Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins

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The *B*, *R*, *C1*, and *Pl* genes regulating the maize anthocyanin pigment biosynthetic pathway encode tissue-specific transcriptional activators. *B* and *R* are functionally duplicate genes that encode proteins with the basic–helix–loop–helix (b-HLH) motif found in Myc proteins. *C1* and *Pl* encode functionally duplicate proteins with homology to the DNA-binding domain of Myb proteins. A member of the b-HLH family (*B* or *R*) and a member of the *myb* family (*C1* or *Pl*) are both required for anthocyanin pigmentation. Transient assays in maize and yeast were used to analyze the functional domains of the B protein and its interaction with C1. The results of these studies demonstrate that the b-HLH domain of B and most of its carboxyl terminus can be deleted with only a partial loss of B-protein function. In contrast, relatively small deletions within the B amino-terminal-coding sequence resulted in no *trans*-activation. Analysis of fusion constructs encoding the DNA-binding domain of yeast GAL4 and portions of B failed to reveal a transcriptional activation domain in the B protein. However, an amino-terminal domain of B was found to recruit a transcriptional activation domain by an interaction with C1. Formation of this complex resulted in the activation of a synthetic promoter containing GAL4 recognition sites, demonstrating that this interaction does not require the normal target promoters for B and C1. B and C1 fusions with yeast GAL4 DNA-binding and transcriptional activation domains were also found to interact when synthesized and assayed in yeast. The domains responsible for this interaction map to a region that contains the Myb homologous repeats of the C1 protein and to the amino terminus of the B protein, which does not contain the b-HLH motif. These studies suggest that the regulation of the maize anthocyanin pigmentation pathway involves a direct interaction between members of two distinct classes of transcriptional activators.

[*Key Words*: Anthocyanin biosynthesis; basic–helix–loop–helix domain; Myb repeats; regulatory genes; protein–protein interactions]

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Genetic analysis of the anthocyanin biosynthetic pathway in maize has identified multiple genes involved in the production of the purple anthocyanin pigments (Coe et al. 1988). Six of these genes have been shown to encode anthocyanin biosynthetic enzymes [*A1*, *A2*, *Bronze1* (*Bz1*), *Bronze2*, *C2*, and *Pr*) and will be referred to as structural genes. Four of the genes (*B*, *R*, *C1*, and *Pl*) encode regulatory proteins that act specifically on the anthocyanin pathway. The *B* gene is required for anthocyanin synthesis in most parts of the plant body (Styles et al. 1973). *R* is required for pigmentation in the aleurone, anthers, and coleoptile (Styles et al. 1973). Both *B* and *R* have extensive allelic diversity (Styles 1970), and in several tissues, certain *R* and *B* alleles can function as duplicate genes with either gene sufficient for pigmentation (Styles et al. 1973). *B* and *R* have been shown to encode homologous proteins (Chandler et al. 1989). *C1* is required for pigmentation of the aleurone and the embryo but is not required for pigmentation of the plant body (Chen and Coe 1977; Coe 1985). *Pl* functions in the pigmentation of most of the plant body (Gerats et al. 1984) and is homologous to *C1* (K. Cone, unpubl.).

Recent molecular evidence supports the proposed regulatory roles of the *B*, *R*, and *C1* gene products (Cone et al. 1986; Paz-Ares et al. 1986, 1987; Chandler et al. 1989; Ludwig et al. 1989; Goff et al. 1990, 1991; Ludwig et al. 1990). *c1*, *r*, or *b* mutations have been found to affect the levels of the mRNAs or enzymes encoded by the anthocyanin structural genes examined. In *r* mutant aleurones, the mRNAs and enzymes encoded by the *A1*

(NADPH-dependent reductase) and the *C2* (chalcone synthase) structural genes are not detectable (Dooner and Nelson 1979; Dooner 1983; Ludwig et al. 1989), and the mRNAs and enzymes of the *A1*, *Bz1* (UDP–glucose flavonol 3-*O*-glucosyl transferase), and *Bronze2* (unknown enzyme activity) structural genes are not detectable in *r*-germinating seedlings (Taylor and Briggs 1990). The presence of high levels of the mRNAs for the *A1* and *Bz1* genes requires a functional *B* gene in husk tissue (Chandler et al. 1989). Introduction of expressed *C1*, *B*, or *R* genes into the appropriate intact colorless maize tissues by microprojectiles results in the activation of the anthocyanin biosynthetic pathway and the appearance of purple pigmented cells (Goff et al. 1990; Ludwig et al. 1990). Microprojectile delivery of cauliflower mosaic virus 35S (CaMV 35S) promoter-expressed *C1* and *B* cDNAs has been shown to lead to the *trans*-activation of specific anthocyanin structural gene promoters (Goff et al. 1990). Transcription assays in isolated nuclei have also been used to demonstrate that control of the anthocyanin structural genes is at the level of transcription (K. Cone, unpubl.). The use of *C1* and *B* cDNAs under the control of a constitutive promoter has shown that one of these regulatory genes does not simply activate transcription of the other (Goff et al. 1990). Taken together, the results of these studies suggest that both a member of the *R/B* gene family and a member of the *C1/Pl* gene family must interact at some level to activate transcription of the anthocyanin biosynthetic pathway structural genes.

Additional evidence for the regulatory roles of these genes was provided by analysis of their coding regions. Sequence analysis of *C1* revealed that it encodes a protein with amino-terminal sequence similarity to the DNA-binding domain of Myb oncoproteins (Paz-Ares et al. 1987). Various Myb homologous proteins are localized to the nucleus and function as transcriptional regulators (Ness et al. 1989; Nishina et al. 1989; Sakura et al. 1989; Howe et al. 1990; Ibanez and Lipsick 1990; Oehler et al. 1990). The *C1*-encoded protein also contains a region rich in acidic amino acid residues (Paz-Ares et al. 1987), similar to the acidic transcriptional activation domains found in many regulatory proteins (Ptashne 1988). The carboxy-terminal acidic region of C1 functions as a transcriptional activation domain when fused to the DNA-binding domain of the yeast transcriptional activator GAL4 (Goff et al. 1991). In addition, the transcriptional activation domain of GAL4 can substitute for the carboxyl terminus of C1 to generate a fusion protein that activates a specific anthocyanin structural gene promoter or the entire anthocyanin biosynthetic pathway (Goff et al. 1991). Activation of anthocyanin structural gene promoters by these C1–GAL4 fusions remains dependent on an expressed *B* gene (Goff et al. 1991).

Sequence analysis of the coding region of *Lc*, a member of the *R/B* gene family, revealed that the Lc protein has homology to the basic-helix–loop–helix (b-HLH) DNA-binding and subunit dimerization domain of Myc oncoproteins (Ludwig et al. 1989). *R-S-*, *B-Peru-*, and *B-I-*coding sequences have also been analyzed and found to be highly homologous to *Lc* (Perrot and Cone 1989; Radicella et al. 1991). Studies on the b-HLH domain of mammalian proteins have shown that the basic motif is responsible for DNA binding, and the HLH motif functions in subunit dimerization (Murre et al. 1989a,b; Davis et al. 1990). In addition to the b-HLH domain, both the R and B proteins have a region rich in acidic amino acids. The presence of regions with homology to known DNA-binding domains and regions with similarity to transcriptional activation domains supports the proposed role of the *B*, *R*, and *C1* gene products as transcriptional regulators of the structural genes of the anthocyanin biosynthetic pathway (Paz-Ares et al. 1987; Chandler et al. 1989; Ludwig et al. 1989). Consistent with a direct mode of structural gene regulation by the C1/Pl and R/B regulatory proteins is the finding that both Myb and b-HLH consensus DNA-binding sites are important for the regulation of the maize *Bz1* gene (Goff et al. 1990; Roth et al. 1991).

To study the *B* gene product in more detail, coding sequences for B-protein deletions and B-protein fusions with heterologous protein domains were constructed. These B derivatives were placed under the control of the constitutive CaMV 35S promoter and introduced into *c1 b r* maize embryogenic callus tissue by use of high-velocity microprojectiles. Constructs were tested for their ability to activate de novo anthocyanin biosynthesis or *trans*-activate a specific anthocyanin structural gene promoter. The results of these studies provide evidence that the regulation of the maize anthocyanin biosynthetic pathway utilizes the direct interaction of two distinct classes of transcriptional regulatory proteins.

Results

Deletion analysis of the B-regulatory protein

Deletion analysis of several mammalian and yeast regulatory proteins has shown that much of the coding sequence for these proteins can be removed without a loss of regulatory function (Hope and Struhl 1986; Ma and Ptashne 1987; Evans 1988; Struhl 1989). To determine which regions of the B protein are not essential for transcriptional activation function, plasmids that express internal deletions of the *B* cDNA under the control of the CaMV 35S promoter were constructed. Each of the B derivatives was tested for its ability to *trans*-activate the *Bz1* structural gene promoter fused to the firefly luciferase reporter gene (Bz1Luc; Fig. 1A). A transformation efficiency control (AdhCAT, Fig. 1A) was included in each transfection. After DNA delivery by microprojectiles, tissues were incubated as described in Materials and methods, homogenized, and assayed for the presence of firefly luciferase and chloramphenicol acetyltransferase (CAT) activity. *Trans*-activation values are expressed as a ratio of luciferase to CAT activity as described previously (Callis et al. 1987). The expressed intact B construct did not activate Bz1Luc when delivered without a CaMV 35S-expressed C1 cDNA (35SC1) but

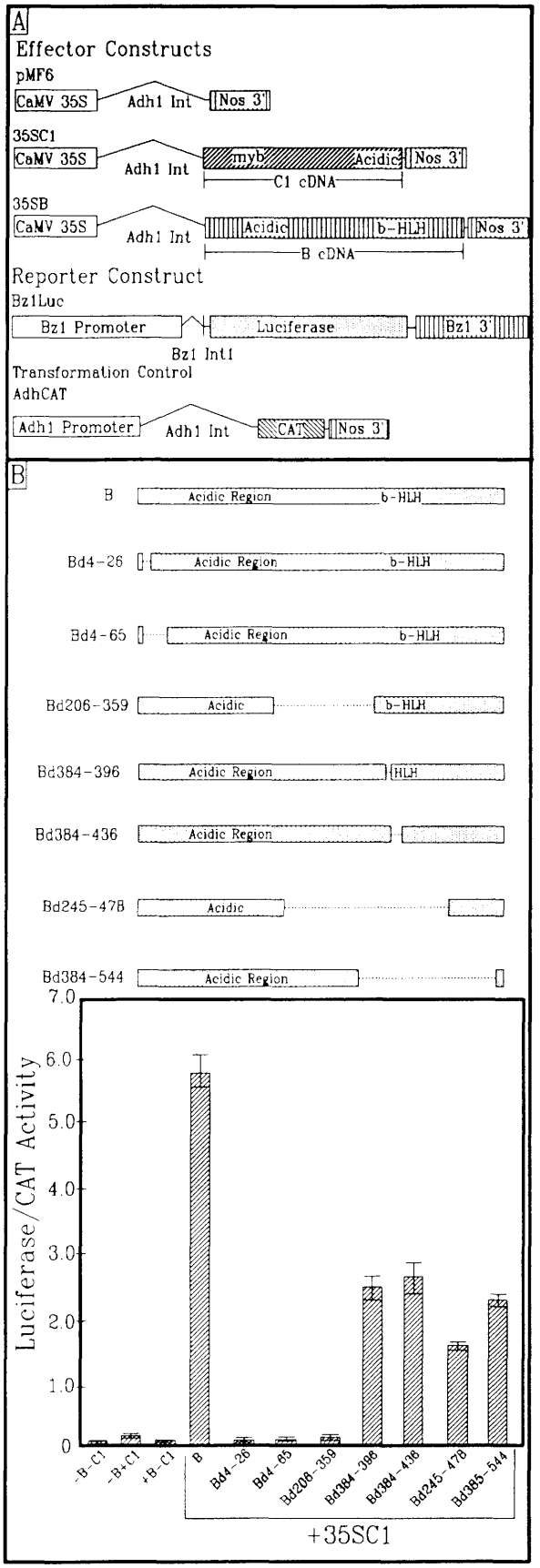


Figure 1. (*A*) Constructs used in this study are not necessarily drawn to scale. Each of these constructs was carried in a pUC plasmid derivative. Components of the various constructs are designated as follows: (CaMV 35S) 0.5-kb 35S promoter from cauliflower mosaic virus; (Adh1 Int) 0.5-kb first intron from the maize *alcohol dehydrogenase-1* (*Adh*) gene; (Nos 3′) 0.25-kb polyadenylation region from the nopaline synthase gene of *Agrobacterium tumefacians* Ti plasmid; (Bz1 promoter) 2.3-kb maize *Bz1* promoter; (Bz1 Int1), 0.15-kb intron from the maize *Bz1* gene; (Luciferase) 1.8-kb firefly luciferase-coding region; (Bz1 3′) polyadenylation region from the maize *Bz1* gene; (Adh1 promoter) maize *Adh* promoter; (CAT) bacterial chloramphenicol transferase gene; (C1 cDNA) 1.1-kb maize *C1* cDNA; (B cDNA) 1.9-kb maize *B-Peru* cDNA. An intron is included in the constitutive expression vectors to enhance the level of expression. (*B*) Plasmids encoding B proteins and B-protein derivatives, with regions missing indicated by a dotted line. The B region rich in acidic amino acids and the b-HLH domain are indicated. The numbers in the construct names designate the missing amino acids. Plasmids encoding B proteins were codelivered to *c1 r b* maize embryogenic callus by microprojectiles with or without the expressed C1 cDNA (35SC1), with the *Bz1* reporter gene (Bz1Luc), and with the transformation control plasmid (AdhCAT) (see *A*). After DNA transfer, callus tissue was incubated at 24°C for 48 hr without illumination, tissues were examined for anthocyanin-producing cells, and extracts were prepared for enzyme activity determination. Activities are expressed as a ratio of firefly luciferase generated from the reporter gene to CAT from the transformation control plasmid. Independent bombardments were analyzed, and data are presented as mean values plus and minus standard errors of the mean. *Trans*-activation by the intact B construct generates between 10^4 and 3×10^5 luciferase light units/10 sec at 25°C.

activated Bz1Luc >500-fold when delivered with 35SC1 (Fig. 1B). 35SC1 alone activated Bz1Luc ~15-fold (Fig. 1B). The internal B-deletion constructs were also assayed for their ability to induce visually detectable pigmentation in recipient maize *b r* aleurone and *b r c1* embryogenic callus cells.

Expressed B-deletion derivatives that lack B-coding sequences from amino acid 245 to the carboxyl terminus encode partially active (20–40%) to fully active transcriptional regulators when codelivered with an expressed *C1* gene. Plasmids encoding either a deletion of the B basic domain (Fig. 1B, Bd384–396) or a deletion of the b-HLH domain (Fig. 1B, Bd384–436) *trans*-activated Bz1Luc >250-fold (Fig. 1B) when delivered with 35SC1. Likewise, larger deletion derivatives spanning the b-HLH domain from amino acid 245 to 478 (Fig. 1B, Bd245–478) or from amino acid 384 to 544 (Fig.1B, Bd384–544) of the intact 562-amino-acid *B*-coding sequence *trans*-activated Bz1Luc >150-fold (Fig. 1B) when delivered with 35SC1. Plasmids encoding B-protein derivatives that lack the B-coding sequence beyond amino acid 551 of the 562-amino-acid protein are fully functional on the Bz1Luc reporter gene. All of these constructs generated pigmented cells, demonstrating their ability to activate the multiple genes required for anthocyanin accumulation (data not shown).

In contrast, plasmids encoding proteins with small alterations or deletions in the amino terminus of B, Bd4–26 (altered for amino acid 3 and deleted for amino acids 4–26, Fig. 1B) and Bd4–65 (altered for amino acid 3 and deleted for amino acids 4–65, Fig. 1B) did not *trans*-acti-

vate the Bz1Luc reporter gene (Fig. 1B) when delivered to intact cells with 35SC1. Likewise, a plasmid encoding a protein with a larger central deletion, Bd206–359 (deleted for amino acids 206–359, Fig. 1B), also failed to *trans*-activate Bz1Luc (Fig. 1B) when delivered to cells with 35SC1. These constructs also failed to stimulate anthocyanin pigment accumulation (data not shown). Because the transformed cells represent a very small percentage of the tissue sampled in each experiment, it is not possible to biochemically detect the B protein, even in the cases where fully active constructs are used. Thus, these negative results do not provide information on the function of the deleted regions. The amino-terminal and central regions of the B protein could be essential for expression of the B protein, proper protein folding, protein stability, and/or B transcriptional activation function.

Analysis of B–GAL4 fusion proteins in maize tissues.

Located within the amino terminus of the B protein is a sequence rich in acidic amino acids (Radicella et al. 1991). Regions rich in acidic amino acids are commonly found within some transcriptional activation domains of regulatory proteins (Ptashne 1988). To determine whether the B protein contains a transcriptional activation domain either within the amino terminus or elsewhere in the B protein, plasmids were constructed that encode the yeast GAL4 DNA-binding domain fused in-frame to various *B*-coding sequences (Fig. 2). The yeast GAL4 protein is a well-characterized transcriptional activator that contains a DNA-binding domain located within its amino terminus (Ma and Ptashne 1987). The DNA-binding domain of GAL4 has been used extensively in fusion constructs to test the transcriptional activation function of attached polypeptide sequences (Giniger et al. 1985; Giniger and Ptashne 1987; Kakidani and Ptashne 1988; Ma et al. 1988; Sadowski et al. 1988; Carey et al. 1990). Plasmids encoding fusion proteins were constructed to express the GAL4 DNA-binding domain fused to either the amino terminus or the carboxyl terminus of B. GAL4–B fusion constructs in an expression vector were codelivered with a minimal CaMV 35S promoter containing GAL4 recognition sites fused to the luciferase reporter (Fig. 2, GALLuc2; Goff et al. 1991). This minimal promoter construct has been shown previously to be activated by GAL4 derivatives in carrot protoplasts (Ma et al. 1988) and by plasmids encoding GAL4–C1 fusion proteins in maize embryogenic callus cells (Goff et al. 1991). The GAL4 DNA-binding domain fused to either the C1 transcriptional activation domain or to the transcriptional activation domain of herpes virus VP16 *trans*-activated GALLuc2 >30-fold (Fig. 2). In this study none of the GAL4–B fusion protein constructs tested alone *trans*-activated the GALLuc2 reporter construct (Fig. 2, open bars, and data not shown). Therefore, this fusion approach did not reveal an independent transcriptional activation domain within the B protein.

Although the B–GAL4 fusion plasmids were not found to induce GALLuc2 when delivered alone, a fusion encoding the GAL4 DNA-binding domain attached at amino acid 551 of the B protein (B1–551–GAL4) did *trans*-activate GALLuc2 >15-fold when codelivered with 35SC1 (Fig. 2). This combination of plasmids did not *trans*-activate a minimal CaMV 35S promoter lacking GAL4-recognition sites (Fig. 2, 35SMin) nor did 35SC1 delivered alone *trans*-activate GALLuc2 (Goff et al. 1991). Because B1–551–GAL4 was unable to activate transcription of GALLuc2 when delivered without 35SC1, this result suggests that B1–551–GAL4 may obtain transcriptional activation function by complex formation with the C1 protein. An interaction between the B and C1 proteins should generate a complex containing the acidic transcriptional activation domain located within the carboxyl terminus of the C1 protein. The C1 transcriptional activation domain, when fused in-frame to the GAL4 DNA-binding domain, activated GALLuc2 independent of B (Fig. 2; Goff et al. 1991) and is therefore not simply converted to a functional form by the B protein.

To localize further a putative C1 interaction domain within the B protein, internal deletion derivatives of the B–GAL4 fusion were tested for their ability to *trans*-activate GALLuc2 with and without 35SC1. A fusion construct containing only amino acids 1–66 of B and the GAL4 DNA-binding domain (B1–66–GAL4) did not *trans*-activate GALLuc2 either with or without an expressed *C1* gene (Fig. 2 and data not shown). Likewise, an internal deletion of amino acids 206–359 within B1–551–GAL4 (Bd206–359–GAL4) failed to *trans*-activate GALLuc2 either alone or when codelivered with 35SC1 (Fig. 2 and data not shown). These data are consistent with the results obtained in maize cells using internal B deletions and the Bz1Luc reporter gene (described above). Derivatives of B1–551–GAL4, which were deleted for B amino acids 245–478 (Bd245–478–GAL4) or 384–544 (Bd384–544–GAL4), *trans*-activated GALLuc2 only in the presence of an expressed *C1* gene (Fig. 2 and data not shown). Thus, if B and C1 interact directly, the interaction domain in B must be located between amino acids 1 and 244.

Activation of GALLuc2 by B–GAL4 fusion proteins and defective C1 proteins

Although the results presented above strongly suggest that the B and C1 proteins interact directly to form a functional transcriptional activator, it is possible that additional activities induced by these regulatory proteins may function in association with B1–551–GAL4, Bd245–478–GAL4, or Bd384–544–GAL4 to *trans*-activate GALLuc2. The functional B–GAL4 constructs, together with the expressed C1 cDNA, were observed to activate both pigment accumulation and the Bz1Luc reporter gene (data not shown). To determine whether induction of GALLuc2 could be achieved in the absence of anthocyanin structural gene *trans*-activation, B–GAL4 fusion constructs were codelivered with defective derivatives of C1 that do not *trans*-activate anthocyanin synthesis. The defective C1 constructs were derivatives of the dom-

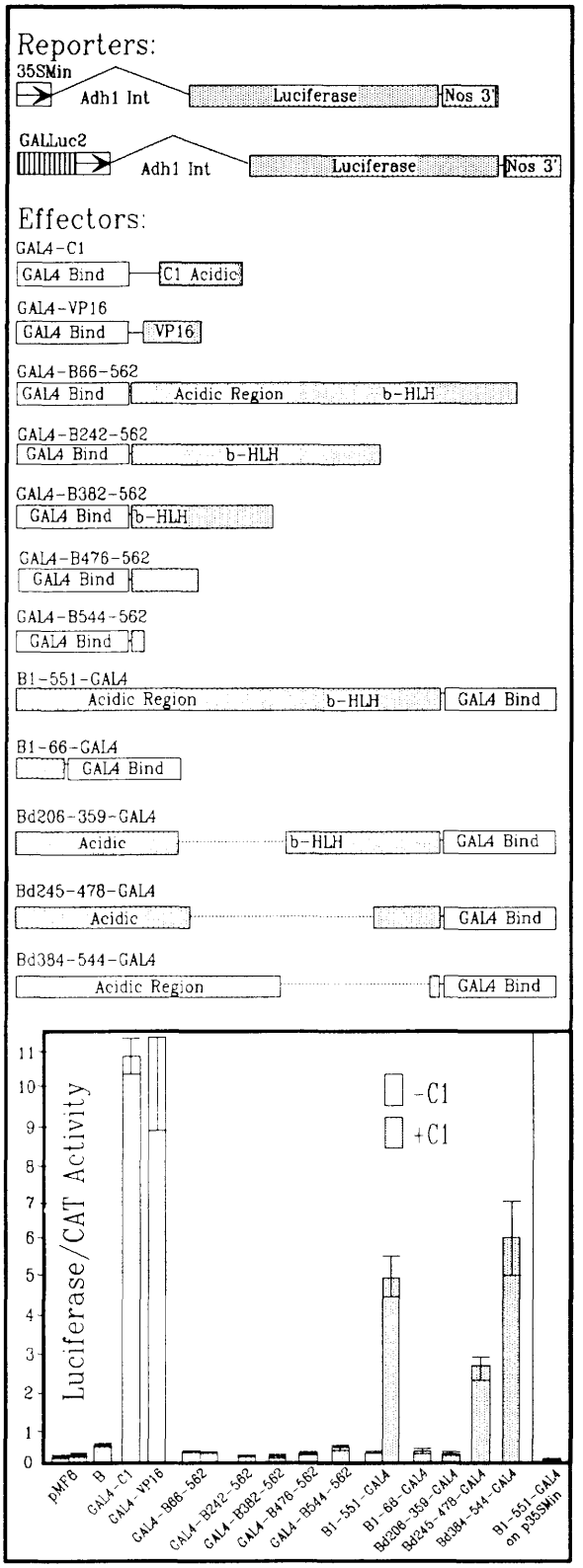


Figure 2. *Trans*-activation by GAL4–B fusion proteins. B-protein fusions with the yeast GAL4 DNA-binding domain are designated by numbers according to the amino acids present from the B protein. Each of the fusions contains the first 147 amino acids of the GAL4 protein. Deletions are named according to the amino acids missing from the 1–551 portion of the B protein and are indicated by the dotted line. (p35SMin) A minimal CaMV 35S promoter (deletion to −90) that contains no GAL4 recognition sequences fused to the firefly luciferase reporter gene. (GALLuc2) The same minimal CaMV 35S promoter with ~10 GAL4 recognition sites (Ma et al. 1988) fused to the firefly luciferase reporter gene. The transformation control plasmid is AdhCAT (Fig. 1A). Plasmids encoding B–GAL4 and GAL4–B fusions were codelivered with or without an expressed C1 cDNA (Fig. 1A, 35SC1) and with reporter plasmid p35SMin or GALLuc2. After DNA transfer, maize callus tissue was incubated for 48 hr without illumination and homogenized for enzyme activity determination. Activities are expressed as a ratio of firefly luciferase generated from the reporter gene to CAT from the transformation control plasmid. Independent bombardments were analyzed, and data are presented as mean values plus and minus standard errors of the mean.

inant inhibitory allele C1-I or inactive mutants of C1. Owing to a number of mutations, the C1-I gene encodes a truncated protein with a nonfunctional acidic domain and several amino acid changes, including a conservative amino acid substitution in the Myb homologous domain (glutamic acid for aspartic acid at position 101). Previous work demonstrated that a combination of the Myb homologous domain of C1-I with the acidic domain of C1 yielded fusion proteins (Fig. 3, CIC1-1 and CIC1-2) that were incapable of *trans*-activating Bz1Luc (Goff et al. 1991). Likewise, a single-amino-acid substitution of glutamic acid for aspartic acid at position 101 in C1 results in an inactive regulatory protein (Goff et al. 1991). When any one of these defective C1 derivatives was codelivered with the B1–551–GAL4 or Bd384–544–GAL4 constructs, GALLuc2 was *trans*-activated (Fig. 3). No GALLuc2 *trans*-activation was observed when B1–551–GAL4 was codelivered with constructs lacking a functional C1 acidic domain (Fig. 3, C1Bind or C1-I). These results support further the hypothesis that GALLuc2 *trans*-activation is dependent on the interaction of B–GAL4 fusion proteins with C1 proteins that contain a functional transcriptional activation domain.

Interaction of B and C1 derivatives in yeast

To obtain further evidence that the B and C1 proteins interact directly to *trans*-activate anthocyanin structural gene promoters, a yeast system developed specifically to test in vivo protein–protein interactions was used. As described by Fields and Song (1989), the detection of protein–protein interactions in yeast is made possible by the attachment of a yeast DNA-binding domain to the first protein in question and a transcriptional activation domain to the second. An interaction between the first and second proteins being examined brings together the DNA-binding and transcriptional activation domains and results in a functional transcriptional regulator that, in turn, stimulates the expression of a reporter gene from a regulated promoter. Plasmid constructs encoding a fusion between the B protein and the DNA-binding domain of GAL4 and deletion derivatives of this fusion were transformed into yeast together with plasmids that

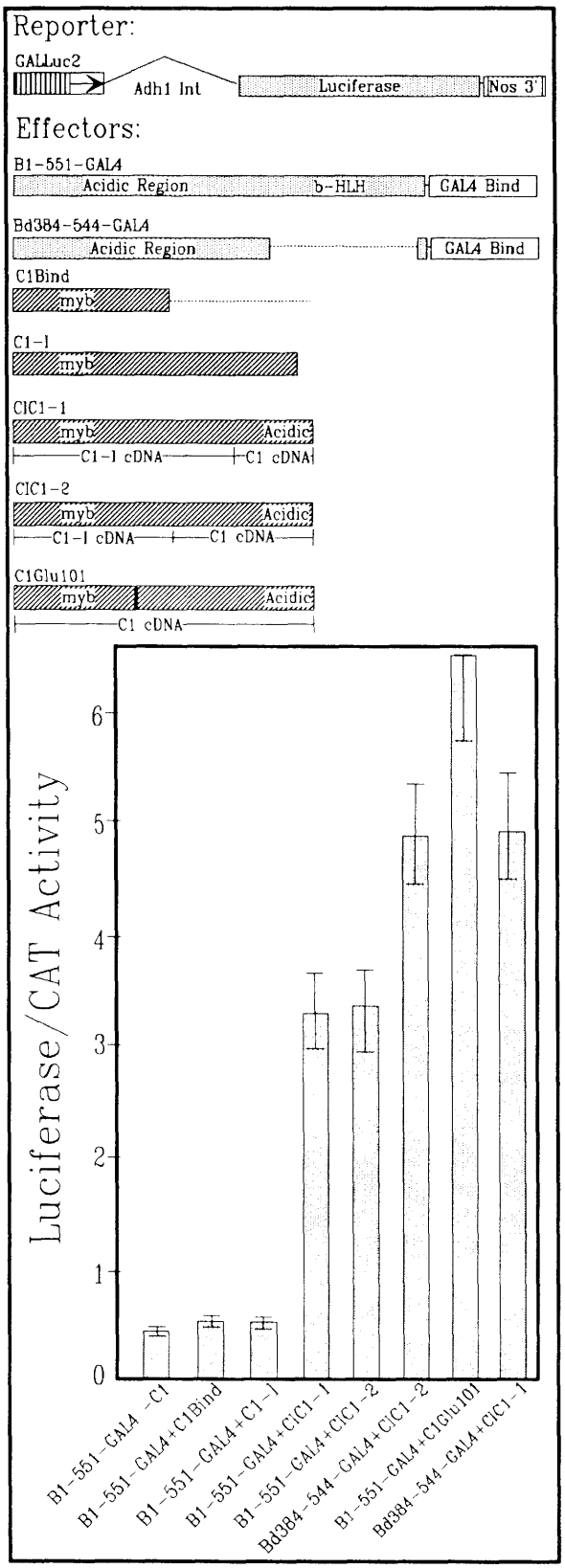
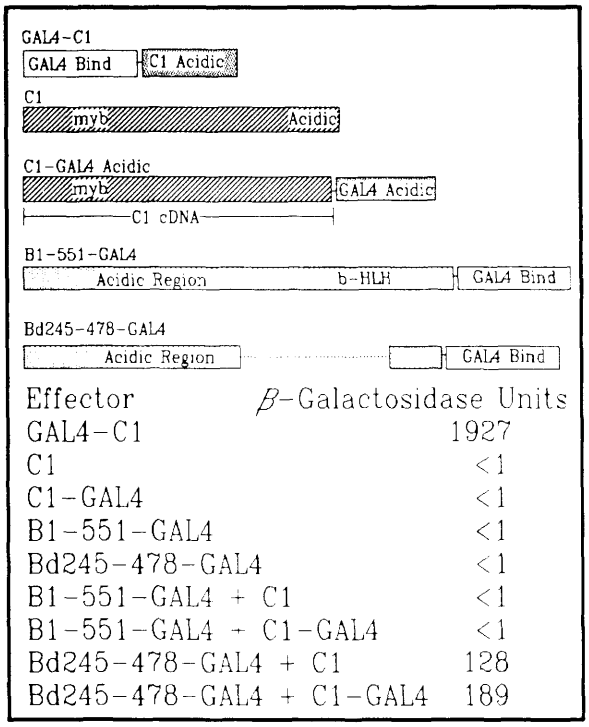


Figure 3. *Trans*-activation by GAL4–B fusion and defective C1 proteins. B-protein fusions with the yeast GAL4 DNA-binding domain are as described in Fig. 2. The C1Bind protein contains amino acids 1–142 of the intact 273-amino-acid C1 protein. The dominant inhibitor protein C1-I has numerous changes within the protein sequence and is 19 amino acids shorter than intact C1 owing to a frameshift and the presence of a stop codon. C1 wild-type and C1-I dominant inhibitor fusion proteins are fused at either amino acid 220 (C1C1-1) or amino acid 142 (C1C1-2) of the C1 protein. C1Glu101 is a single-amino-acid substitution of glutamic acid for aspartic acid at position 101 in the C1 protein. The dotted line represents amino acids missing in deletion constructs. Plasmids encoding B fusions with the GAL4 DNA-binding domain were codelivered with the expressed C1 cDNA derivatives or C1-I inhibitor–C1 fusion constructs, the GAL4-regulated reporter gene (GALLuc2), and the transformation control (Fig. 1A, AdhCAT). After DNA transfer, maize callus tissue was incubated for 48 hr without illumination and homogenized for enzyme activity determination. Activities are expressed as a ratio of firefly luciferase generated from the reporter gene to CAT generated from the transformation control plasmid. Independent bombardments were analyzed, and data are presented as mean values plus and minus standard errors of the mean.

express either the intact maize C1 protein or C1 fused to the transcriptional activation domain of GAL4. β-Galactosidase synthesized from an endogenous GAL4-regulated promoter in response to the interaction of the various B and C1 derivatives was assayed. Western analysis of extracts from the transformed yeast cultures was used to verify the presence of the B and C1 derivatives (data not shown). The results of these studies demonstrate that B–GAL4, C1, or C1–GAL4 fusions alone are not active on the GAL4-regulated reporter construct (Fig. 4). In addition, the B–GAL4 fusion containing the b-HLH domain was unable to activate the reporter gene in the presence of an expressed C1 gene or C1–GAL4 fusion construct (Fig. 4). However, a B–GAL4 internal deletion fusion (deleted for B amino acids 245–478, which includes the B b-HLH domain) was found to interact with either an intact C1 protein or a C1 protein fused to the transcriptional activation domain of the yeast GAL4 protein. This interaction resulted in the *trans*-activation of the GAL4-regulated promoter and the synthesis of β-galactosidase (Fig. 4). These results provide evidence for a direct interaction between B and C1 in the regulation of their target promoters. Furthermore, these results indicate that the presence of the carboxy- terminal B sequence containing the b-HLH blocks the ability of the B and C1 proteins to activate transcription in yeast.

Discussion

The B protein, together with the C1 or Pl protein, controls the coordinate transcription of the structural genes in the maize anthocyanin pathway. Sequence analysis of the B protein revealed two conserved regions: an area rich in acidic amino acids, which, by analogy with other transcriptional activators, may serve as a transcriptional activation domain and a b-HLH region, which may serve as a DNA-binding and subunit dimerization domain. To determine if, or how, these two regions of the B protein contribute to its function as a transcriptional activator, a number of B derivatives, including both deletion and fu-



Effector	β-Galactosidase Units
GAL4–C1	1927
C1	<1
C1–GAL4	<1
B1–551–GAL4	<1
Bd245–478–GAL4	<1
B1–551–GAL4 + C1	<1
B1–551–GAL4 + C1–GAL4	<1
Bd245–478–GAL4 + C1	128
Bd245–478–GAL4 + C1–GAL4	189

Figure 4. Interaction of B and C1 fusion proteins in yeast. The GAL4–C1 fusion protein contains amino acids 1–147 of GAL4 and 173–273 of the maize C1 protein. The C1 protein is the intact 273-amino-acid maize protein. The C1–GAL4 acidic protein contains amino acids 1–258 of C1 fused to transcriptional activation domain II of the GAL4 protein. The B–GAL4 fusion proteins are those described in Fig. 2. The dotted line represents amino acids missing in deletion constructs. Yeast cells transformed with replicating plasmids encoding B–GAL4 (DNA-binding domain) fusion proteins, with and without either C1 or C1–GAL4 (transcriptional activation domain), were assayed for *trans*-activation of a GAL4-regulated reporter gene (GAL1–LacZ; Fields and Song 1989). A transformed cell with a plasmid encoding the GAL4 DNA-binding domain fused to the C1 transcriptional activation domain (GAL4–C1) was used as a positive control. Units of β-galactosidase were determined and are expressed as the average units obtained in the assay of 3–10 independent transformants.

sion proteins, were tested for their ability to activate transcription of appropriate target promoters.

The results of this study, discussed in greater detail below, are consistent with the following model. We propose that the B protein functions by a direct interaction between the amino terminus of B and the maize C1 protein. Such an interaction should generate a B/C1 complex that contains both the b-HLH DNA-binding and subunit dimerization domain and the Myb homologous DNA-binding domain. The B protein may not need a transcriptional activation domain, as a B/C1 complex would contain a functional transcriptional activation domain provided by the C1 protein. The interaction of the C1 protein with B derivatives missing the b-HLH domain may allow defective B proteins to retain partial transcriptional activation function, possibly by the tethering of the complex to target promoters through the Myb homologous DNA-binding domain present in the C1 protein. The C1 protein must rely on the interaction with the B protein to gain transcriptional activation function, as the C1 protein alone does not efficiently *trans*-activate structural gene promoters despite the fact that it contains a Myb homologous DNA-binding domain and a transcriptional activation domain. The formation of a B/C1 complex may increase the specificity and/or affinity of the individual DNA-binding domains supplied by each protein.

The B-deletion experiments demonstrated that the carboxy-terminal half of B can be removed without a total loss of transcriptional activation function in transient expression assays. Deletion of amino acids 245–478 or amino acids 384–544 resulted in B derivative proteins able to induce Bz1Luc at ~25–50% of the induction observed with the intact B protein. Deletion of the B basic or b-HLH domain generated proteins with activities similar to the more extensive carboxy-terminal deletions. These results demonstrate that the B protein b-HLH region is not essential for transcriptional activation in transient expression assays. Furthermore, these results suggest that either the B protein contains a second DNA-binding domain capable of recognizing the target structural gene promoters or that DNA binding is not required for B-protein function, possibly because it interacts with a second protein capable of DNA binding.

We favor the hypothesis that the b-HLH deletion derivatives are still partially active because they interact with another DNA-binding protein. Three lines of evidence support our hypothesis that the acidic amino terminus of B is required for the interaction with the other regulatory factor C1. First, plasmids that encode the amino-terminal region of B (at least amino acids 1–244) fused to the GAL4 DNA-binding domain *trans*-activate a reporter gene containing a minimal CaMV 35S promoter with GAL4 recognition sites (GALLuc2), only when codelivered with an expressed *C1* cDNA. Second, plasmids that encode defective C1 proteins, which are unable to activate the anthocyanin structural genes but retain a functional C1 transcriptional activation domain, are able to *trans*-activate GALLuc2 with B–GAL4 fusion constructs. Third, a B-protein fusion with the GAL4 DNA-binding domain synthesized in yeast is able to recruit the C1 protein and *trans*-activate a GAL4-regulated promoter. Consistent with the proposal that the amino terminus plays an important role in B-protein function is the high degree of conservation of this region among the members of the B and R protein family. Also consistent is the observation that alterations and deletions within the amino terminus completely eliminated B activity on Bz1Luc in plant transient assays. Likewise, the B derivative deleted for amino acids 206–359 was not found to activate transcription in either yeast or plant assays. Verification of the presence of a B derivative deleted for amino acids 206–359 was only possible in yeast, and it therefore remains possible that in plant tissues, amino-terminal alterations are affecting synthesis or stability rather than B-protein function.

It is clear from the accumulated genetic evidence that there is an interaction at some level between the *B* and *C1* genes. Previously described substitution analysis of the C1 protein has demonstrated that amino acids 1–117 of C1 fused to the transcriptional activation domain of yeast GAL4 could activate anthocyanin biosynthesis and *trans*-activate the *Bz1* promoter in maize only in the presence of the B protein (Goff et al. 1991). Together with the results presented in this study, these findings suggest that the interaction between the B and C1 proteins requires amino acids within 1–117 of C1 and 1–244 of B. An interaction between the B and C1 proteins does not appear to depend on their recognition of anthocyanin structural gene promoters because the B–GAL4 and C1 proteins retain the ability to interact when directed to the GAL4-regulated minimal CaMV 35S reporter gene in maize cells and to interact in yeast cells lacking the anthocyanin structural genes. B-deletion derivatives, such as Bd245–478 or Bd384–544, which are likely to provide neither a B DNA-binding function nor a detectable transcriptional activation domain to an active B/C1 complex, must achieve functional activity by another mean. The interaction of the B and C1 proteins may alter the Myb homologous DNA-binding domain of C1 to generate a functional DNA recognition complex in the absence of an obvious B DNA-binding domain. Such a role could be achieved by the induction of conformational changes in the C1 and/or B proteins or by providing additional DNA recognition specificity.

A transcriptional activation domain was not detected using B–GAL4 fusion constructs containing the DNA-binding domain of the yeast GAL4 protein and various B regions, including the acidic domain. None of the fusion proteins activated a minimal CaMV 35S promoter containing GAL4 recognition sequences (GALLuc2) when delivered to plant tissues in the absence of an expressed C1 gene. In the presence of an expressed C1 gene, several of the B–GAL4 fusion constructs were found to activate GALLuc2 in plant cells. B–GAL4 fusion proteins were detected by antibodies in extracts of yeast transformed with the appropriate plasmids, suggesting that the failure of specific constructs to *trans*-activate reporters is not the result of an absence of these proteins. Thus, we conclude that the B protein is not able to supply an independent transcriptional activation domain in B–GAL4 fusion proteins.

The most prominent structural feature of the B protein revealed by protein sequence comparisons is the b-HLH domain. Such b-HLH domains mediate the interaction between proteins in the formation of either homodimers or heterodimers via the association of HLH regions (Lassar et al. 1989; Murre et al. 1989a,b; Davis et al. 1990). This association brings together two adjacent basic regions that compose the functional DNA-binding domain (Murre et al. 1989a;b; Davis et al. 1990; Voronova and Baltimore 1990). Thus the HLH domain is functionally analogous to the leucine zipper dimerization domain (Gentz et al. 1989; Turner and Tjian 1989; Vinson et al. 1989), which allows efficient recognition of the target DNA sequence by subunit dimerization (Davis et al. 1990; Jones 1990; Voronova and Baltimore 1990). The specificity of protein subunit interaction has been localized to the putative helices and is apparently independent of the loop connecting them (Davis et al. 1990; Voronova and Baltimore 1990).

b-HLH proteins recognize a consensus DNA-binding site with the core sequence CANNTG (Murre et al. 1989a; Beckmann et al. 1990; Davis et al. 1990; Henthorn et al. 1990). Recent deletion analysis of the *Bz1* promoter has identified a region of ~65 bp responsible for the regulation of *Bz1* by B/R and C1/Pl (Roth et al. 1991). Located within this regulatory region are both b-HLH and Myb consensus DNA-binding sites (Biedenkapp et al. 1988; Murre et al. 1989a; Beckmann et al. 1990; Davis et al. 1990; Henthorn et al. 1990; Roth et al. 1991). Mutations within the b-HLH or the Myb consensus DNA-binding sites reduce *trans*-activation of the *Bz1* promoter to 1% and 10% of the wild-type promoter, respectively (Roth et al. 1991). In addition, multimerized synthetic 38-bp oligonucleotides containing the *Bz1* b-HLH and Myb consensus DNA-binding sites confer B and C1 regulation on an inactive core CaMV 35S promoter (Roth et al. 1991). These results suggest that the region of the *Bz1* promoter containing the b-HLH consensus DNA-binding site is very important for *Bz1* promoter activity. However, deletion of the B b-HLH domain, which we postulate recognizes this consensus DNA-binding site, has only a twofold effect on *trans*-activation of Bz1Luc. A possible explanation for this apparent contradiction is that the core Myb and Myc consensus DNA-binding sites located within the *Bz1* promoter are only separated by a few base pairs. The mutations generated in the previous study altered the entire core consensus DNA-binding site and therefore may have influenced the binding of not only one but both regulatory proteins. Consistent with this hypothesis is the observation that a mutation introduced between the b-HLH and Myb consensus DNA-binding sites also blocks *trans*-activation by the regulatory proteins (Roth et al. 1991). In vitro studies in progress should provide further evidence for the use and requirements of these b-HLH and Myb consensus DNA-binding sites by the B/R and C1/Pl regulatory proteins.

The induction of GALLuc2 by B1–551–GAL4 and C1 derivatives carrying C1-I amino-terminal-coding sequences provides a clue to the possible mechanism of *C1-I* dominant inhibition. The C1-I/C1 fusion derivatives tested in this study retain their ability to interact with the B–GAL4 proteins and activate transcription of a heterologous promoter in plant cell transient assays. We propose that it should also be possible for C1-I/B complexes to form in maize genotypes carrying a single copy of the dominant inhibitory allele C1-I. Such complex formation may thereby prevent active C1/B complex formation and result in dominant inhibition of the wild-type C1 allele.

Unlike the situation in maize cells, the intact B–GAL4 fusion containing amino acids 245–478, which includes the b-HLH domain, when synthesized in yeast, was not observed to activate the GAL4-regulated reporter gene in

the presence of C1. However, a B–GAL4 fusion protein missing an internal region of B was able to activate the GAL4-regulated reporter gene when a functional C1 protein was present. A variety of explanations may account for this result. The presence of this region in the B–GAL4 fusion protein may block proper protein folding or prevent translocation of the fusion protein to the nucleus in yeast but not in maize. Alternatively, an endogenous yeast b-HLH protein may block B-protein function by the formation of an inactive heterodimer or protein complex. Yeast proteins with b-HLH domains have been described (Cai and Davis 1990; Ogawa and Oshima 1990), and it is possible that other b-HLH proteins exist in yeast. Regardless of the mechanism that prevents B–GAL4 from functioning with C1, this result suggests that attempts to demonstrate protein–protein interactions in yeast using fusion proteins may, in some cases, be difficult and require the use of constructs encoding a variety of test protein–GAL4 fusions.

The anthocyanin biosynthetic pathway consists of several enzymatic steps requiring a number of structural gene-encoded enzymes. The expression of many, if not all, of these structural genes requires both a functional member of the *B/R* family and a member of the *C1/Pl* family of regulatory genes (Dooner and Nelson 1979; Dooner 1983; Chandler et al. 1989; Ludwig et al. 1989; Taylor and Briggs 1990). Although the promoter regions of several structural genes have been sequenced, they do not contain an apparent block of conservation with that region of the *Bz1* promoter shown to be responsible for regulation by B and C1. It is therefore possible that the regulation of the different structural genes may exhibit differential dependence on specific properties of the two classes of transcriptional activators controlling these genes. Future analysis of the regulation of a number of the *B/R*- and *C1/Pl*-regulated structural gene promoters by B and C1 derivatives should provide a unique opportunity to gain valuable insights into the transcriptional control of an entire set of coordinately induced biosynthetic pathway genes.

Materials and methods

Plant materials

An immature maize 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 diam.) on agarose plates containing N6 media (Lowe et al. 1985). *C1 b r* kernels were surface sterilized with 70% ethanol and soaked overnight in sterile water, and the pericarp was removed to expose the aleurone for gene transfer. Dissected kernels were placed on agarose plates containing N6 media for microprojectile delivery of DNA.

Plasmids

The structures of plasmid inserts used in this study are presented in Figures 1–4. All inserts are carried in plasmid vectors derived from pUC (Vieira and Messing 1982). The construction of the plasmids in Figure 1A has been described previously: the *Bz1* promoter–luciferase reporter, Bz1Luc (Klein et al. 1989); the maize expression vector pMF6 used for the constitutive expression of all effectors in the maize transient expression assays (Callis et al. 1987); the *B* cDNA isolated from the *B-Peru* genotype (Radicella et al. 1991) and expressed in pMF6 (Goff et al. 1990); the expressed C1 cDNA (35SC1; Goff et al. 1990, 1991); the Adh1 promoter/CAT transformation control, AdhCAT, referred to previously as pAI1CN (Callis et al. 1987).

The constructs containing the B amino-terminal deletions were constructed as follows: *Pst*I–*Hin*dIII and *Bgl*II–*Hin*dIII restriction fragments containing the B amino acids 27–562 and 66–562, respectively, and 20 nucleotides of 3′-untranslated sequence were subcloned into a cassette that provided an in-frame translational start codon and 23 nucleotides of the B cDNA leader sequence. DNA sequence analysis was used to verify the resulting B reading frame, and the expression constructs were generated by insertion of these coding sequences into pMF6. When translated, these constructs should produce B proteins with the third amino acid changed from leucine to proline and missing amino acids 4–26 (Bd4–26) or 4–65 (Bd4–65).

Bd206–359 was constructed by deletion of an internal *Nae*I fragment of the B cDNA and is missing nucleotides encoding amino acids 206–359. Bd245–478 was constructed by deletion of an internal *Nco*I fragment from the B cDNA and is missing nucleotides encoding amino acids 245–478. Bd384–544 was constructed by an internal deletion of a *Nar*I fragment from the B cDNA and is missing nucleotides encoding amino acids 384–544. Bd384–396 was generated by first using the oligonucleotide 5′-ACA TCT CGT TAA GCT TCT CCG-3′ for site-directed mutagenesis (Kunkel et al. 1987) to create a *Hin*dIII site at nucleotides corresponding to amino acid 396. The DNA containing the resulting silent mutation was digested at the *Hin*dIII site and at an internal *Nar*I site, the ends were made blunt using the Klenow fragment of DNA polymerase I, and the DNA was religated. Bd384–436 was generated in a similar manner by using the oligonucleotide 5′-TTG CCT CCT GGA CTC GAG TTC TTG TAC C-3′ to create an *Xho*I site at nucleotides corresponding to amino acid 436. The DNA containing the resulting silent mutation was digested at the *Xho*I site and at an internal *Nar*I site, the ends were made blunt as described above, and the DNA was religated.

Plasmids containing the yeast GAL4 gene (pMA210 and pMA563) were provided by Jun Ma and Mark Ptashne. The minimal 35S reporter gene and 35S reporter containing GAL4-recognition sites (Giniger et al. 1985) were described previously (Ma et al. 1988; Goff et al. 1991). The expressed GAL4 DNA-binding region contains the coding sequence for amino acids 1–147 of GAL4 (*Bam*HI–*Cla*I) inserted into the expression vector pMF6. GAL4–B66–562 was constructed by ligation of the GAL4 DNA-binding coding sequence (encoding amino acids 1–147) to the B cDNA at the coding sequence for amino acid 66; GAL4–B242–562 was constructed by ligation of GAL4(1–147) to the B cDNA at the coding sequence for amino acid 242; GAL4–B382–562 was constructed by ligation of GAL4(1-147) to the B cDNA at the coding sequence for amino acid 382; GAL4–B476–562 was constructed by ligation of GAL4(1–147) to the B cDNA at the coding sequence for amino acid 476; and GAL4–B544–562 was constructed by ligation of GAL4(1–147) to the B cDNA at the coding sequence for amino acid 544.

B1–551–GAL4 was constructed from a PCR-derived GAL4 DNA-binding domain generated by the oligonucleotide 5′-CCC GAT CCA GAT GAA GCT ACT GTC TTC TAT C-3′ and a universal primer on a subclone derived from pMA210. The fragment generated was subcloned and later inserted into the B cDNA at the *Bcl*I site. B1–66–GAL4 was constructed by ligating the GAL4 DNA-binding domain subclone described above to

the *Bgl*II site in the B cDNA. Bd206–359–GAL4 was generated by deletion of the internal *Nae*I fragment from B1–551–GAL4. Bd245–478–GAL4 was generated by deletion of the internal *Nco*I fragment of B1–551–GAL4. Bd384–544–GAL4 was generated by deletion of the internal *Nar*I fragment from B1–551–GAL4. For all yeast expression constructs described below, a stop codon was inserted after the GAL4 DNA-binding domain by the insertion of a streptomycin resistance gene flanked by stop codons in all frames and subsequent removal of the streptomycin resistance fragment as described by Frey and Krisch (1985). For plant expression constructs, translation of the fusion containing the GAL4 DNA-binding domain attached to the carboxyl terminus of B terminated in the Nos 3′ polyadenylation region.

The GAL4 DNA-binding region fused to the acidic region of C1 (Fig. 2, GAL4-C1) contains coding sequence for amino acids 1–147 of GAL4 fused to coding sequence for amino acids 173 to 273 of C1 as described previously (Goff et al. 1991). The GAL4 DNA-binding domain fused to the herpes simplex virus VP16 transcriptional activation domain (Fig. 2, pGAL4–VP16) contains the coding sequence for amino acids 1–147 of GAL4 fused to a coding sequence for 80 amino acids of VP16 (Triezenberg et al. 1988) under the control of the CaMV 35S promoter as described previously (Goff et al. 1991). The expressed C1-I cDNA (Fig. 3, C1-I) was described previously (Goff et al. 1991). C1-I/C1 fusion constructs were generated by fusion of the amino-terminal-encoding C1-I cDNA to the carboxy-terminal-encoding C1 cDNA at either the *Pst*I site (CIC1-1, C1-I amino acids 1–220 fused to C1 amino acids 221–273, Fig. 3) or *Aat*II site (CIC1-2, C1-I amino acids 1–142 fused to C1 amino acids 143–273, Fig. 3). C1Glu^{101} (Fig. 3) was generated by oligonucleotide-mediated, site-directed mutagenesis as described previously (Goff et al. 1991) and codes for glutamic acid rather than aspartic acid at C1 amino acid position 101.

The yeast constructs were generated by ligation of the appropriate B construct into a yeast-replicating vector (2μ origin, HIS3$^-$ selectable marker) with the phosphoglycerol kinase (PGK) promoter and a PGK terminator. In all cases, the ligation was at the start codon for the B constructs, effectively removing the *B* 5′-untranslated region. The C1 and C1 fusion constructs were ligated into a yeast-replicating vector (CEN/ARS origin, LEU2$^+$ selectable marker) with the glyceraldehyde phosphate dehydrogenase promoter and PGK terminator derived from pG-1 (Schena et al. 1991). Forty-five nucleotides of 5′-untranslated region from the *C1* gene remained in these constructs.

High-velocity microprojectile bombardment

Plasmids used for high-velocity microprojectile delivery were purified by equilibrium centrifugation in a cesium chloride gradient. Plasmid DNA was delivered to intact tissues by high-velocity microprojectiles using a custom built microprojectile gun (S. Goff, unpubl.) essentially as described previously (Klein et al. 1988, 1989). A total of 8 μg of plasmid DNA (at 1.0 mg/ml) was precipitated onto microprojectiles by the addition of 25 μl of tungsten powder (1.0 μm average diam., 50 mg/ml), followed by the addition of 25 μl of 1.0 M $CaCl_2$ and 10 μl of 100 mM spermidine free base. Microprojectiles were allowed to settle for ~3–5 min at room temperature, and 25 μl of overlying solution was discarded. As an alternate procedure, a fivefold increase in all components was used, and 300 μl of the supernatant was discarded. The resulting microprojectile solution was vortexed, and 2.5 μl of this slurry was loaded onto each macroprojectile for bombardment. Decreasing the standard amount of effector plasmid DNA used per preparation by as much as 10-fold did not significantly alter the results of the *trans*-activation experiments. Through the use of several independent preparations of plasmids encoding either the wild-type B or C1 cDNAs under the CaMV 35S promoter control, we have observed no significant differences in effector plasmid quality. Transformed tissues were analyzed 48 hr after bombardment following incubation at 24°C without illumination.

Enzyme assays

Anthocyanin biosynthesis was determined by visual detection of pigmented cells with 20- to 50-fold magnification under a dissecting microscope. Luciferase was assayed in tissue extracts as described previously (Callis et al. 1987). Luciferase activity is expressed as light units detected by an Analytical Scientific Instruments model 3010 Luminometer at room temperature for 10 sec. CAT activity was determined by the conversion of ^{14}C-labeled acetyl-CoA to ethyl acetate soluble counts per minute for 1 hr at 37°C and counted in scintillation fluor as described previously (Sleigh 1986). CAT activity is expressed as counts per minute.

Yeast transformation and growth

Yeast cultures were grown in YEP supplemented with 2% glucose to prepare cells for transformation. Transformation was accomplished using the lithium acetate procedure (Ito et al. 1983). Transformants were selected at 30°C on plates containing synthetic media supplemented with 2% glucose and lacking the appropriate amino acid (either −Leu, −His, or −Leu −His). Transformants were grown in synthetic media supplemented with glucose and transferred by a 1 : 25 dilution into synthetic media supplemented with 2% galactose and 2% raffinose, minus the appropriate amino acid. Cells were then grown at 30°C to log phase, and 200 μl of cell suspension was transferred to 800 μl of Z buffer, 50 μl of 0.1% SDS, and 2 drops of chloroform, vortexed for 20 sec, and assayed at 28°C with 200 μl of 4 mg/ml of ortho-nitrophenyl-β-D-galactopyranoside as described previously (Miller 1972). Values of β-galactosidase activity were calculated and are expressed according to Miller (1972).

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