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Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues

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The *C1*, *B* and *R* genes regulating the maize anthocyanin biosynthetic pathway encode tissue-specific regulatory proteins with similarities to transcriptional activators. The *C1* and *R* regulatory genes are usually responsible for pigmentation of seed tissues, and the *B-Peru* allele of *B*, but not the *B-I* allele, can substitute for *R* function in the seed. In this study, members of the *B* family of regulatory genes were delivered to intact maize tissues by high velocity microprojectiles. In colorless *r* aleurones or embryos, the introduction of the *B-Peru* genomic clone or the expressed cDNAs of *B-Peru* or *B-I* resulted in anthocyanin-producing cells. Luciferase produced from the *Bronze1* anthocyanin structural gene promoter was induced 100-fold when co-introduced with the expressed *B-Peru* or *B-I* cDNAs. This quantitative transactivation assay demonstrates that the proteins encoded by these two *B* alleles are equally able to transactivate the *Bronze1* promoter. Analogous results were obtained using embryogenic callus cells.These observations suggest that one major contribution towards tissue-specific anthocyanin synthesis controlled by the various alleles of the *B* and *R* genes is the differential expression of functionally similar proteins.

Key words: anthocyanin biosynthesis/microprojectile bombardment/regulatory genes/transactivation/transcription factors

Introduction

Extensive genetic analysis of the anthocyanin biosynthetic pathway in maize has identified a minimum of 20 loci involved in anthocyanin production (Coe *et al.*, 1988). At least four of these genes are believed to encode regulatory proteins that act specifically on the anthocyanin pathway. *Pl* is required for anthocyanin pigmentation in most of the plant body (Gerats *et al.*, 1984) although strong light can induce pigmentation in *pl* mutants (Coe *et al.*, 1988). *C1* is required for anthocyanin pigmentation of the aleurone and the embryo but is not required for pigmentation of 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). The *B* gene is required for anthocyanin synthesis in most parts of the plant body (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). For example, a functional *R* gene is normally required for pigmented aleurones, and specific *B* alleles, such as *B-Peru*, can substitute for *R* in this tissue. However, the *B-I* allele does not support pigmentation of the aleurone or embryo but does condition intense pigmentation in many other plant tissues independent of *R* (Styles *et al.*, 1973).

Molecular evidence in support of the proposed regulatory roles of *C1*, *R* and *B* is accumulating (Cone *et al.* 1986; Paz-Ares *et al.*, 1987; Chandler *et al.*, 1989; Ludwig *et al.*, 1989). *c1*, *r* or *b* mutations have been found to affect the levels of the mRNAs or enzymes encoded by some of the anthocyanin structural genes examined (Cone *et al.*, 1986; Dooner and Nelson, 1979; Dooner, 1983). *c1* mutants have low steady-state levels of the mRNAs and enzymes encoded by the *A1* (NADPH-dependent reductase) and *Bronze1* (*Bz1*, UDP glucose flavonol 3-O-glucosyl transferase) genes in aleurones (Cone *et al.*, 1986; Dooner and Nelson, 1979; Dooner, 1983). In *r* mutant aleurones, the mRNAs and enzymes encoded by the *A1* and the *C2* (chalcone synthetase) structural genes are not detectable (Ludwig *et al.*, 1989; Dooner and Nelson, 1979; Dooner, 1983), and the mRNAs and enzymes of the *A1*, *Bronze1* and *Bronze2* (unknown enzyme activity) structural genes are not detectable in *r* germinating seedlings (K.C.Cone, unpublished results; Taylor and Briggs, 1990). The presence of high levels of the mRNAs for the *A1* and *Bz1* genes requires a functional *B* gene in ear husk tissue (Chandler *et al.*, 1989).

Additional evidence for the regulatory roles of these genes comes from sequence analysis of their coding regions. Both *C1* (Paz-Ares *et al.*, 1986; Cone *et al.*, 1986) and *Lc*, a member of the *R* gene family (Ludwig *et al.*, 1989), have been cloned using transposon tagging strategies. Sequence analysis of *C1* revealed that it encodes a protein with amino-terminal homology to the DNA binding region of members of the *myb* oncogene family (Paz-Ares *et al.*, 1987). The *C1* protein also contains a region rich in acidic amino acid residues similar to the acidic activating domains found in many transcriptional regulatory proteins (Ptashne, 1988). Sequence analysis of the coding region of *Lc* revealed that the *Lc* gene product has homology to the helix–loop–helix domain of *myc* oncogene proteins (Ludwig *et al.*, 1989). *R-S* (Perrot and Cone, 1989), *B-Peru*, and *B-I* coding sequences have also recently been analyzed and found to be highly homologous to the *Lc* member of the *R* family (V.L.Chandler, unpublished results; Ludwig *et al.*, 1989). Studies on the helix–loop–helix domain of *myc*-like

immunoglobulin enhancer-binding proteins have shown that this conserved domain is required for DNA-binding and subunit dimerization (Murre *et al.*, 1989a and b). Both the *Lc* and the *B* proteins have regions rich in acidic amino acids. The presence of regions with homology to known DNA-binding domains and of regions with similarity to transcriptional activation domains supports the proposed role of the *C1*, *R* and *B* gene products as direct regulators of the structural genes of the anthocyanin biosynthetic pathway (Paz-Ares *et al.*, 1987; Ludwig *et al.*, 1989; Chandler *et al.*, 1989).

In order to study the *B* gene product in more detail, we developed an *in vivo* functional assay system for the *B-Peru* gene and the expressed *B-Peru* and *B-I* cDNAs. These DNAs were introduced into intact aleurone and embryo tissues by high velocity microprojectiles since this method was previously shown to give regulated expression of the *A1* and *Bz1* anthocyanin biosynthetic genes in these tissues (Klein *et al.*, 1989). The ability of the transferred *B-Peru* and *B-I* regulatory genes to activate *de novo* anthocyanin biosynthesis in *r* aleurones and embryos was examined. Additionally, the *B-Peru* and *B-I* gene products were tested for their ability to transactivate the *A1* and *Bz1* promoters, which are known to be under *B-Peru* regulation in *r* aleurones (Styles *et al.*, 1973). Embryogenic callus was examined as an alternative tissue in which the anthocyanin regulatory genes' function could be assayed.

Results

Complementation of r regulatory mutations

Different *B* alleles show extensive allelic diversity in both the timing of pigment synthesis during development and the tissue-specificity of pigment synthesis. Most alleles of *B* (such as *B-I*) do not complement *r* mutations in the aleurone or in the embryo. Therefore, we tested whether the delivery and expression of either the *B-Peru* gene, *B-Peru* cDNA or *B-I* cDNA into intact *r* aleurones and embryos could cause recipient cells to produce anthocyanin pigments. The structures of these constructs are shown in Figure 1. The *B-Peru* gene (p*B-Peru*gen) is expressed using its own promoter, while the *B-Peru* cDNA (p35S*B-Peru*) and *B-I* cDNA (p35S*B-I*) are expressed from the cauliflower mosaic virus 35S promoter as described in Materials and methods. The pMF6 expression vector used had been shown to express other coding regions such as that of firefly luciferase in aleurones, embryos, embryogenic callus and BMS cells (M.E.Fromm, unpublished results).

As expected, *r* aleurone or embryo tissues bombarded with the pMF6 vector plasmid lacking a *B-I* or *B-Peru* cDNA insert displayed no anthocyanin producing cells (Figure 2A and data not shown). *r* aleurones and embryos receiving p*B-Peru*gen, p35S*B-Peru*, or p35S*B-I* exhibited numerous single cells containing pigment (Figure 2B, C and D, and data not shown). These results demonstrate that the requirement for an *R* regulatory gene in aleurones and embryos can be bypassed via expression of an introduced *B-Peru* gene or cDNA as expected from genetic studies. Pigment formation in *r* tissue by the expression of the *B-I* cDNA demonstrates that the *B-I* protein can functionally substitute for the *R* gene product in aleurones. Thus this approach demonstrates that the *B-I* gene product can function in tissues where, by genetic evidence, the *B-I* gene does not induce the anthocyanin pathway.

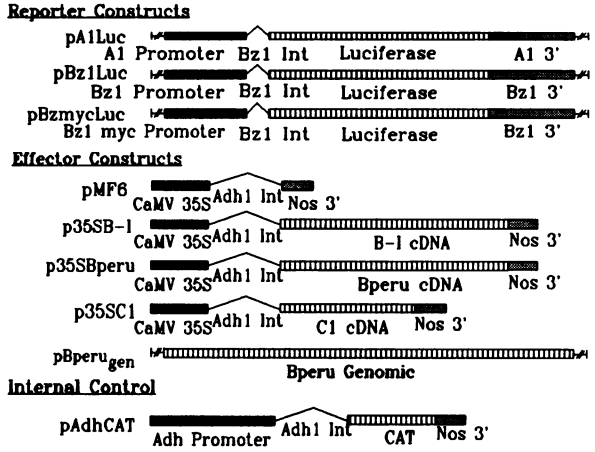

Fig. 1. Plasmid constructs. Plasmids used in this study are described in Materials and methods. Plasmid constructs are not drawn to scale. The BzmycLuc construct contains the intact 2.3 kb *Bronze1* promoter with the 6 bp substitution of the myc-like binding site (see Figure 3).

Transactivation of Bz1 and A1 in aleurones and embryos

Although accumulation of anthocyanin pigments in transformed cells demonstrates that the *B-Peru* and *B-I* proteins can activate *de novo* pigment synthesis, it is difficult to compare regulatory protein function quantitatively with this visual assay. In an attempt to develop a quantitative assay for the function of the *B* regulatory proteins, we examined their ability to *trans*-activate specific promoters. Luciferase production from a single anthocyanin structural gene promoter, either *Bz1* or *A1*, gives a simpler and more sensitive functional assay for *B-Peru* or *B-I* regulation than does anthocyanin pigmentation, which requires co-ordinate induction of many genes. This transactivation assay also provides a quantitative approach to compare various regulatory gene expression plasmids or structural gene promoter constructs, and allows analysis of specific transcriptional components. p*Bz1*Luc (Figure 1), which expresses luciferase from the *Bz1* promoter, was co-introduced into aleurones or embryos with the *B* constructs to determine the ability of the *B* regulatory proteins to induce structural gene promoters.

As an internal control for the efficiency of gene transfer, the p*Adh*CAT plasmid (Figure 1), containing the alcohol dehydrogenase 1 (*Adh1*) promoter driving the chloramphenicol acetyltransferase (CAT) coding region, was included in each DNA sample used for bombardment. The values of *B-Peru* or *B-I* transactivation of p*Bz1*Luc are therefore expressed as a ratio of the luciferase enzymatic activity to the CAT enzymatic activity generated from the pAdhCAT internal control plasmid. Tissues receiving only the p*Bz1*Luc reporter plasmid and p*Adh*CAT contained low ratios of luciferase to CAT, whereas tissues co-bombarded with either p*B-Peru*gen, p35S*B-Peru* or p35S*B-I* had 55- to 128-fold higher ratios of luciferase to CAT (Table I). Aleurones and embryos receiving the p*A1*Luc reporter plasmid and a *B* effector plasmid displayed, on average, 166- and 26-fold higher levels of luciferase:CAT respectively than did tissues receiving only p*A1*Luc and p*Adh*CAT (Table I).

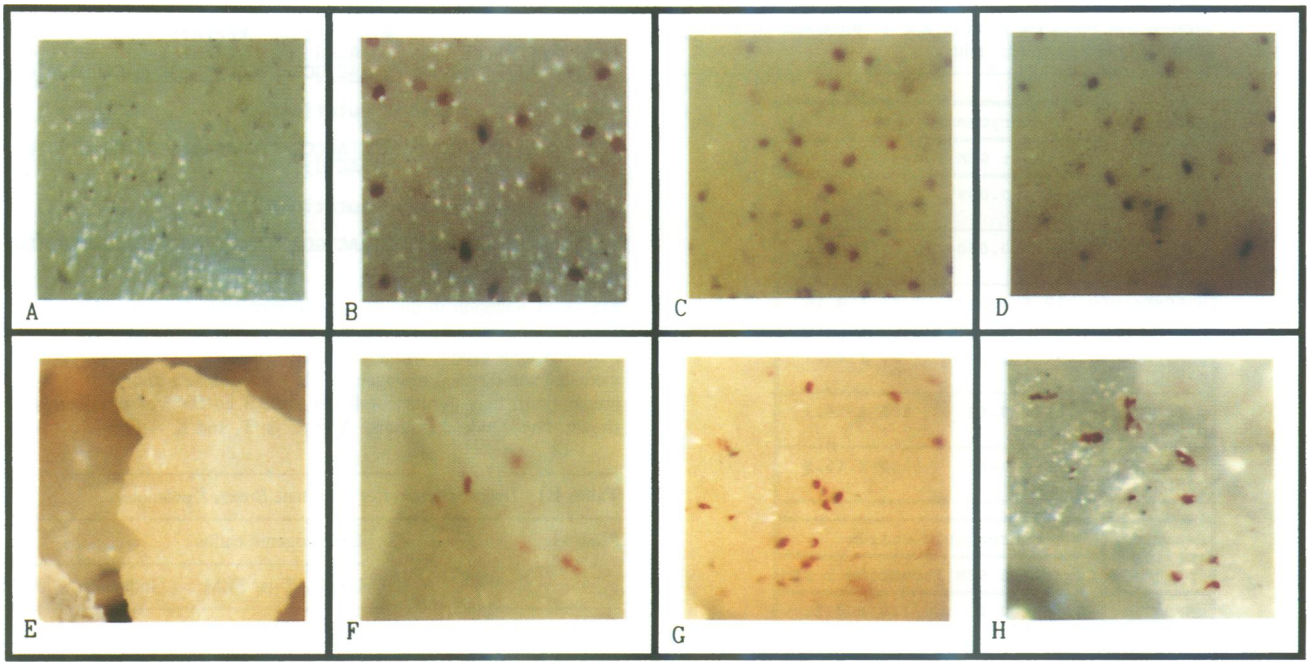

Fig. 2. Transactivation of anthocyanin synthesis following delivery of the *B* genes. Plasmid DNAs were delivered by high velocity microprojectile bombardment to aleurones and embryogenic callus as described in Materials and methods. Tissues were photographed after 24–48 h incubation. **Panel A**, *r* aleurone with pMF6; **panel B**, *r* aleurone with p*B-Peru*gen; **panel C**, *r* aleurone with p35S*B-Peru* cDNA; **panel D**, *r* aleurone with p35S*B-I* cDNA; **panel E**, embryogenic callus with p35S*C1* and pMF6; **panel F**, *r c1* embryogenic callus with p35S*C1* and p*B-Peru*gen; **panel G**, *r c1* embryogenic callus with p35S*C1* and p35S*B-Peru*; **panel H**, *r c1* embryogenic callus with p35S*C1* and p35S*B-I*.

Table I. *Trans*-activation of *Bronze1* and *A1* promoters by *B* regulatory proteins in aleurones and embryos.

Plasmids + p*Bz1*Luc + p*Adh*CAT	*C1 r b* aleurones		*C1 r b* embryos	
	Luc:CAT ± SEM	Induction ± SEM	Luc:CAT ± SEM	Induction ± SEM
pMF6	0.02 ± 0.006 n = 6	1.02 ± 0.29	0.02 ± 0.003 n = 8	0.98 ± 0.14
p*BPeru*gen	1.8 ± 0.5 n = 6	92 ± 19.1	1.40 ± 0.19 n = 8	70 ± 9.3
p35S*BPeru*	2.5 ± 0.2 n = 6	118 ± 9.9	1.10 ± 0.14 n = 9	55 ± 7.2
p35S*B-I*	2.7 ± 0.6 n = 6	128 ± 26.9	1.27 ± 0.30 n = 9	64 ± 14.8
	C1 r b aleurones		*C1 r b* embryos	
Plasmids + p*A1*Luc p*Adh*CAT	Luc:CAT ± SEM	Induction ± SEM	Luc:CAT ± SEM	Induction ± SEM
pMF6	0.03 ± 0.008 n = 5	0.99 ± 0.24	0.08 ± 0.018 n = 6	0.99 ± 0.23
p35S*BPeru*	5.3 ± 1.4 n = 3	166 ± 42.6	2.0 ± 0.33 n = 5	26 ± 4.3

Plasmid constructs were delivered to aleurones and embryos by high velocity microprojectile bombardment. Luciferase and CAT activities were determined as described in Materials and methods and are expressed as ratios of luciferase to CAT (Luc:CAT). Induction levels were determined by dividing the Luc:CAT ratio of each independent bombardment by the average Luc:CAT of the experiments using the control expression vector pMF6. Independent bombardments (n) were averaged and all values are expressed as the average plus or minus the standard errors of the mean (SEM).

The induced levels of luciferase:CAT from the *A1* and *Bz1* promoters are comparable (Table I).

Complementation in cultured cell lines

In an attempt to simplify and extend the usefulness of the system described above, we tested for *trans*-activation of the anthocyanin pathway by transferring the *B* genes into embryogenic callus cells. Following delivery of either p*B-Peru*gen, p35S*B-Peru*, or p35S*B-I* into embryogenic callus, no pigmented cells were observed (data not shown). Since this callus was derived from *r c1* embryos, we delivered p35S*C1* with each of the *B* plasmids. This combination results in the activation of the anthocyanin biosynthetic pathway and pigmented cells were observed (Figure 2F, G

Table II. *Trans*-activation of the *Bronze1* and *A1* promoters by *B* regulatory proteins in embryogenic callus.

Plasmids +pBz1Luc pAdhCAT	Embryogenic Callus	
	Luc/CAT ± SEM	Induction ± SEM
pMF6	0.044 ± 0.009 n=6	1.00 ± 0.20
pMF6 p35SC1	0.146 ± 0.030 n=6	3.31 ± 0.93
p35SBPeru pMF6	0.029 ± 0.007 n=6	0.65 ± 0.16
pBPerugen p35SC1	3.54 ± 1.09 n=6	80 ± 24.7
p35SBPeru p35SC1	4.64 ± 0.62 n=6	105 ± 14.1
p35SB-I p35SC1	5.95 ± 0.60 n=6	135 ± 13.5

Plasmids +pA1Luc pAdhCAT	Embryogenic Callus	
	Luc/CAT ± SEM	Induction ± SEM
pMF6 p35SC1	0.36 ± 0.10 n=6	1.0 ± 0.26
p35SB-I p35SC1	5.69 ± 0.34 n=6	15.7 ± 0.93

Plasmid DNAs were delivered to embryogenic callus cells by high velocity microprojectile bombardment as described in Materials and methods. Luciferase (Luc) and chloramphenicol acetyltransferase (CAT) activities in extracts of bombarded embryogenic callus are expressed as described in Table I.

and H). Anthocyanin synthesis was not restricted to a particular cell type. Tissue receiving the expression vector pMF6 or p35S*C1* alone displayed no pigmented cells (Figure 2E and data not shown). These results demonstrate that the expression of both the *B-Peru* (or *B-I*) and *C1* genes is required to activate the anthocyanin biosynthetic pathway in *r c1* embryogenic callus.

Transactivation of Bz1 and A1 in embryogenic callus cells

Transactivation of the p*Bz1*Luc and p*A1*Luc reporter genes by the *B* constructs was also examined in embryogenic callus. The results of these experiments were generally consistent with the anthocyanin pigmentation results described above, since the *B-I* or *B-Peru* gene alone was insufficient to *trans*-activate either p*Bz1*Luc or p*A1*Luc reporter plasmids delivered to embryogenic callus tissue (Table II and data not shown). Likewise, the p35S*C1* construct delivered alone did not result in an increase in luciferase activity from the p*Bz1*Luc reporter plasmid. *Trans*-activation of the p*Bz1*Luc of p*A1*Luc reporter constructs required delivery of one of the *B* genes with an expressed *C1* cDNA (Table II). In embryogenic callus, an 80- to 135-fold *trans*-activation of p*Bz1*Luc and a 15-fold *trans*-activation of p*A1*Luc were observed. The magnitude of induction of the *A1* promoter appears lower than that of the *Bz1* promoter, but this is due to higher basal luciferase activity produced by the p*A1*Luc construct. Thus embryogenic callus appears similar to aleurones and embryos in this *trans*-activation assay.

Analysis of a mutant Bz1 promoter defective in induction

Deletion and linker-scanning analysis of the *Bz1* promoter has identified a 45 bp region essential for *R* and *C1* mediated induction (B.Roth and M.Fromm, in preparation).

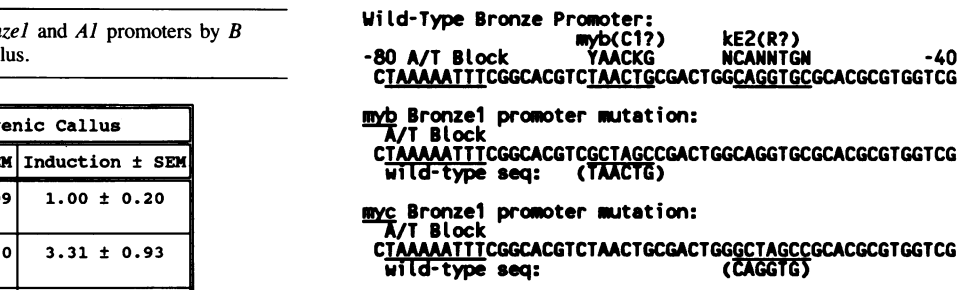

Fig. 3. A mutation in the *Bronze1* promoter blocks transactivation. Sequence of wild-type and mutant *Bronze1* promoters used in transactivation assays. Mutations were generated by oligonucleotide-mediated site-directed mutagenesis (B.Roth and M.Fromm, in preparation); only 40 nucleotides of the 2.3 kb *Bronze1* promoter are shown. Nucleotide definitions: Y = C or T, and K = G or T.

Table III. *Trans*-activation of a mutant *Bronze1* promoter.

Plasmids	Embryogenic callus	
	Luc:CAT ± SEM	Induction ± SEM
pMF6 p*Bz1*Luc	0.041 ± 0.006	0.98 ± 0.17
p*Adh*CAT	n = 9	
p35S*BPeru* p*Bz1*Luc	4.4 ± 0.43	107 ± 13
p35S*C1* p*Adh*CAT	n = 9	
p35S*BPeru* p*Bz*mybLuc	2.55 ± 0.61	62 ± 15
p35S*C1* p*Adh*CAT	n = 6	
p35S*BPeru* p*Bz*mycLuc	0.15 ± 0.02	3.7 ± 0.5
p35S*C1* p*Adh*CAT	n = 6	

Luciferase (Luc) and chloramphenicol acetyltransferase (CAT) activities in extracts of bombarded embryogenic callus were determined as described in Materials and methods. Activities are expressed as ratios of luciferase to chloramphenicol acetyltransferase (Luc:CAT). The p*Bz*mycLuc and p*Bz*mybLuc plasmids are altered in 6 nucleotides which constitute a myc or myb consensus DNA-binding site respectively.

Interestingly, this region contains sequences similar to *myb* (Biedenkapp *et al.*, 1988) and immunoglobulin kE2 enhancer binding sites (*myc*-related) (Sen and Baltimore, 1986) as shown in Figure 3. These observations are intriguing given the homology between *C1* and *myb*-related proteins and between *B/R* and *myc*-related proteins. We therefore asked whether mutations in these sites interfered with the ability of the *B* constructs to *trans*-activate the *Bz1* promoter. The sequences of the altered promoters (referred to as p*Bz*mybLuc and p*Bz*mycLuc) are shown in Figure 3. Embryogenic callus receiving p35S*B-Peru*, p35S*C1* and the p*Bz*mycLuc reporter contained only 5% of the induced level of luciferase to CAT activity relative to callus receiving p35S*B-Peru*, p35S*C1* and p*Bz1*Luc (Table III). Similar results were obtained using *C1 b r-g* aleurones as the target tissue (data not shown). Substitution of p35S*B-I* for p35S*B-Peru* in such an experiment also resulted in low levels of induced luciferase to CAT activity (data not shown). These observations demonstrate that this 6 bp region is critical for induction of the *Bronze1* promoter not only by the *R*-like *B-Peru* regulatory protein but also by the product of the *B-I* gene. Mutagenesis of the *myb*-like binding site decreased the *B*-mediated induction by only two-fold. Since the effect of the *myb* DNA binding site mutation is small, more experiments will be required to determine whether the *myb* consensus binding site within the *Bz1* promoter is important for transcription.

Discussion

The introduction of the *B-Peru*gen, p35S*B-Peru* or p35S*B-I* DNAs into *r* aleurone cells *trans*-activates the anthocyanin biosynthetic pathway. Co-transformation of the *B-Peru* or *B-I* genes with the p*Bz1*Luc or p*A1*Luc reporter constructs results in activation of the *Bz1* or *A1* promoters in *r* aleurones or embryos. Co-transformation of p*Bz1*Luc or p*A1*Luc with p35S*C1* and one of the *B* genes leads to activation of the *Bz1* or *A1* promoters in *c1 r* embryogenic callus cells. These results establish that the *B* clones used in this study contain functional coding regions. These assays alone would not distinguish between an *R* gene and any of the *B-Peru* or *B-I* genes. However, other evidence such as chromosome map position (i.e. *R* is located on chromosome 10, while *B* is on chromosome 2) and transposon mutation and reversion data help to confirm the identity of the *B-Peru* gene as well as the *B-Peru* and *B-I* cDNAs as functional *B-Peru* or *B-I* coding sequences (Chandler *et al.*, 1989). It has also recently been demonstrated that microprojectile delivery of the *Lc* gene to various maize tissues results in pigmented cells (Ludwig *et al.*, 1990).

The purple pigmented cells observed in *r* aleurones and embryos indicates that the inactive set of anthocyanin biosynthetic genes in these cells is activated following delivery of the *B-Peru* gene or an expressed cDNA for this gene. Furthermore, the activation of anthocyanin biosynthetic genes in aleurones and embryos by the expression of the introduced *B-I* cDNA demonstrates that the *B-I* regulatory protein, which is normally employed in the plant body, is functionally equivalent to, and can substitute for the *R* gene product in aleurones and embryos. This observation suggests that the control of anthocyanin biosynthesis in particular tissues is achieved by the tissue-specific expression of the *B* or *R* regulatory protein rather than by the functional specificity of the *B* or *R* polypeptides. Consistent with this hypothesis, the differential distribution of anthocyanin pigments in husks and seeds of *B-Peru* and *B-I* plants correlates with the differential steady-state levels of *B* mRNA (Chandler *et al.*, 1989; J.P.Radicella and V.Chandler, unpublished results).

Previous investigations have shown that it is possible to use a transient expression assay to study the effects of mutations in the *cis*-acting sites of the *Bz1* promoter (B.Roth and M.Fromm, in preparation). In that study, a 6 nucleotide region with homology to the consensus DNA-binding site for *myc*-like proteins (Sen and Baltimore, 1986) was identified as critical for *Bz1* promoter function. It was proposed that this *myc* consensus site was the site at which *R* might bind based on the homology of *R* to *myc*. The results presented in this study demonstrate that this same mutation in the *myc* DNA-binding consensus sequence also prevents *trans*-activation of the *Bz1* promoter in the presence of the *B-I* or *B-Peru* gene products, which are also homologous to *myc* (Chandler *et al.*, 1989). These results suggest that the *R* and *B* polypeptides mediate their effects through the same site in the *Bz1* promoter. Although the results presented in this study do not provide evidence that members of the *B* family of proteins interact directly with the *Bronze* or *A1* promoters, they are consistent with and provide support for such an interpretation. Further *in vitro* and *in vivo* studies will be necessary to obtain definitive evidence for a direct interaction of the *B* and *C1* proteins with the genes they regulate.

The activation of the *Bronze* and *A1* promoters or the genes for the entire anthrocyanin biosynthetic pathway in embryogenic callus tissue indicates that this system is a valid alternative to the time-consuming process of isolating aleurones and/or embryos. If a *c1 r* embryogenic callus genotype is used, such as A188XB73 material, then many combinations of permissive or non-permissive genotypes are available by inclusion of the appropriate set of *C1*, *B* or *R* plasmids. Complementation of *c1* and *r* by the *C1* and *B* genes in embryogenic callus allows visible detection of the cells that received the plasmid DNAs. Although the majority of these cells only transiently express the acquired DNA, a low percentage of the cells are expected to incorporate the introduced genes stably. It may therefore be possible to visually screen for rare, stably transformed sectors of embryogenic callus and regenerate these sectors to give mature plants.

Several previous studies with gene transfer by high velocity microprojectiles into intact plant tissues have demonstrated that the transferred genes were properly regulated (Bruce *et al.*, 1989; Klein *et al.*, 1989). The *A1* and *Bz1* anthocyanin genes exhibited the appropriate genetic and tissue-specific expression in aleurones and embryos of different *R* and *C1* genotypes (Klein *et al.*, 1989). An analysis of the expression of an extensive series of deletions and linker-scanning mutations of the *Bz1* promoter localized the critical regulatory region to ~ 45 bp (B.Roth and M.Fromm, in preparation). High velocity microprojectile delivery of DNA has also been used to demonstrate that the oat phytochrome promoter is properly regulated by red/far red light conditions in rice coleoptiles (Bruce *et al.*, 1989), and that the promoter of a tomato pollen-specific gene is highly expressed in pollen but not in leaf tissues (Twell *et al.*, 1989). The present studies extend the use of gene transfer by high velocity microprojectiles to the analysis of regulatory genes. The transferred regulatory genes appear to regulate the appropriate endogenous genes or co-introduced reporter gene constructs. Such assays can serve to confirm the identity and integrity of cloned regulatory genes in cases where a phenotypic response can be observed at the single cell level. Alternatively, the proper regulation of a co-transferred structural gene can also be used as an indicator of a functional regulatory gene. This *in vivo* functional assay can be extended to study the effect of coding region mutations and protein domain substitutions on plant regulatory proteins.

Materials and methods

Plant materials

Aleurones and embryos were isolated from *C1 b r-g* immature kernels as previously described (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 medium (Lowe *et al.*, 1985).

Plasmids

The pMF6 expression vector consists of the cauliflower mosaic virus (CaMV) 35S promoter (0.5 kb), the maize alcohol dehydrogenase 1 (*Adh1*) first intron (0.5 kb) and the nopaline synthase (*nos*) polyadenylation region (0.25 kb) as previously described (Callis *et al.*, 1987), with additional restriction sites between the *Adh1* intron and *nos* fragments. The *B-Peru* genomic clone was isolated as previously described (Chandler *et al.*, 1989). The 11 kb clone contains the following: ~4 kb 5′ to the start of translation; ~3.5 kb of coding region and ~3.5 kb 3′ to the polyadenylation signal. The multiple introns present in the *B-Peru* genomic clone are not shown. The

genomic clone was used to isolate *B-Peru* and *B-I* cDNA clones (each 1.9 kb) by screening λgt11 libraries prepared from *B-Peru r-g* or *B-I r-g* husks (J.P.Radicella, D.Turks and V.Chandler, unpublished results) by standard techniques. The identity of the clones was confirmed by DNA sequencing. The C1 cDNA (1.1 kb) was isolated from a λgt11 library prepared from poly A selected mRNA from color-converted W22 (Brink derivation) 30 days post-pollination (K.C.Cone, unpublished data). The *B* cDNAs and the *C1* cDNA were expressed via insertion into the expression vector pMF6. The *Bronze1* promoter–luciferase (p*Bz1*Luc) and *A1* promoter–luciferase (p*A1*Luc) plasmids were previously described (Klein *et al.*, 1989). The alcohol dehydrogenase promoter–chloramphenicol acetyltransferase plasmid (p*Adh*CAT) used in this study was previously described as pAI1CN (Callis *et al.*, 1987). Structures of all plasmids used in this study are presented in Figure 1. Plasmids transferred into cells were isolated by equilibrium ultracentrifugation in a cesium chloride gradient as previously described.

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 μg of plasmid DNA was used in each microprojectile preparation. For delivery to aleurone or embryos, 3 μg of effector plasmid, 3 μg of reporter plasmid and 2 μg of internal control plasmid (p*Adh*CAT) were used. For delivery to embryogenic callus, 2 μg of each plasmid was used. Plasmid DNA was precipitated onto 25 μl of 50 mg/ml 1.0 μm tungsten microprojectiles by the addition of 25 μl 1.0 M $CaCl_2$ and 10 μl 100 mM spermidine free base. Particles and DNA were allowed to settle, and 50 μl of the solution was removed. 2.5 μl of the resulting particle suspension was used per bombardment. Tissues were analyzed after 24–48 h incubation at 24°C. Aleurones and embryos were incubated with illumination, and embryogenic callus cells were incubated without illumination.

Enzyme assays

In each experiment, the *r* aleurones or embryos were bombarded with a mixture of the appropriate plasmid DNAs and incubated under illumination for 24–48 h 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 (Luc) was assayed in tissue extracts as previously described (Callis *et al.*, 1987), and is expressed as the number of light units detected in 10 s at 25°C per 100 μl of extract. Chloramphenicol acetyltransferase (CAT) activity was determined by the conversion of ^{14}C-labeled acetyl-CoA to ethyl acetate soluble CPM for 1 h at 37°C and counted in EcoScint scintillation fluor as previously described (Sleigh, 1986), and are expressed as counts per minute generated from 25 μl of extract.

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