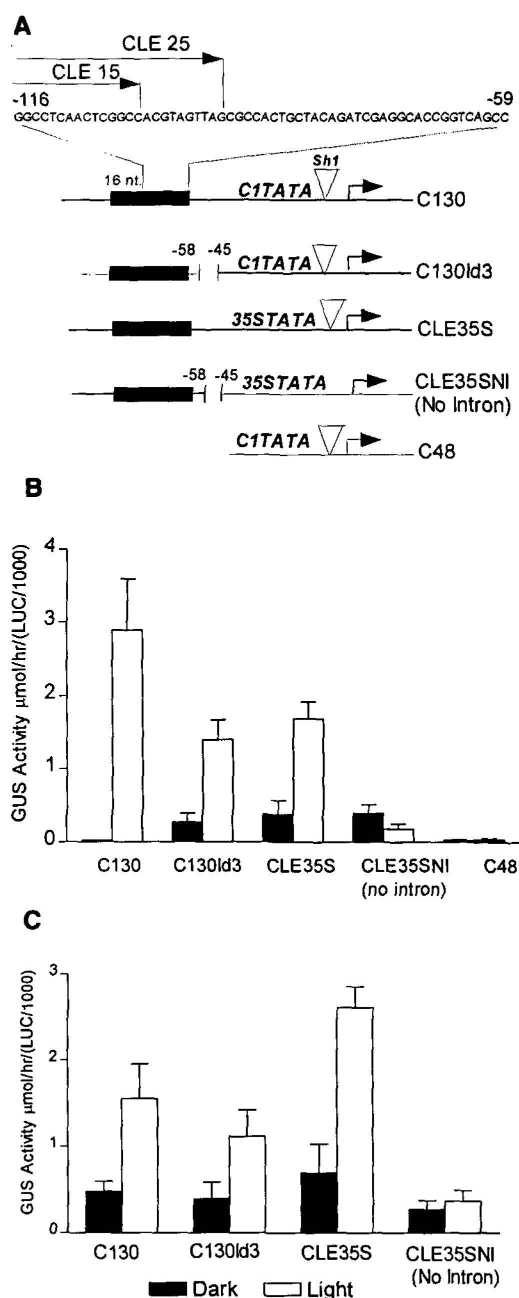

Figure 8. Gain-of-Function Tests of the Light-Responsive Element in Particle Bombardment.

(A) The light-responsive element (76 bp) was combined with either the *C1* TATA (C130 and C130ld3) or the 35S TATA (CLE35S and CLE35SNI [No Intron]) box. CLE35S includes the *Sh1* first intron; CLE35SNI (No Intron) does not have the intron. nt., nucleotide.

(B) and **(C)** Light responses of the constructs shown in **(A)** in leaf and developing embryo tissues, respectively. The CLE15 and CLE25 oligonucleotides indicated in **(A)** were also tested in the *C1* minimal promoter construct (C48) with negative results (data not shown). LUC, luciferase.

acid–regulated low pI α-amylase promoter (TCCATGC) (Whittier et al., 1987).

In the native *C1* promoter in which a single copy of the VP1/ABA *cis* element is present, VP1 and ABA show a less than additive interaction (Hattori et al., 1992). However, a dimer of the VP1/ABA *cis* element (Sph dimer) confers a synergistic response to VP1 and ABA in the minimal promoter construct (Figure 2). One possibility is that dimerization allows cooperative interactions that may not occur in the normal context. In any case, the G-box–mediated VP1 response shows a similar requirement for multiple elements in minimal promoter constructs (Vasil et al., 1995), suggesting that multimerization substitutes for an as yet undefined aspect of the native promoter contexts of these elements.

We have localized the light *cis*-acting element of *C1* gene to a 56-bp region. This 56-bp region includes a dyad sequence (GGCCTCAACTCGGCC) that overlaps an adjacent G-box motif. This structure bears some similarity to the box II/box I (unit I) complex of the chalcone synthase gene promoter of parsley. Both are composed of an ACGT motif adjacent to a GGCC-X_7-GGCC palindromic sequence. The functional importance of these features, including the spacing within the palindromic, is supported by the fine structure analysis of the *chs* complex (Block et al., 1990). In the *C1* gene, additional sequences located 3′ end to the structure are also necessary (Figure 7). One of the maize chalcone synthase genes (*C2*), which is regulated by the *C1* gene in the aleurone and scutellum, also includes a box II–like element in the promoter region (Franken et al., 1991). It has been shown that the maize *C2* mRNA can be light activated in seedlings with all possible combinations of upstream regulatory mutants (*R/rg*, *B/b*, and *Pl/pl*) (Taylor and Briggs, 1990). These results suggest that a common light regulatory pathway can potentially affect both the regulatory (*C1*) and structural genes (*C2*) in the pathway and that, unlike developmental control, light regulation of the anthocyanin pathway may not be hierarchical. However, Scheffler et al. (1994) have shown that the light enhancement of *C2* expression in the aleurone of germinating seeds requires a functional *C1* allele.

Our data indicate that the interaction of light and developmental factors is mediated at least in part by the regulatory elements in the *C1* promoter. A synergistic interaction was observed in light plus VP1 and light plus ABA treatments (Figure 5C). Thus, light treatment also enhances VP1 and ABA activation of *C1* in transient expression assays. This finding is consistent with evidence that these signals interact significantly during seed maturation. For example, wild-type seeds grown in total darkness fail to accumulate anthocyanins, even though VP1, ABA, and a responsive *C1* allele are all present (Cone et al., 1993; Dooner and Ralston, 1994). Therefore, a light signal seems to be required in addition to VP1 and ABA signals for induction of the pathway during seed maturation. Conversely, the maturation phenotypes of the *c1-p* allele and *vp1* mutants indicate that light alone is insufficient to activate *C1* in the absence of a functional ABA response element and the VP1 factor, respectively. These interactions suggest that the combinatorial arrangement of ABA, VP1, and light-responsive elements in the *C1* promoter plays a role in the integration of these signals. In the transient expression assays, however, the requirement for the signals acting in combination is at least partially relaxed. It is possible that not all modes of *C1* regulation are recovered in our system. On the other hand, the effect of changes in *C1* expression at the level of phenotype may be strongly threshold dependent, in which case the effects observed here might account for the qualitative features of anthocyanin regulation.

An important feature distinguishing the VP1 and light responses that we detected in the transient expression assay system is the contrast in tissue specificity. VP1 transactivation of C1-Sh-GUS is detected only in embryo-derived suspension culture protoplasts and seed tissues, whereas this same construct is light activated in all tissues tested, including those of leaves and roots (Figure 5). The endogenous *C1* gene is expressed exclusively in embryo and aleurone tissue and does not functionally overlap with the plant-specific *pl* gene in light regulation of anthocyanins in nonseed tissues (Cone et al., 1993). Therefore, although both light and VP1 regulation of the *C1* gene are normally seed-specific responses, our results indicate that the tissue specificity observed in each case is determined by different mechanisms. In contrast, the wheat *Em* promoter is activated by VP1 in both seed and leaf tissues (Figure 5B). Consistent with the broad specificity of the Em-GUS response that we see in the maize transient expression systems, Parcy et al. (1994) have shown that several *Em*-related *LEA* genes are activated in vegetative tissues by ectopic expression of the ABI3 protein in transgenic Arabidopsis. The limited tissue specificity of the C1-Sh-GUS transactivation response, on the other hand, implies that additional seed-specific factors are required for activation of the *C1* gene.

The universal light activation mediated by the minimal *C1* light response element is consistent with the fact that these sequences are highly conserved in the plant-specific *pl* promoter, whereas the VP1/ABA response region is divergent in *pl* (Cone et al., 1993). However, these results also reveal an important limitation of the transient expression assay. It is possible that separate determinants of tissue specificity for light regulation may reside in 5′ or 3′ flanking regions that were not included in our reporter construct. On the other hand, other possible modes of control that involve chromatin structure or other epigenetic mechanisms are not likely to be recovered in a transient expression assay.

METHODS

Protoplast Electroporation

Protoplasts were prepared from a 4- or 5-day-old embryo-derived suspension cell line (DK XL80 hybrid), as described by Vasil et al. (1989).

Effector and/or reporter plasmid DNA (20 μg per 6 × 10^6) protoplasts were transferred into freshly isolated protoplasts by using a cell porator (Bethesda Research Laboratory) at 180 V, 800 μF. Each experiment was replicated in triplicate. After being cultured in growth media (Vasil et al., 1989), protoplasts were harvested, and β-glucuronidase (GUS) enzyme activity (Jefferson, 1987) and protein content (Bradford, 1976) were assayed as described by Hattori et al. (1992).

Particle Bombardment

Developing embryos dissected from *viviparous1* (*vp1*) kernels at 17 to 22 days after pollination or the third to fifth leaf of aseptically germinated W22 seedlings were used as material for helium gun bombardment. The preparation of plasmid DNA mixture as well as the helium gun conditions were described by Taylor and Vasil (1991). Each bombardment assay contained four embryos or a 1-cm leaf section. After bombardment, samples were split; one part (two embryos or half of the leaf section) was kept in the dark, and the other part was left in the light, as indicated. The different wavelength treatments were achieved by putting different wavelength cutoff filters (gelatin filter; Eastman Kodak Co., Rochester, NY) on the Petri dishes and wrapping the dishes with alumium foil to make sure the only light source was through the filter. Each experiment was replicated at least in triplicate. The materials were harvested after 40 hr for abscisic acid (ABA) and VP1 treatments and after 24 hr for light experiments. The GUS and luciferase enzyme activities were assayed as described by Hattori et al. (1992). GUS activity was normalized to luciferase activity.

Plasmid Constructions

Construction of C609, C235, C130, DG96, DG181, DG, MG96, C235ld1, C235ld2, 8283, 8081, 7879, 3274, and 7677 was described by Hattori et al. (1992). To construct the multiple-base-change mutants 112, 118, 138, 120, 134, 128, and 126, the EcoRI-XbaI fragment of C165 was cloned into pSelect (Promega), and the Altered Site in Vitro Mutagenesis System (Promega) was used to generate the site-directed mutants. The synthetic single-strand DNA for each mutant is as follows: for mutant 112, 5′-CCCATGACAGATCTGTCGTGTCG-3′; for 118, 5′-ATGACAGCAGCTGCGTGTCGTCC-3′; for 138, 5′-CATGACAGCAGTGTTAACTCGTCCATGCATGC-3′; for 120, 5′-AGTGTCGTGTTAACCATGCATGC-3′; for 134, 5′-GTGTCG-TGTCGTCGA- CGCATGCACTTTA-3′; for 128, 5′-TGTCGTCCATTACGGCACTTTAGG-3′; and for 126, 5′-GTCCATGCATTACCTTTAGGTGC-3′. To construct the Sph dimer, the following complementary pair of oligonucleotides was used: 5′-GATCCGTGTCGTCCATGCAT-3′ and 5′-GATCATGCATGGACGACACG-3′. The oligonucleotides were phosphorylated with T4 polynucleotide kinase and annealed. The compatible BamHI sites on the ends of this annealed pair were cloned into the pSelect BamHI site. The HindIII-EcoRI fragment from the pSelect plasmid was then cloned into the cauliflower mosaic virus (CaMV) 35S (−46) minimal promoter (Weisshaar et al., 1991) upstream of *GUS*. To construct C48, C235 was digested with SmaI and SalI, and the backbone was religated. To construct C130ld3, the SphI-EcoRI fragment from C609ld3 was cloned into pSelect. The 700-bp HindIII fragment excised from the pSelect clone was ligated to the HindIII-digested C609 backbone fragment. For CLE35S, synthetic complementary oligonucleotides, including the 35S TATA region, and the transcription start site with a 5′ SalI site and a 3′ XbaI site–compatible sequence, were made. After being phosphorylated, the annealed pair was cloned in place of the 50-bp SalI-XbaI fragment of C130. To construct CLE35SNI (no intron), the SphI-NaeI fragment from C165 was cloned into pSelect, and the HindIII-EcoRI fragment from the resulting pSelect plasmid was cloned in front of the CaMV 35S minimal promoter. All final plasmid constructs were sequenced to confirm the expected alteration of sequences.

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