Identification of functional domains in the maize transcriptional activator C1: comparison of wild-type and dominant inhibitor proteins

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Genes encoding fusions between the maize regulatory protein C1 and the yeast transcriptional activator GAL4 and mutant C1 proteins were assayed for their ability to *trans*-activate anthocyanin biosynthetic genes in intact maize tissues. The putative DNA-binding region of C1 fused to the transcriptional activation domain of GAL4 activated transcription of anthocyanin structural gene promoters in *c1* aleurones, *c1 Rscm2* embryos, and *c1 r* embryogenic callus. Cells receiving these constructs accumulated purple anthocyanin pigments. The C1 acidic region fused to the GAL4 DNA-binding domain activated transcription of a GAL4-regulated promoter. An internal deletion of C1 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 C1 cDNA and the dominant inhibitor allele C1-I cDNA were used to identify the amino acid changes in C1 responsible for the C1-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]

Received October 16, 1990; revised version accepted December 13, 1990.

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). *C1* 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). *c1* (colorless) mutants have 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 *C1* (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 *C1* 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 C1 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 *c1* alleles, a dominant inhibitory allele *C1-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 quantify 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 pMF6 alone (data not shown). *c1* aleurones receiving plasmids with frameshifts 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 I 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 AdhI 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

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 (p35SC1fs26 or p35SC1fs27, respectively; Fig. 1) were not observed to

trans-activate the *Bz1* promoter (Table 1). These results clearly demonstrate that the presence of the C1 gene product activates the *Bz1* promoter in *c1* 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 C1

To obtain evidence that the basic amino-terminal region of C1 (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 C1-coding sequence with an internal deletion (amino acids 117–144) was constructed and placed under control of the CaMV 35S promoter. This C1 internal deletion (pC1del3; Fig. 1) was delivered into *c1* 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 pC1del3 to *c1* aleurones, purple cells were observed (Fig. 2). Likewise, codelivery of pC1del3 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 C1 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 C1 protein (Table 1). This observation provides evidence that the C1 amino-terminal basic region and the C1 carboxy-terminal acidic region can function as independent domains.

Activation of a GAL4-regulated promoter by a GAL4–C1 fusion protein

The carboxy-terminal acidic region of the C1 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 C1 (amino acids 173–273) and placed under CaMV 35S promoter control (pGALC1; 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 (pGALBind1; Fig. 1). pGALC1 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

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 50 $\times$ magnification: (A) *C1* genomic plasmid, pC1gen. (B) An expressed *C1* cDNA, p35SC1. (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 pGALC1 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 *Bz1* 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 *Bz1* reporter

Table 1. Trans-activation of pBz1Luc by C1 constructs

Plasmids + pBz1Luc + pAdhCAT	<i>c1 Rscm2</i> embryos		<i>c1</i> aleurones	
	Luc/CAT	induction	Luc/CAT	induction
pMF6	0.05 ± 0.01 <i>n</i> = 12	1.0	0.04 ± 0.01 <i>n</i> = 12	1.0
p35SC1	4.18 ± 0.94 <i>n</i> = 12	85 ± 19	3.64 ± 0.68 <i>n</i> = 9	91 ± 17
pC1gen	16.6 ± 3.87 <i>n</i> = 6	339 ± 79	13.8 ± 5.02 <i>n</i> = 6	346 ± 125
pC1del3	1.23 ± 0.06 <i>n</i> = 3	25 ± 1.3	ND	ND
pC1fs26	0.07 ± 0.03 <i>n</i> = 3	1.35 ± 0.6	0.02 ± 0.00 <i>n</i> = 3	0.40 ± 0.03
pC1fs27	0.17 ± 0.02 <i>n</i> = 3	3.44 ± 0.3	0.04 ± 0.00 <i>n</i> = 3	0.82 ± 0.05

Plasmid constructs are described in Fig. 1 and were delivered to *c1 Rscm* 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 firefly 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 pBz1Luc by the *C1* deletion pC1del3 was not determined (ND) in *c1* aleurones.

plasmid pBz1Luc into *c1 r* embryogenic callus cells 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). pC1GAL10 was also observed to *trans*-activate pBz1Luc >100-fold in *c1* aleurones (data not shown). However, both pC1GAL7.1 and pC1GAL6.1 *trans*-activated pBz1Luc on the average of only 10- to 20-fold in *c1* aleurones (data not shown). The cause of this variability in pBz1Luc *trans*-activation by the different C1–GAL4 fusions in *c1* aleurones versus *c1 r* embryogenic callus is not yet understood. *Trans*-activation of pBz1Luc by these C1–GAL4 fusions remains dependent on the presence of an expressed *B* gene in *c1 r* embryogenic callus (data not shown), as was the case for *trans*-activation of pBz1Luc by wild-type *C1* (Goff et al. 1990).

A frameshift mutation at amino acid 258 of the *C1* coding sequence (p35SC1fs27) 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 pBz1Luc (Table 1). Taken together 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 pBz1Luc

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, p35SC1fs27) was codelivered with an expressed *C1* construct (either p35SC1 or pC1gen) and the *Bz1* reporter plasmid pBz1Luc. Codelivery of equal amounts (by weight) of an expressed *C1* construct and p35SC1fs27 reduced pBz1Luc *trans*-activation by ~50% (Fig. 3); and at higher amounts of p35SC1fs27, 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 pBz1Luc *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*-activa-

Table 2. Trans-activation of pBz1Luc by GAL4-C1 and C1-GAL4 fusion constructs

Plasmids + pAdhCAT	GAL4-C1 <i>c1 r</i> Embryogenic callus	
	Luc/CAT	induction
pMF6	0.42 ± 0.12	1.0
p35SMin	<i>n</i> = 6	
pGALBind1	2.14 ± 0.34	5.1 ± 0.8
p35SMin	<i>n</i> = 6	
pGALVP16	0.50 ± 0.02	1.2 ± 0.0
p35SMin	<i>n</i> = 6	
pGALC1	0.73 ± 0.13	1.7 ± 0.3
p35SMin	<i>n</i> = 6	
pMF6	0.61 ± 0.05	1.0
pGALLuc2	<i>n</i> = 6	
pGALBind1	2.15 ± 0.15	3.5 ± 0.2
pGALLuc2	<i>n</i> = 6	
pGALVP16	32.3 ± 2.8	52.7 ± 4.5
pGALLuc2	<i>n</i> = 6	
pGALC1	46.5 ± 11.7	76 ± 19
pGALLuc2	<i>n</i> = 6	
C1-GAL4		
Plasmids + pBz1Luc + pAdhCAT p35SBPeru	<i>c1 r</i> Embryogenic callus	
	Luc/CAT	induction
pMF6	0.03 ± 0.01	1.0
	<i>n</i> = 6	
p35SC1	2.8 ± 0.3	93 ± 9
	<i>n</i> = 6	
pC1GAL7.1	1.9 ± 0.4	63 ± 13
	<i>n</i> = 6	
pC1GAL6.1	2.4 ± 0.2	80 ± 7
	<i>n</i> = 6	
pC1GAL10	4.3 ± 0.9	145 ± 30
	<i>n</i> = 6	

Plasmid constructs are described in Fig. 1 and were delivered to *c1r* embryogenic callus tissue by 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-C1 constructs, plasmids delivered are those listed plus the internal control plasmid pAdhCAT. In C1-GAL4 constructs, plasmids delivered are those listed plus pBz1Luc, pAdhCAT, and p35SB-Peru.

tion of pBz1Luc. The expressed intact C1-I protein (p35SC1-I; Fig. 1) fails to trans-activate pBz1Luc (Table 3), and inhibits C1-mediated pBz1Luc trans-activation by as much as 95% (Table 4). A construct encoding amino acids 1–220 of C1 fused to the corresponding carboxy-terminal segment of C1-I (amino acids 221–252, pC1CI-1; Fig. 1) encodes a protein with amino acid substitutions at position 228 (P for L) and 249–252 (SWTT for DVRA) and terminates following amino acid 252 rather than at the wild-type position following amino acid 273 (Paz-Ares et al. 1990). pC1CI-1 was observed to

trans-activate pBz1Luc at only 11% of the wild-type C1 level (Table 3) and was not observed to generate anthocyanin-producing cells (data not shown). Codelivery of pC1CI-1 with intact C1 resulted in 60% inhibition of pBz1Luc trans-activation (Table 4).

A complementary fusion construct between amino acids 1 and 142 of C1-I and amino acids 143 and 273 of C1 (pC1CI-2; Fig. 1) has two alterations relative to C1. Amino acid Asp¹⁰¹ within the basic *myb* homology domain is changed to Glu¹⁰¹, and amino acids Ala¹²²–Gly¹²³ are deleted (Paz-Ares et al. 1990). pC1CI-2 trans-activated pBz1Luc at <1% of the wild-type C1 level (Table 3) and inhibited C1-mediated trans-activation of pBz1Luc by ~85% (Table 4). These results demonstrate that changes within both the basic DNA-binding domain and the acidic transcriptional activation domain can block the trans-activation function of C1. In addition, they demonstrate that the inhibitory property of C1-I is not due to a single change within one functional domain of the C1 protein but, rather, that the C1-I dominant inhibitor phenotype is most likely due to an additive effect of multiple alterations.

To test the possibility that the single conservative change within the basic domain with *myb* homology is partially responsible for the inhibitory phenotype of C1-I, site-directed mutagenesis was used to change C1

Figure 3. Inhibition of pBz1Luc trans-activation by a frame-shifted construct. Inhibition of C1-mediated trans-activation of pBz1Luc was determined by codelivery of a construct encoding a frameshift at amino acid 258 of the C1 cDNA (p35SC1fs27) with a wild-type C1 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., $\mu\text{g}/\mu\text{g}$). Zero (0) represents no frameshifted construct present in the bombardment.

Table 3. Trans-activation of pBz1Luc by C1-I and C1/C1-I fusion constructs

Plasmids + pAdhCAT pBz1Luc p35SB-Peru	c1 r Embryogenic callus		
	Luc/CAT	induction	wild type (%)
pMF6	0.04 ± 0.01 n = 6		
p35SC1	7.6 ± 0.22 n = 3	180 ± 7.5	100.0
p35SC1-I	0.04 ± 0.00 n = 3	0.95 ± 0.12	0.0
pC1CI-1	0.85 ± 0.01 n = 3	20.2 ± 0.14	11.2
pC1CI-2	0.06 ± 0.01 n = 3	1.44 ± 0.11	0.8
pC1Glu ¹⁰¹	0.17 ± 0.02 n = 6	3.95 ± 0.49	2.2

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 pBz1Luc, 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.

amino acid Asp¹⁰¹ to Glu¹⁰¹ (pC1Glu¹⁰¹; Fig. 1). This single amino acid change in the C1 protein was observed to decrease trans-activation of pBz1Luc to ~2% of the wild-type level (Table 3). Reversion of this mutant coding sequence to Asp¹⁰¹ by site-directed mutagenesis restored the wild-type level of C1-mediated trans-activation (data not shown). Although C1-mediated pBz1Luc 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

Table 4. Inhibition of pBz1Luc trans-activation by C1-I and C1/C1-I fusion constructs

Plasmids + pAdhCAT pBz1Luc p35SB-Peru	Ratio	c1 r Embryogenic callus		
		Luc/CAT	induction	Wild type (%)
pMF6		0.03 ± 0.01 n = 6		
pMF6	3	2.05 ± 0.22 n = 6	63.9 ± 6.8	100.0
pC1gen	1			
p35SC1-I	3	0.09 ± 0.02 n = 6	2.7 ± 0.51	4.2
pC1gen	1			
p35SC1fs27	3	0.79 ± 0.14 n = 6	24.8 ± 4.2	38.8
pC1gen	1			
pMF6		0.04 ± 0.01 n = 6		
pMF6	3	11.5 ± 1.00 n = 6	279 ± 23.8	100.0
p35SC1	1			
pC1CI-1	3	4.7 ± 0.33 n = 6	110 ± 7.9	39.4
p35SC1	1			
pMF6		0.02 ± 0.01 n = 3		
pMF6	3	1.70 ± 0.28 n = 6	106 ± 17.4	100.0
pC1gen	1			
pC1CI-2	3	0.26 ± 0.04 n = 6	16.0 ± 2.4	15.1
pC1gen	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 pBz1Luc, 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 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 *Bz1* reporter construct resulted in the activation of *pBz1Luc*. 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 *pBz1Luc* 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 (Giniger 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 *pBz1Luc* 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 *Bz1* 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 *Bz1* 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 *Bz1* 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 *pBz1Luc* and is a potent inhibitor of *C1*-mediated *pBz1Luc trans*-activation. A fusion between *C1* 1–220 and *C1-I* 221–252 (*pC1C1-I*), and a frameshift within the *C1* acidic region at amino acid 258 (*p35SC1fs27*) both encode proteins with altered transcriptional activation domains. Like *C1-I*, both of these constructs failed to *trans*-activate *pBz1Luc* 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 (*p35SC1fs27*) and the *C1-C1* fusion (*pC1C1-I*) encode proteins that weakly inhibit *C1*-mediated *pBz1Luc trans*-activation relative to the potent inhibitor encoded by *C1-I*.

The *C1-I* protein and these altered *C1* proteins could inhibit *pBz1Luc 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). Expression

of a C1 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 C1 protein normally functions as a subunit in a heterodimer, the C1-I-, pC1C1-1-, or p35SC1fs27-encoded proteins could block the wild-type protein or protein complex by the formation of mixed dimers [Herskowitz 1987]. The predicted polypeptide encoded by p35SC1fs27 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 C1-I 1-142 and C1 143-273 (pC1C1-2) contains a conservative substitution within the *myb* homology domain (Asp¹⁰¹ to Glu¹⁰¹) and a deletion of amino acids Ala¹²² to Glu¹²³ [Paz-Ares et al. 1990], and *trans*-activates pBz1Luc at ~1% the level of the wild-type protein. This construct was shown to inhibit *trans*-activation of pBz1Luc by intact C1, although not to the extent of the intact C1-I protein. Together with the results described above, these observations provide support for the interpretation that the inhibitory phenotype of C1-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 pBz1Luc *trans*-activation and contribute to C1-I-mediated inhibition. Such a model is consistent with the genetic evidence that reversion of the C1-I allele to the wild-type C1 allele occurs extremely infrequently if ever [Coe 1962] and also with the numerous sequence differences found between C1-I and C1 [Paz-Ares et al. 1990]. Together, the available evidence suggests that the dominant inhibitory C1-I allele arose by multiple events that alone only partially eliminated C1 *trans*-activation or generated C1-I inhibition.

Site-directed mutagenesis of C1 Asp¹⁰¹ to Glu¹⁰¹ (pC1Glu¹⁰¹) decreased pBz1Luc *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 C1 can have very dramatic effects on transcriptional activation function. It is possible that this conservative amino acid change decreases C1 DNA binding; however, the C1-I protein (with Glu¹⁰¹) 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 pC1Glu¹⁰¹ 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 pC1C1-2, however, pC1Glu¹⁰¹ was not observed to inhibit pBz1Luc *trans*-activation by intact C1. This result suggests that the inhibitory property of pC1C1-2 results from either the Ala¹²² to Gly¹²³ deletion adjacent to the *myb*-homologous region or from the combination of this deletion and the Asp¹⁰¹ to Glu¹⁰¹ substitution.

Although there is no definitive evidence that the products of C1 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 C1 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, TFIID [Stringer et al. 1990]. The C1 DNA-binding domain fused to the transcriptional activation domain of GAL4 remains dependent on an expressed B gene to achieve *trans*-activation of pBz1Luc, as does C1. Neither C1, C1-GAL4, nor B/R alone can activate pBz1Luc, 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 C1 (or C1-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 C1 and B/R is required for pBz1Luc *trans*-activation, the interacting region in C1 must be localized to the amino terminus. Alternatively, additional adaptor or coactivator proteins may recognize R/B and the amino terminus of C1. 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 C1, since the C1 protein has not been found to contain such a motif. In vitro analysis of the interactions between the C1 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 *C1* cDNA (1.1 kb) was isolated from a λ gt11 library prepared from poly(A)⁺-selected mRNA from color-converted W22 (Brink derivation) kernels 30 days postpollination (K.C. Cone, unpubl.). The *C1-I* cDNA was isolated from a λ gt11 library (K.C. Cone, unpubl.) prepared from homozygous *C1-I* kernels (Coe 1962). The *C1* genomic clone (pC1gen) used was described previously (Cone et al. 1988) and contains ~8 kb of genomic DNA harboring the *C1* promoter, introns, and coding sequence. The *Bz1* promoter-luciferase (pBz1Luc) plasmid was described previously (Klein et al. 1989). The *Adh1* promoter/chloramphenicol acetyltransferase plasmid (pAdhCAT) used in this study was described previously as pA11CN (Callis et al. 1987). The frame-shifts in *C1* to generate p35SC1fs26 and p35SC1fs27 were constructed by filling in restriction sites located at the coding region for amino acids 32 (*NcoI*) and 258 (*XhoI*), respectively. The fusions of the GAL4 transcriptional activation domain to the *C1* cDNA to generate pC1GAL7.1, pC1GAL6.1, and pC1GAL10 were at the restriction endonuclease sites at positions 117 (*NarI*), 144 (*NarI*), and 258 (*XhoI*) of the *C1*-coding sequence, respectively. pC1del3 was deleted between the *C1*-coding region for amino acids 117 (*NarI*) to 144 (*NarI*). The expressed GAL4 DNA-binding region contains the coding sequence for amino acids 1–147 of GAL4 (to *ClaI*) inserted into the expression vector pMF6. The GAL4 DNA-binding region fused to the acidic region of *C1* contains coding sequence for amino acids 1–147 of GAL4 fused to coding sequence for amino acids 173–273 of *C1* (*ClaI* to *NarI* ligation). The expressed *B-Peru* cDNA was described previously (Goff et al. 1990). The GAL4 DNA-binding domain fused to the herpes simplex virus VP16 transcriptional activation domain (pGALVP16) contains the coding sequence for amino acids 1–147 of GAL4 fused to a coding sequence for 80 amino acids of VP16. The VP16 transcriptional activation domain was kindly provided by S. Triezenberg (Triezenberg et al. 1988). The expressed *C1-I* cDNA (p35SC1-I) contains a 1.0-kb cDNA with the entire coding region inserted into the pMF6 expression vector. Fusions between the *C1* and *C1-I* cDNAs were used to generate pC1C1-1 (*C1* 1–220, *EcoRI*–

PstI fused to *C1-I* 221–252, *PstI*–*EcoRI*) and pC1C1-2 (*C1-I* 1–142, *EcoRI*–*AatII* fused to *C1* 143–273, *AatII*–*EcoRI*), and contain the 3'- and 5'-untranslated regions of the respective *C1* and *C1-I* cDNAs. pC1Glu¹⁰¹ was generated by site-directed mutagenesis (Kunkel et al. 1987) of the wild-type *C1* cDNA and codes for glutamic acid at position 101 rather than the wild-type asparagine. The minimal 35S reporter and 35S reporter containing GAL4 recognition sites (Giniger et al. 1985) were constructed from pMA556 and pMA558 (Ma et al. 1988) by ligation of the vector and CaMV 35S promoter region (*PstI*–*HindIII*) of pMA556 (without GAL4 recognition sites) and pMA558 (with ~10 GAL4 recognition sequences) to a *PstI*–*SacI* *Adh* intron-luciferase fragment and a *SacI*–*HindIII* *nos* 3' end fragment. All inserts are carried in plasmid vectors derived from pUC. Plasmids transferred into cells were isolated by equilibrium ultracentrifugation.

High-velocity microprojectile bombardment

Plasmid DNA was delivered to intact tissues by high-velocity microprojectiles using the Biolistics device as described (Klein et al. 1988, 1989). A total of 8 μ g of plasmid DNA (at 1.0 mg/ml) was precipitated onto microprojectiles by the addition of 25 μ l of 50 mg/ml 1.0 μ m tungsten, followed by the addition of 25 μ l of 1.0 M CaCl₂ and 10 μ l 100 mM spermidine free base. Microprojectiles were allowed to settle for ~5 min at room temperature, and 25 μ l of overlying solution was removed. As an alternate procedure, a fivefold increase in all components was used, and 300 μ l of the supernatant was removed. The resulting microprojectile solution was vortexed, and 2.5 μ l of this slurry was loaded onto each macroprojectile for bombardment. Tissues were analyzed after 24–48 hr incubation at 24°C. Aleurones and embryos were incubated with illumination, and embryogenic callus was incubated without illumination (see table footnotes).

Enzyme assays

In each experiment, the *c1 R* aleurones or *c1 Rscm* embryos were bombarded with a mixture of the appropriate plasmid DNAs and incubated under illumination for 24–48 hr before assaying for luciferase and CAT activity. Anthocyanin biosynthesis was determined by visual detection of pigmented cells using 20- to 50-fold magnification under a Zeiss dissecting microscope. Photographs were also taken at the same magnification using Kodak tungsten film (ASA 160) developed by standard procedures. Luciferase was assayed in tissue extracts as described previously (Callis et al. 1987). Luciferase activity is expressed as light units detected by an Analytical Luminescence model 2001 Luminometer for 10 sec at 25°C. CAT activity was determined by the conversion of ¹⁴C-labeled acetyl-CoA to ethylacetate soluble counts per minute for 1 hr at 37°C and counted in EcoScint scintillation fluor as described previously (Sleigh 1988) (CAT activity is expressed as cpm).

Acknowledgments

We thank Bradley A. Roth and Theodore M. Klein for assistance in this work and for plasmid constructs. We are grateful to Vicki L. Chandler, Jun Ma, Mark Ptashne, David Ow, and Steven J. Triezenberg for various plasmid constructs. We thank Jutta A. Tuerck, Sheila McCormick, David Twell, and Brenda Lowe for critical reading of the manuscript. We also thank E.I. DuPont Medical Products Division for the use of the microprojectile particle delivery apparatus. This work was supported by a U.S.

Department of Agriculture C.R.G.O. grant 89-37280-4898 to M.E.F.

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S A Goff, K C Cone and M E Fromm

Genes Dev. 1991, **5**:

Access the most recent version at doi:[10.1101/gad.5.2.298](https://doi.org/10.1101/gad.5.2.298)

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