Identification of functional domains in the maize transcriptional activator CI: comparison of wild-type and dominant inhibitor proteins

Stephen A. Goff,1,2,4 Karen C. Cone,3 and Michael E. Fromm2,5

1Plant Gene Expression Center, U.S. Department of Agriculture/University of California at Berkeley, Albany, California 94710 USA; 2Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211 USA

Genes encoding fusions between the maize regulatory protein CI and the yeast transcriptional activator GAL4 and mutant CI proteins were assayed for their ability to trans-activate anthocyanin biosynthetic genes in intact maize tissues. The putative DNA-binding region of CI fused to the transcriptional activation domain of GAL4 activated transcription of anthocyanin structural gene promoters in ci aleurones, ci Rscm2 embryos, and ci r embryogenic callus. Cells receiving these constructs accumulated purple anthocyanin pigments. The CI acidic region fused to the GAL4 DNA-binding domain activated transcription of a GAL4-regulated promoter. An internal deletion of CI also induced pigmentation; however, frameshifts in either the amino-terminal basic or carboxy-terminal acidic region blocked trans-activation, and the latter generated a dominant inhibitory protein. Fusion constructs between the wild-type CI cDNA and the dominant inhibitor allele CI-I cDNA were used to identify the amino acid changes in CI responsible for the CI-I inhibitory phenotype. Results from these studies establish that amino acids within the myb-homologous domain are critical for transcriptional activation.

[Key Words: Anthocyanins; trans-activation; transcriptional activators; dominant inhibition]

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Genetic analysis of maize has identified a large number of genes, including at least four regulatory loci, involved in the synthesis of the purple anthocyanin pigments (Coe et al. 1988). For example, the regulatory genes B (Styles et al. 1973) and Pl (Gerats et al. 1984) are required for pigmentation in most of the plant body, although strong light can partially overcome the requirement for Pl (Coe et al. 1988). CI is required for pigmentation of the aleurone and the embryo but is not required in the plant body (Chen and Coe 1977; Coe 1985). A functional R gene product is required for anthocyanin pigmentation in the aleurone, anthers, and coleoptile (Styles et al. 1973), although the B-Peru allele of B can substitute for R in the aleurone (Styles et al. 1973). The Rscm2 allele of R conditions anthocyanin pigmentation in the maize embryo (Styles et al. 1973). r or b mutations have been found to affect the levels of the mRNAs and/or enzymes encoded by some of the anthocyanin structural genes examined (Dooner and Nelson 1979; Dooner 1983; Chandler et al. 1989; Ludwig et al. 1989). CI-I, a colorless allele, has low levels of the mRNAs and enzymes encoded by the A1 [NADPH-dependent reductase] and Bronze 1 [Bz1, UDP-glucose flavonol 3-O-glucosyl transferase] structural genes in aleurones [Dooner and Nelson 1979; Dooner 1983; K.C. Cone, unpubl.].

Sequence analysis of the coding regions of the R (Ludwig et al. 1989; Perrot and Cone 1989) and CI (Paz-Ares et al. 1987) genes provides molecular evidence in support of the proposed regulatory roles of the encoded proteins [Cone et al. 1986; Paz-Ares et al. 1986; Ludwig et al. 1989]. The B gene is homologous to the R gene [Chandler et al. 1989], and both B and R encode proteins with homology to the helix-loop-helix subunit dimerization region of the myc family of proteins [Chandler et al. 1989; Ludwig et al. 1989; Perrot and Cone 1989]. The CI gene encodes a protein with amino-terminal homology to the DNA-binding domain of members of the myb oncogene family [Paz-Ares et al. 1987]. The CI protein also contains a carboxy-terminal region rich in acidic amino acid residues analogous to the acidic transcriptional activation domains found in many transcriptional activators [for review, see Ptashne 1988]. In addition to the various recessive mutant ci alleles, a dominant inhibitory allele CI-I has been identified (Emerson et al. 1935; Coe 1962). This dominant inhibitor allele was not deliberately isolated by maize geneticists but is believed to originate

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from ancient cultivated lines (East and Hayes 1911). The presence of this allele blocks pigmentation of the aleurone in C1/C1-I heterozygotes (Emerson et al. 1935; Coe 1962), and pale pigmentation is occasionally observed in the aleurone of maize lines carrying an increased dosage of the wild-type C1 allele (Coe 1962). C1-I aleurone cells contain higher levels of C1-I mRNA relative to the level of C1 mRNA found in wild-type cells (Cone et al. 1988). Recent sequence analysis of this inhibitory allele revealed that amino acid substitutions, insertions, and small deletions are present in the C1-I-coding region (Paz-Ares et al. 1990). An 8-bp insertion near the 3' end of the gene is predicted to result in premature termination of the C1-I-encoded polypeptide (Paz-Ares et al. 1990). In vitro transcription/translation of a full-length C1-I mRNA results in the production of a polypeptide that is smaller than the wild-type protein (K.C. Cone, unpubl.).

High-velocity microprojectile delivery of DNA has been used to develop a system to further analyze the regulation of the anthocyanin biosynthetic genes. The A1 and Bz1 genes have been shown to complement a1 and bz1 mutants when delivered into nonpigmented aleurone cells [Klein et al. 1989]. This approach has been used to demonstrate C1- and R-regulated expression of introduced A1 and Bz1 structural gene promoters [Klein et al. 1989] and trans-activation of the A1 and Bz1 promoters by the B regulatory genes [Goff et al. 1990]. Delivery of a Bz1-luciferase reporter construct has also been used to define the cis-acting sequences of the Bz1 promoter required for C1- and R-regulated expression [B.A. Roth and M.E. Fromm, unpubl.]. Recently, microprojectile delivery of DNA has been used to demonstrate that the entire anthocyanin biosynthetic pathway can be induced by the delivery of the regulatory genes R [Ludwig et al. 1990] and B [Goff et al. 1990], and to demonstrate that the B-I [B-Intense] allele, which is not expressed in the seed, and the B-Peru allele, which is expressed in the seed, encode functionally equivalent B regulatory proteins [Goff et al. 1990]. The present study was undertaken to analyze the functional domains of the C1 protein.

A number of studies on yeast, invertebrate, and mammalian transcriptional activators have demonstrated that these proteins are modular in structure and that specific domains can function independently and can be repositioned within the protein or exchanged between proteins without a loss of function [Ptashne 1988; Dynan 1989]. Codelivery of C1 constructs with anthocyanin structural gene reporters was used in this study to quantitate the functional activity of various C1 constructs encoding deletion, frameshift, and fusion proteins. To obtain evidence that the amino-terminal and carboxy-terminal domains of C1 can function independently of one another, we have tested the trans-activation function of an internal C1 deletion. The C1 basic and acidic domains were also analyzed for trans-activation function in fusion constructs with the yeast transcriptional activator GAL4 basic DNA-binding and acidic transcriptional activation domains. Similar fusions with these GAL4 domains have been used to identify the transcriptional activation domain of the herpesvirus VP16 protein [Sadowski et al. 1988; Chasman et al. 1989] and specific amino acid requirements within the Drosophila bicoid DNA-recognition domain [Hanes and Brent 1989].

In addition to the analysis of the functional domains of wild-type C1, fusions between C1 and the dominant inhibitor encoded by the C1-I allele were used to analyze the changes in C1 responsible for the dominant inhibitory phenotype of C1-I. The results of these studies provide evidence that a highly conserved amino acid present in the C1 myb-like DNA-binding region, but altered in C1-I, is critical to the trans-activation function of C1 and, therefore, may be essential to the function of other myb-like proteins.

Results

Complementation of c1 aleurones and embryos

The activity of the C1 genomic subclone [pC1gen; for plasmid constructs, see Fig. 1] and the cauliflower mosaic virus 35S [CaMV 35S] promoter-expressed C1 cDNA [p35SC1] were tested by gene transfer into c1 aleurones or c1 Rscm2 embryos using high-velocity microprojectiles as described previously [Klein et al. 1989; Goff et al. 1990]. Following incubation, pigmented cells were observed in c1 aleurones receiving the C1 DNA constructs (Fig. 2) but not in c1 aleurones or c1 Rscm2 embryos receiving the control vector pM6F6 alone [data not shown]. c1 aleurones receiving plasmids with frame-shifts in the C1-coding region were also unpigmented [data not shown]. These observations demonstrate that the C1 genomic and the CaMV 35S C1 cDNA constructs used in this study encode functional proteins able to complement the c1 regulatory mutation following delivery by microprojectile bombardment.

Trans-activation of the maize Bz1 promoter by C1 constructs

To quantify the activation of anthocyanin structural gene promoters by the transferred C1 constructs, a reporter plasmid carrying the Bz1 promoter driving the synthesis of firefly luciferase [pBz1Luc; Fig. 1] was codelivered with either pC1gen or p35SC1. An internal control plasmid expressing chloramphenicol acetyltransferase [CAT] from the alcohol dehydrogenase 1 promoter [pAdhCAT; Fig. 1] was included in each bombardment as a control for the efficiency of DNA transfer. Within the limits of detection, the activity of the Adhl promoter is not influenced by anthocyanin regulatory loci [data not shown]. The activation of the pBz1Luc reporter by the C1 effector plasmids is therefore expressed as a ratio of luciferase to CAT activity. Homogenates of c1 aleurones or c1 Rscm2 embryos receiving the pBz1Luc reporter plasmid without a C1 expression plasmid contained low ratios of luciferase to CAT activity, whereas tissues receiving the pBz1Luc reporter plasmid as well as a C1 effector plasmid contained, on average, 85- to >300-fold.

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Figure 1. Plasmid structures. Plasmids used in this study are not necessarily drawn to scale. Components of plasmids are described in Materials and methods. Effector plasmids encode trans-acting factors. Reporter plasmids were used to determine the activity of effector constructs. The bombardment control plasmid was used to normalize efficiency of gene delivery. Abbreviations are as follows: (Adh1 Int) Maize alcohol dehydrogenase 1 intron 1; (Nos 3') nopaline synthase 3' polyadenylation region; (35SMin) minimal cauliflower mosaic virus 35S promoter. The remaining abbreviations are described in the text.

higher ratios of luciferase to CAT activity (Table 1). Constructs with frameshift mutations in either the amino-terminal or carboxy-terminal coding region (p35SClf26 or p35SClf27, respectively; Fig. 1) were not observed to trans-activate the Bz1 promoter (Table 1). These results clearly demonstrate that the presence of the CI gene product activates the Bz1 promoter in cl aleurones and in c1 Rscm2 embryos, as expected from previous genetic studies (Dooner and Nelson 1979, Dooner 1983; Cone et al. 1986).

Complementation and trans-activation with an internal deletion of CI

To obtain evidence that the basic amino-terminal region of CI (amino acids 1–114), with homology to the DNA-binding domain of the myb oncogene products, and the carboxy-terminal acidic region (amino acids 234–273) can function as independent domains, a CI-coding sequence with an internal deletion (amino acids 117–144) was constructed and placed under control of the CaMV 35S promoter. This CI internal deletion (pCIdel3; Fig. 1) was delivered into cl aleurones and c1 Rscm2 embryos and tested for its ability to complement the c1 genotype as well as trans-activate the Bz1 reporter pBz1Luc. Following delivery of pCIdel3 to cl aleurones, purple cells were observed (Fig. 2). Likewise, codelivery of pCIdel3 with the pBz1Luc reporter into c1 Rscm2 embryos resulted in a 25-fold increase of luciferase to CAT activity relative to cells receiving control plasmids (Table 1). These results demonstrate that amino acids 117–144 of the CI protein are not essential for either trans-activation of a specific structural gene promoter nor activation of the entire anthocyanin biosynthetic pathway, although this deletion polypeptide appears to be less efficient at Bz1 promoter trans-activation than the intact CI protein (Table 1). This observation provides evidence that the CI amino-terminal basic region and the CI carboxy-terminal acidic region can function as independent domains.

Activation of a GAL4-regulated promoter by a GAL4–CI fusion protein

The carboxy-terminal acidic region of the CI protein was tested for its ability to functionally replace the transcriptional activation domain of the yeast GAL4 protein. The coding sequence for the GAL4 DNA-binding domain, amino acids 1–147 (Ma and Ptashne 1987), was fused to the coding sequence for the carboxyl terminus of CI (amino acids 173–273) and placed under CaMV 35S promoter control (pGALCl; Fig. 1). As a positive control, a plasmid encoding a fusion between the DNA-binding domain of GAL4 and the transcriptional activation domain of herpes simplex virus VP16 was constructed (pGALVP16; Fig. 1). As a negative control, a plasmid encoding only the GAL4 DNA-binding domain was also constructed (pGAIBind1; Fig. 1). pGALCl and pGALVP16 were tested for their ability to trans-activate reporter plasmids containing a minimal 35S promoter with and without GAL4 recognition sites (pGALLuc2 and p35SMin, respectively; Fig. 1). Delivery of either pGALLuc2 or p35SMin without an effector plasmid into embryogenic callus tissue resulted
Trans-activation of anthocyanin pigmentation

Figure 2. Induction of anthocyanin synthesis in maize aleurones. Maize c1 displaying purple anthocyanin-producing cells. Aleurones were bombarded with the following plasmids, described in Materials and methods, and photographed at 50x magnification: [A] C1 genomic plasmid, pC1gen. [B] An expressed C1 cDNA, p3SSC1. [C] An expressed internal deletion of the C1 cDNA, pC1del3. [D] A fusion between the C1 amino-terminal basic coding sequence at C1 amino acid 117 and the GAL4 acidic transcriptional activation domain, pC1GAL7.1. [E] A fusion between the C1 amino-terminal basic coding sequence at C1 amino acid 144 and the GAL4 acidic transcriptional activation domain, pC1GAL6.1. [F] A fusion between the C1 amino-terminal basic coding sequence at C1 amino acid 258 and the GAL4 acidic transcriptional activation domain, pC2GAL10.

in low levels of luciferase to CAT activity (Table 2). Embryogenic callus tissue has been shown to be a reliable substitute for aleurones or embryos in such trans-activation assays when the appropriate regulatory genes are transferred into these cells (Goff et al. 1990). Codelivery of p35SMin with any of the effector plasmids also resulted in low ratios of luciferase to CAT activity (Table 2). The expressed GAL4 DNA-binding domain alone was observed to induce reporter constructs with and without GAL4 DNA-binding sites approximately fivefold (Table 2), therefore, this induction is considered nonspecific. Codelivery of pGALVP16 or pGALCI with a reporter containing GAL4 DNA-recognition sites resulted in a 53- and 76-fold increase in luciferase to CAT activity, respectively (Table 2). These observations demonstrate that the C1 acidic region can function as a transcriptional activation domain when fused to a well-characterized heterologous DNA-binding domain.

Complementation and trans-activation with C1–GAL4 fusion proteins

To obtain more definitive evidence that the C1 protein functions as a transcriptional activator, a coding sequence for the transcriptional activation domain of the yeast regulatory protein GAL4 (region II, amino acids 768–881 [Ma and Ptashne 1987]) was fused to C1 at amino acids 117 [pC1GAL7.1], 144 [pC1GAL6.1], or 258 [pC1GAL10]. These constructs express fusion proteins with the putative DNA-binding region of C1 and varying lengths of the remainder of C1 (the intact C1 protein is 273 amino acids) fused to a known transcriptional activation domain (Fig. 1). Each of these constructs was delivered to c1 aleurones by high-velocity microprojectile bombardment, and each was observed to induce pigmentation of recipient cells (Fig. 2). Constructs expressing the GAL4 transcriptional activation domain fused in close proximity to the putative DNA-binding region of C1 (pC1GAL7.1 and pC1GAL6.1) resulted in proteins that stimulate only moderate pigment accumulation, whereas constructs expressing either the intact C1 protein or a fusion of the GAL4 transcriptional activation domain at amino acid 258 of C1 [pC1GAL10] stimulate intense pigmentation (Fig. 2).

Previous work has demonstrated that both an expressed C1 gene and an expressed B gene are required for pigment induction or trans-activation of the Bzl promoter in c1 r embryogenic callus (Goff et al. 1990). Codelivery of plasmids containing an expressed B gene and various C1–GAL4 fusion proteins with the Bzl reporter
plasmid pBzILuc into c1 embryos resulted in a 63- to 145-fold increase in the ratio of luciferase to CAT activity relative to cells receiving reporter and control plasmids [Table 2]. pClGAL10 was also observed to trans-activate pBzILuc >100-fold in c1 embryos (data not shown). However, both pClGAL7.1 and pClGAL6.1 trans-activated pBzILuc on the average of only 10- to 20-fold in c1 embryos [data not shown]. The cause of this variability in pBzILuc trans-activation by the different C1-GAL4 fusions in c1 embryos versus c1 embryos is not yet understood. Trans-activation of pBzILuc by these C1-GAL4 fusions remains dependent on the presence of an expressed B gene in c1 embryos (data not shown), as was the case for trans-activation of pBzILuc by wild-type C1 (Goff et al. 1990).

A frameshift mutation at amino acid 258 of the C1 coding sequence [p35SChs27] generates a C1 protein with an additional 74 amino acids fused to amino acid 258 of C1. The protein encoded by the gene with this frameshift mutation neither stimulated pigmentation [data not shown] nor trans-activated pBzILuc [Table 1].

When compared with the data presented above, these results demonstrate that the C1 carboxy-terminal acidic region is essential for C1-mediated trans-activation but can be replaced by the acidic transcriptional activation domain of a known yeast regulatory protein. The C1-GAL4 fusion construct encodes not only a functional activator of a specific anthocyanin structural gene promoter but also the entire pigmentation pathway. In addition, these C1-GAL4 fusion constructs remain dependent on the presence of an expressed B construct [data not shown].

A frameshift mutant of C1 inhibits trans-activation of the pBzILuc
A genetically identified dominant inhibitory allele of C1, designated C1-I [Emerson et al. 1935; Coe 1962], blocks anthocyanin biosynthesis in aleurones and encodes a protein with several amino acid changes. The most obvious alteration is that the C1-I protein is truncated at its carboxyl terminus and thus lacks 21 amino acids that comprise much of the acidic domain [Paz-Ares et al. 1990]. This raises the possibility that disruption of the transcriptional activation domain not only leads to a failure to trans-activate but also generates the inhibitory phenotype of C1-I. To test this possibility, the C1 construct containing a frameshift in the acidic domain [amino acid 258, p35SChs27] was codelivered with an expressed C1 construct [either p35SCh or pClgen] and the Bz1 reporter plasmid pBzILuc. Codelivery of equal amounts [by weight] of an expressed C1 construct and p35SChs27 reduced pBzILuc trans-activation by ~50% [Fig. 3]; and at higher amounts of p35SChs27, inhibition of trans-activation approached 90% [Fig. 3]. These results demonstrate that it is possible to generate a dominant inhibitor of anthocyanin structural gene transactivation by the introduction of a frameshift in the carboxy-terminal acidic transcriptional activation domain of C1. Analogous experiments using control plasmids lacking C1 [either pUC18 or pMF6] or a construct with a frameshift mutation in the coding sequence at amino acid 32 of C1 [p35SC126] did not result in a decrease in pBzILuc trans-activation [data not shown].

### Analysis of the alterations in C1-I that confer dominant inhibition

To determine the location of the amino acid change, or changes, in the C1 protein that generates the C1-I dominant inhibition, in-frame exchanges were made between the coding sequence of the C1 and C1-I cDNAs at various positions, and the resulting constructs were tested for both trans-activation and inhibition of trans-activation.

| Table 1. Trans-activation of pBzILuc by C1 constructs |
|--------------------------------------------------------|
| **Plasmids** + pBzILuc + pAdhCAT | **c1 Rscm2 embryos** | **c1 aleurones** |
| Luc/CAT | induction | Luc/CAT | induction |
| pMF6 | 0.05 ± 0.01 | 1.0 | 0.04 ± 0.01 | 1.0 |
| p35SC1 | 4.18 ± 0.94 | 85 ± 19 | 3.64 ± 0.68 | 91 ± 17 |
| pClgen | 16.6 ± 3.87 | 339 ± 79 | 13.8 ± 5.02 | 346 ± 125 |
| pC1del3 | 1.23 ± 0.06 | 25 ± 1.3 | ND | ND |
| pC1hs26 | 0.07 ± 0.03 | 1.35 ± 0.6 | 0.02 ± 0.00 | 0.40 ± 0.03 |
| pC1hs27 | 0.17 ± 0.02 | 3.44 ± 0.3 | 0.04 ± 0.00 | 0.82 ± 0.05 |

Plasmid constructs are described in Fig. 1 and were delivered to c1 embryos and to c1 aleurones by microprojectiles. Following incubation for 24–48 hr with illumination, tissues were homogenized and enzyme activities determined. Activities are expressed as a ratio of luciferase [Luc] to CAT. Independent bombardments were analyzed and data are presented as mean values of n repetitions plus or minus S.E.M. Induction values are determined as the ratio of luciferase to CAT activities to those activities observed with control plasmids. Induced activities are expressed as mean values of n repetitions plus or minus S.E.M. Induction of pBzILuc by the C1 deletion pC1del3 was not determined [ND] in c1 aleurones.
Table 2. Trans-activation of pbz1Luc by GAL4–C1 and Cl–GAL4 fusion constructs

| Plasmids | GAL4–Cl    | Cl r Embryogenic callus |
|----------|------------|-------------------------|
| + pA/CAT | Luc/CAT    | induction               |
| pMF6     | 0.42 ± 0.12| 1.0                     |
| p35SC1   | 2.14 ± 0.34| 5.1 ± 0.8               |
| pGALBind1| 2.50 ± 0.02| 1.2 ± 0.0               |
| pGALMin  | 0.73 ± 0.13| 1.7 ± 0.3               |
| pMF6     | 0.61 ± 0.05| 1.0                     |
| pGALuc2  | 2.15 ± 0.15| 3.5 ± 0.2               |
| pGALuc2  | 32.3 ± 2.8 | 52.7 ± 4.5              |
| pGALuc2  | 46.5 ± 11.7| 76 ± 19                 |
| pGALuc2  | n = 6      |                         |

Plasmid constructs are described in Fig. 1 and were delivered to Cl r callus using microprojectiles. Following incubation for 24–48 hr without illumination, tissues were homogenized and enzyme activities were determined. Activities are expressed as described in the footnote to Table 1. In GAL4–Cl constructs, plasmids delivered are those listed plus the internal control plasmid pADhCAT. In Cl–GAL4 constructs, plasmids delivered are those listed plus pbz1Luc, pA/CAT, and p35SC-Peru.

Figure 3. Inhibition of pbz1Luc trans-activation by a frameshifted construct. Inhibition of Cl-mediated trans-activation of pbz1Luc was determined by codelivery of a construct encoding a frameshift at amino acid 258 of the Cl cDNA (p35SC1fs27) with a wild-type Cl construct (either p35SC1 or pC1gen). The results of individual bombardments and enzyme assays are expressed as a ratio of firefly luciferase activity to CAT activity above the background activity of tissues receiving expression vector plasmid without an inserted coding sequence. Determination of the enzyme is as described in Materials and methods. The ratios of frameshifted construct to intact coding sequence construct are weight ratios (i.e., μg/μg). Zero (0) represents no frameshifted construct present in the bombardment.
Table 3. Trans-activation of pBzlLuc by C1-I and C1/C1-I fusion constructs

| Plasmids + pAdhCAT | pBzlLuc p35SB-Peru | c1 r Embryogenic callus | Luc/CAT | induction | wild type (%) |
|-------------------|-------------------|------------------------|--------|-----------|--------------|
| pMF6              | 0.04 ± 0.01       | 180 ± 7.5              | n = 6  | 100.0     |
| p35SC1            | 7.6 ± 0.22        | 0.95 ± 0.12            | n = 3  | 0.0       |
| p35SC1-I          | 0.04 ± 0.00       | 20.2 ± 0.14            | n = 3  | 11.2      |
| pCICl-1           | 0.85 ± 0.01       | 1.44 ± 0.11            | n = 3  | 0.8       |
| pCICl-2           | 0.06 ± 0.01       | 3.95 ± 0.49            | n = 6  | 2.2       |
| pCIClGlu<sup>101</sup> | 0.17 ± 0.02 | 180 ± 7.5              | n = 6  | 100.0     |

Plasmid constructs are described in Fig. 1 and were delivered to c1 r embryogenic callus tissue by microprojectiles. Following incubation for 24–48 hr without illumination, tissues were homogenized and enzyme activities determined. Plasmids delivered are those listed plus pBzlLuc, pAdhCAT, and p35SB-Peru. Activities are expressed as described in the footnote to Table 1. The induction is expressed as the percentage of induction observed relative to the intact C1 cDNA construct.

The single amino acid Asp<sup>101</sup> to Glu<sup>101</sup> (pCIClGlu<sup>101</sup>, Fig. 1). This single amino acid change in the C1 protein was observed to decrease trans-activation of pBzlLuc to ~2% of the wild-type level (Table 3). Reversion of this mutant coding sequence to Asp<sup>101</sup> by site-directed mutagenesis restored the wild-type level of C1-mediated trans-activation (data not shown). Although C1-mediated pBzlLuc trans-activation is decreased dramatically by this single mutation, we have not observed an inhibitory phenotype associated with this single mutation (data not shown).

Discussion

Microprojectile delivery of a C1 genomic clone or an expressed C1 cDNA into c1 R aleurone or c1 Rscm2 embryo tissues activated the anthocyanin biosynthetic pathway.

Table 4. Inhibition of pBzlLuc trans-activation by C1-I and C1/C1-I fusion constructs

| Plasmids + pAdhCAT | pBzlLuc p35SB-Peru | c1 r Embryogenic callus | Luc/CAT | induction | Wild type (%) |
|-------------------|-------------------|------------------------|--------|-----------|--------------|
| pMF6              | 0.03 ± 0.01       | 63.9 ± 6.8             | n = 6  | 100.0     |
| pMF6              | 2.05 ± 0.22       | 2.7 ± 0.51             | n = 6  | 4.2       |
| p35SC1            | 0.99 ± 0.02       | 0.79 ± 0.14            | n = 6  | 38.8      |
| p35SC1fs27        | 0.04 ± 0.01       | 11.5 ± 1.00            | n = 6  | 100.0     |
| pMF6              | 11.5 ± 1.00       | 279 ± 23.8             | n = 6  | 100.0     |
| p35SC1            | 4.7 ± 0.33        | 110 ± 7.9              | n = 6  | 39.4      |
| p35SC1            | 0.02 ± 0.01       | 106 ± 17.4             | n = 3  | 15.1      |
| pMF6              | 1.70 ± 0.28       | 106 ± 17.4             | n = 6  | 100.0     |
| pCICl-1           | 0.26 ± 0.04       | 16.0 ± 2.4             | n = 6  | 15.1      |
| pCIClgen          | 0.26 ± 0.04       | 16.0 ± 2.4             | n = 6  | 15.1      |

Plasmid constructs are described in Fig. 1 and were delivered to c1 r embryogenic callus tissue by microprojectiles. Following incubation for 24–48 hr without illumination, tissues were homogenized and enzyme activities determined. Activities are expressed as described in the legend to Table 1. Plasmids delivered are those listed plus pBzlLuc, pAdhCAT, and p35SB-Peru. The induction is expressed as the percentage of induction observed relative to the intact C1 cDNA construct, divided into sections to represent values obtained in individually controlled experiments.
pathway and resulted in purple pigmented cells. This complementation of the c1 mutation by the cloned C1 gene and expressed C1 cDNA confirms the identity of these constructs as functional C1 regulatory genes. These, and previously published results using the R [Ludwig et al. 1990] or B [Goff et al. 1990] anthocyanin gene regulatory genes, demonstrate that a complex biosynthetic pathway can be activated by microprojectile delivery of the appropriate regulatory genes. The introduced C1 gene functions in aleurones, embryos and, as described previously, embryogenic callus [Goff et al. 1990]. Codelivery of an expressed C1 gene and a BzI reporter construct resulted in the activation of pBzILuc. This quantitative trans-activation assay allows for more detailed studies on the structure and function of plant regulatory gene products.

An internal deletion of the C1 protein that does not remove any of the amino-terminal basic region or the carboxy-terminal acidic region trans-activated pBzILuc and stimulated anthocyanin synthesis. Such deletion analysis has shown previously that up to 80% of either of the yeast regulatory proteins GAL4 or GCN4 can be eliminated without a total loss of regulatory function [Hope and Struhl 1986; Ma and Ptashne 1987]. The internal deletion of C1 used in this study demonstrates that amino acids 117–144 are not crucial for C1 trans-activation function and provides evidence that the amino-terminal basic region and the carboxy-terminal acidic region can function as independent domains.

A fusion containing the DNA-binding domain of GAL4 and the acidic carboxy-terminal domain of C1 [pGALC1] was found to stimulate transcription of a minimal CaMV 35S promoter containing GAL4 DNA recognition sites. An expressed GAL4 DNA-binding domain alone [pGALBind1] did not activate this promoter, and neither pGALC1 nor pGALBind1 activated a minimal CaMV 35S promoter lacking GAL4 DNA recognition sites. These results demonstrate that the C1 acidic region can function as a transcriptional activation domain when directed to a target promoter by the yeast GAL4 DNA-binding domain. Many types of transcriptional activation domains have been identified [Ptashne 1988]. Domains rich in glutamine, proline, and acidic amino acid residues are commonly found in regulatory proteins [Ptashne 1988; Brendel and Karlin 1989]. Acidic transcriptional activation domains have no apparent rigid amino acid sequence requirements but are high in negative charge and capable of forming amphipathic helices [Ginger and Ptashne 1987; Ma and Ptashne 1987]. The sequence of the acidic region of C1 is proposed to be capable of forming an amphipathic helix between residues 246 and 260 [Paz-Ares et al. 1990], in agreement with the transcriptional activation properties of the C1 protein.

Fusion proteins containing the myb-homologous basic domain of C1 and the transcriptional activation domain of yeast GAL4 [region II; Ma and Ptashne 1987] were found to stimulate transcription from pBzILuc and activate anthocyanin biosynthesis. These observations demonstrate that the acidic region of C1 can be replaced by an acidic transcriptional activation domain of a well-studied transcriptional regulator. These results, together with the results discussed above, provide in vivo evidence that the product of the C1 gene functions as a transcriptional activator that utilizes a myb-like DNA-binding domain with an acidic transcriptional activation domain. The basic domain of myb oncogene products consists of three imperfect repeats of 51–52 amino acids [Gerondakis and Bishop 1986; Rosson and Reddy 1986], and this domain has been shown to bind both specifically and nonspecifically to DNA [Oehler et al. 1990]. Deletion analysis of the myb DNA-binding region was used to demonstrate that the first repeat [missing from the maize C1 protein] is not essential for DNA binding, whereas the second and third repeats are crucial for specific site recognition [Howe et al. 1990; Oehler et al. 1990]. Several myb oncogene products have been reported to function as transcriptional activators that act through specific DNA-binding sites [Ness et al. 1989; Nishina et al. 1989; Sakura et al. 1989; Ibanez and Lipsick 1990]. It is noteworthy that a BzI promoter mutation in a 6-bp region with homology to the myb consensus DNA-binding site has recently been demonstrated to decrease expression of the BzI promoter in C1 R maize embryos (B.A. Roth and M.E. Fromm, unpubl.). Multimers of the myb-consensus binding site [together with a putative binding site for R or B] placed upstream of an inactive CaMV 35S core promoter, confer C1 and B inducibility to this promoter (S.A. Goff and M.E. Fromm, unpubl.). Together, these data strongly suggest that C1-regulated expression of the BzI promoter is mediated through a myb consensus DNA-binding site.

Fusions between the wild-type C1 allele and the dominant inhibitor C1-I allele were used to analyze some of the numerous amino acid changes present in C1-I. Results from these experiments demonstrate that changes in both the amino-terminal basic and carboxy-terminal acidic domains of C1 can decrease or eliminate the trans-activation function of the resulting gene products. As expected from genetic studies, the intact C1-I protein fails to activate pBzILuc and is a potent inhibitor of C1-mediated pBzILuc trans-activation. A fusion between C1-1–220 and C1-I 221–252 [pC1CI-I], and a frameshift within the C1 acidic region at amino acid 258 [p35SClfs27] both encode proteins with altered transcriptional activation domains. Like C1-I, both of these constructs failed to trans-activate pBzILuc efficiently. Lack of trans-activation by these constructs provides further evidence that the intact C1 acidic region is essential for efficient transcriptional activation. Both the frameshift (p35SClfs27) and the C1–Cl fusion [pC1CI-I] encode proteins that weakly inhibit C1-mediated pBzILuc trans-activation relative to the potent inhibitor encoded by C1-I. The C1-I protein and these altered C1 proteins could inhibit pBzILuc trans-activation via one of several different mechanisms. They do not simply block wild-type C1 gene expression since they inhibit CaMV 35S-expressed C1 products, and C1/C1-I cells contain both C1 and C1-I transcripts [Paz-Ares et al. 1990].
of a CI DNA-binding domain lacking a transcriptional activation domain could sterically block the function of the intact protein, as demonstrated for both GCN4 and GAL4 DNA-binding domains synthesized without activation domains (for review, see Struhl 1989), and for the *Escherichia coli* lac repressor when a repressor binding site is located within a heterologous promoter (Deuschle et al. 1990). Alternatively, if the CI protein normally functions as a subunit in a heterodimer, the CI-I, pCI1-1, or p35SClfs27-encoded proteins could block the wild-type protein or protein complex by the formation of mixed dimers (Herskowitz 1987). The predicted polypeptide encoded by p35SClfs27 contains a basic carboxyl terminus (net charge of +3) rather than the wild-type acidic domain (net charge of −9). It is also possible, therefore, that this basic carboxyl terminus directly disrupts essential components of the transcriptional machinery. Further studies of these proteins are necessary to distinguish between the possible modes of inhibition.

A fusion between CI-1 1–142 and CI 143–273 (pCIC1-2) contains a conservative substitution within the myb homology domain (Asp^{101} to Glu^{101}) and a deletion of amino acids Ala^{122} to Glu^{123} (Paz-Ares et al. 1990), and trans-activate pBziLuc at ~1% the level of the wild-type protein. This construct was shown to inhibit trans-activation of pBziLuc by intact CI, although not to the extent of the intact CI-I protein. Together with the results described above, these observations provide support for the interpretation that the inhibitory phenotype of CI-I results from the additive effects of changes within different domains of the protein. Mutations within both the basic DNA-binding and acidic transcriptional activation domains decrease pBziLuc trans-activation and contribute to CI-I-mediated inhibition. Such a model is consistent with the genetic evidence that reversion of the CI-I allele to the wild-type CI allele occurs extremely infrequently if ever (Coe 1962) and also with the numerous sequence differences found between CI-I and CI (Paz-Ares et al. 1990). Together, the available evidence suggests that the dominant inhibitory CI-I allele arose by multiple events that alone only partially eliminated CI trans-activation or generated CI-I inhibition.

Site-directed mutagenesis of CI Asp^{101} to Glu^{101} (pCIGlu^{101}) decreased pBziLuc trans-activation to ~2% of the wild-type level. This result shows that a single conservative amino acid substitution in the DNA-binding domain of CI can have very dramatic effects on transcriptional activation function. It is possible that this conservative amino acid change decreases CI DNA binding, however, the CI-I protein (with Glu^{101}) is believed to bind DNA (cited in Paz-Ares et al. 1990). This Asp residue is conserved evolutionarily between the various members of the myb oncogene family (Bergmann et al. 1981; Klempnauer et al. 1982, 1986; Gonda et al. 1985; Boyle et al. 1986; Shen-Ong et al. 1986; Peter et al. 1987) and may occupy a position in the DNA-recognition helix (Ohlendorf et al. 1982; Lehming et al. 1987; Aggarwal et al. 1988, Jordan and Pabo 1988; Otting et al. 1988). This conservation suggests that an analogous change in any of the various mammalian or insect myb-homologous domains could also decrease the transcriptional activation function of these proteins dramatically. Detailed studies on the structure of the pCIGlu^{101} DNA-binding domain could provide insight into the mechanism of myb-mediated transcriptional activation. Single amino acid substitutions within the helix-turn-helix DNA-binding domain of bacteriophage λ repressor have been shown to dramatically decrease transcriptional activation (Bushman et al. 1989), possibly by alteration of an RNA polymerase direct contact point. Specific amino acid residues located within the MyoD1 DNA-binding domain are also believed to be critical for transcriptional activation function (Davis et al. 1990). Unlike pCIC1-2, however, pCIGlu^{101} was not observed to inhibit pBziLuc trans-activation by intact CI. This result suggests that the inhibitory property of pCIC1-2 results from either the Ala^{122} to Gly^{123} deletion adjacent to the myb-homologous region or from the combination of this deletion and the Asp^{101} to Glu^{101} substitution.

Although there is no definitive evidence that the products of CI and B/R act directly on anthocyanin structural gene promoters, the results to date strongly suggest such a mode of action. It is noteworthy that the CI transcriptional activation domain can activate transcription of a GAL4-regulated promoter independent of the B or R gene products when fused to the GAL4 DNA-binding domain. Mounting evidence suggests that transcriptional activation domains may function by contact with additional proteins, designated coactivators or adaptors (Berger et al. 1990, Kelleher et al. 1990, Liu and Green 1990, Pugh and Tjian 1990), or by direct contact with the TATA-binding factor, TFIIID (Stringer et al. 1990). The CI DNA-binding domain fused to the transcriptional activation domain of GAL4 remains dependent on an expressed B gene to achieve trans-activation of pBziLuc, as does CI. Neither CI, CI–GAL4, nor B/R alone can activate pBziLuc, suggesting that a synergistic interaction of these regulatory proteins may be necessary to achieve anthocyanin structural gene promoter activity. Interactions between the rat glucocorticoid receptor and GAL4 derivatives (Kakidani and Ptashne 1988), or the mammalian transcriptional activator ATF and GAL4 derivatives have been described recently to synergistically activate their respective target promoters (Carey et al. 1990, Lin et al. 1990). Likewise, the *BAS1* gene of yeast, which contains a myb-like DNA-binding domain, requires an additional protein encoded by *BAS2* to activate *HIS* biosynthetic genes (Tice-Baldwin et al. 1989). Both an expressed CI [or CI–GAL4 fusion] and B gene are required for activity of a minimal CaMV 35S target promoter with a synthetic trimer of a myb and myc consensus DNA-binding site (B.A. Roth, S.A. Goff, and M.E. Fromm, in prep.). The results of this study demonstrate that if an interaction between CI and B/R is required for pBziLuc trans-activation, the interacting region in CI must be localized to the amino terminus. Alternatively, additional adaptor or coactivator proteins may recognize R/B and the amino terminus of CI. Although B and R have been shown to encode proteins with a potential helix–
loop–helix dimerization motif, it is not likely that R/B proteins use this motif to interact with CI, since the CI protein has not been found to contain such a motif. In vitro analysis of the interactions between the CI and R/B proteins and DNA may clarify the nature of their mutual dependence.

Materials and methods

Plant materials

Aleurones and embryos were isolated from c1 or c1 Rscm2 immature kernels as described previously [Klein et al. 1989]. An immature ear of A188 crossed with B73 was used as the source of 1-mm embryos for initiating the embryogenic callus as described [Kamo and Hodges 1986]. Embryogenic callus tissue was prepared for gene transfer by spreading a thin “lawn” of tissue in a circular area (3-cm diameter) on agarose plates containing N6 media [Lowe et al. 1985].

Plasmids

Structures of all plasmid inserts used in this study are presented in Figure 1. The pMF6 expression vector consists of the CaMV 35S promoter [0.5 kb], the Adh1 first intron [0.5 kb], and the nopaline synthase [nos] polyadenylation region [0.25 kb] as described previously [Callis et al. 1987], with additional restriction sites between the Adh1 intron and nos fragments. The CI cDNA [1.1 kb] was isolated from a ϕX174 library prepared from poly[A]⁺-selected mRNA from color-converted W22 (Brink et al. 1988) and contains ~8 kb of genomic DNA harbor-
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