Molecular Consequences of *Ds* Insertion Into and Excision From the Helix-Loop-Helix Domain of the Maize *R* Gene

Yanhong Liu,*,[1] Liangjiang Wang,* Jerry L. Kermicle[‡] and Susan R. Wessler*,[†]

**Department of Botany and [†]Department of Genetics, University of Georgia, Athens, Georgia 30602 and [‡]Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706*

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ABSTRACT

The R and B proteins of maize are required to activate the transcription of several genes in the anthocyanin biosynthetic pathway. To determine the structural requirements for *R* function *in vivo*, we are exploiting its sensitive mutant phenotype to identify transposon (*Ds*) insertions that disrupt critical domains. Here we report that the ability of the *r-m1* allele to activate transcription of at least three structural genes is reduced to only 2% of wild-type activity because of a 396-bp *Ds* element in helix 2 of the basic helix-loop-helix (bHLH) motif. Residual activity likely results from the synthesis of a mutant protein that contains seven additional amino acids in helix 2. This protein is encoded by a transcript where most of the *Ds* sequence has been spliced from pre-mRNA. Two phenotypic classes of stable derivative alleles, very pale and extremely pale, condition <1% of wild-type activity as a result of the presence of two- and three-amino-acid insertions, respectively, at the site of *Ds* excision. Localization of these mutant proteins to the nucleus indicates a requirement for an intact bHLH domain after nuclear import. The fact that deletion of the entire bHLH domain has only a minor effect on R protein activity while these small insertions virtually abolish activity suggests that deletion of the bHLH domain may bypass a requirement for bHLH-mediated protein-protein interactions in the activation of the structural genes in the anthocyanin biosynthetic pathway.

THE anthocyanin biosynthetic pathway is proving to be an ideal system for the study of gene regulation in higher plants. The presence or absence of pigment is a sensitive, nonlethal phenotype that has been exploited in the identification of >10 loci required for expression of the wild-type purple color (Coe *et al.* 1988). The pathway is controlled by the composition of two small gene families, *R/B* and *C1/Pl.* One functional gene from each family is required, in addition to the structural genes, for pigmentation of a given tissue. The *R/B* gene family consists of numerous alleles at both the *R* locus, located on the long arm of chromosome *10*, and the *B* locus, located on the short arm of chromosome *2* (reviewed in Ludwig and Wessler 1990; Dooner and Robbins 1991).

R and B proteins are functionally homologous. *Lc*, the first member of the *R* gene family to be sequenced (Ludwig *et al.* 1989), shares ~97% amino acid identity with the R proteins encoded by other members of the *R* gene family, including *Sn*, S, *R-sc*, and *P* (Perrot and Cone 1989; Consonni *et al.* 1993; M. Alleman, J. Kermicle and S. Dellaporta, personal communication), and >80% amino acid identity with *B-Peru* and *B-I* (Radicella *et al.* 1991, 1992). Transient transformation studies have demonstrated that the constitutive expression of R/B proteins is sufficient to activate the anthocyanin pathway in virtually all tissues (Goff *et al.* 1990; Ludwig *et al.* 1990).

Consistent with their role as regulatory proteins, R/B proteins contain the basic helix-loop-helix (bHLH) domain (Chandler *et al.* 1989; Ludwig *et al.* 1989). *R* also contains three nuclear localization sequences (NLSs, designated NLS-A, NLS-M, NLS-C; see Figure 2A), defined after bombardment of onion cells with the reporter gene *GUS* fused to parts of the *R* gene (Shieh *et al.* 1993).

R/B proteins act in concert with the C1 protein, which contains a *myb*-domain, to activate transcription of at least four structural genes in the anthocyanin pathway (Paz-Ares *et al.* 1987; Goff *et al.* 1990; Ludwig and Wessler 1990; Roth *et al.* 1991; Bodeau and Walbot 1992). There is no activation unless both R/B and C1 proteins are present simultaneously. Transient transformation assays have been used extensively to identify important structural motifs in the C1 and B proteins and to establish that they interact (Goff *et al.* 1992; Sainz *et al.* 1997a,b). These studies involve the cotransformation into maize tissue of the luciferase reporter gene fused to the *bz1* structural gene promoter with constitutively expressed but mutant *B* or *C1* genes. Surprisingly, deletion of the entire bHLH domain and two of the three NLSs results in only a twofold reduction in the ability of *B* to activate reporter gene transcription in this assay system (Goff *et al.* 1992).

Corresponding author: Susan R. Wessler, Department of Genetics, Life Sciences Bldg., University of Georgia, Athens, GA 30602.
E-mail: sue@dogwood.botany.uga.edu

[1]*Current address:* Waksman Institute, Rutgers University, P.O. Box 759, Piscataway, NJ 08855.

As an alternative approach, we have been using a collection of 43 *Ds*-induced alleles of the *R-sc* gene (Kermicle *et al.* 1989; Alleman and Kermicle 1993) to determine which structural motifs are important for *R* function. In a previous study, we demonstrated that a *Ds* insertion in the *r-m9* allele led to the synthesis of a truncated R protein that conditioned only 5% of wild-type activity because of the deletion of the C-terminal NLS-C (Liu *et al.* 1996). In this study, we report that the *r-m1* allele has an insertion of a *Ds* element in the bHLH domain. Characterization of this allele and its stable derivatives indicates that two- and three-amino-acid insertions in helix 2 of the bHLH domain dramatically reduce *R* activity both *in vivo* and after transient transformation of mutant constructs into maize cells. The fact that deletion of the entire bHLH domain had only a minor effect on activity in transient transformation assays led others to conclude that the bHLH domain is not required for transactivation (Goff *et al.* 1992; Tuerck and Fromm 1994). Taken together, the data now suggest that bHLH-mediated protein-protein interactions are required for R protein to activate the structural genes in the anthocyanin pathway, and that deletion of the bHLH domain bypasses this requirement.

MATERIALS AND METHODS

Plant stocks: All strains used in this study were in the W22 background and homozygous for the designated allele. The *r-m1* allele was isolated as described previously (Kermicle 1980). The *R-sc* allele conditions deep purple pigmentation in the aleurone of the mature seed. The *R-g:8 pale* allele conditions stable dilute seed color and a colorless plant. The *r-r* allele pigments the plant parts. *P-vv* conditions red-striped (variegated) pericarp and cob, whereas *P-wr* produces colorless pericarp and colored cob. The *wx* gene conditions amylose synthesis in the endosperm.

D1–D5 were obtained by pollinating homozygous *r-m1; P-vv* (red striped pericarp, *Ac* allele) with *R-g:8 pale* (stable pale seed color); *wx* in a detasseling plot isolated from other maize. Single *R-sc* kernels were selected. *R-g:8 pale* and *wx*, which served as pollen contamination markers, were segregated in the progeny to confirm male parentage. *D6–D12* were isolated as follows: the *r-m1, P-vv* stock was crossed to *R-g:8 pale; P-wr*, spotted kernels were selected and pollinated with *r-r*. Of the 228 plants tested, 21 displayed no spotted kernels. The colorless (*D6–D9*) and pale (*D10–D12*) kernels were chosen for this study.

Anthocyanin extraction and quantification: Anthocyanins were extracted from 10 mg of dissected aleurones, as described previously (Rabino and Mancinelli 1986). Absorbance at 530 nm was used to measure anthocyanin content. For each sample, three independent experiments were performed. All values are normalized to the value for *R-sc*. Anthocyanin content was calculated for each sample as follows: $[A_{530}\text{-}A_{530(r\text{-}g)}]/[A_{530(R\text{-}sc)}\text{-}A_{530(r\text{-}g)}]$.

DNA extraction and PCR amplification: Maize genomic DNA was isolated as described previously from 2-wk-old seedlings (Shure *et al.* 1983). The *Ds* insertion sites in *r-m1* and *D1–D12* were determined after PCR amplification of genomic DNA using *Lc (R)* gene-specific primer 1 [5′CCTCGTCCTC AAGTCACTGC3′ corresponding to positions +1531 to +1550 of the *Lc* sequence (Ludwig *et al.* 1989)] and primer 2 [5′GCA GACCTCCTTCCTCACAC3′, positions +1722 to +1741 of the *Lc* sequence (Ludwig *et al.* 1989)]. PCR conditions were as described (Weil and Wessler 1993). PCR products were cloned into the TA cloning vector (Invitrogen, Carlsbad, CA), and sequences were determined by the Molecular Genetics Instrumentation Facility at the University of Georgia.

RNA isolation and blot analysis: Total RNA was extracted from immature kernels 25 days after pollination (DAP) as described (Fedoroff *et al.* 1983) and poly(A)$^+$ RNA was purified with PolyATract mRNA Isolation Systems (Promega, Madison, WI) according to the manufacturer's directions. RNA blots were performed as described previously (Ludwig *et al.* 1989) and probed with a *Kpn*I/*Pst*I fragment from the *a1* gene (O'Reilly *et al.* 1985), a 1.5-kb *Eco*RI fragment of *c2* cDNA (Niesbach-Klosgen *et al.* 1987), a 0.8-kb *Pst*I fragment of *Lc* cDNA (Ludwig *et al.* 1989), or *pMAc1* containing part of the maize actin gene (Shah *et al.* 1983). Probes were labeled using random primers with [α-^{32}P]dCTP and [α-^{32}P]dATP (Feinberg and Volgelstein 1983). Hybridization signals were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA) to determine the intensity of hybridization to the probe of interest and dividing by the signal of the actin control.

3′ RACE and PCR amplification of cDNA: 3′ RACE system (GIBCO BRL, Gaithersburg, MD) was used as described (Frohman *et al.* 1988) with modifications. Approximately 2 μg poly(A)$^+$ RNA from kernels isolated 25 DAP was used for first-strand cDNA synthesis. For reverse transcription, 0.5 μg of an oligo(dT) adapter primer (5′GACTCGAGTCGACAT GCT_{17}3′) was used. Primer 1 [5′CCTCGTCCTCAAGTCAC TGC3′ corresponding to positions +1531 to +1550 of the *Lc* sequence (Ludwig *et al.* 1989)] and the adapter primer without 17 dT residues were used to amplify target cDNAs. PCR samples were cycled 35 times through 1 min at 94°, 2 min at 56°, and 3 min at 72°. After final extension at 72° for 10 min, PCR products were cloned and sequenced.

Protein extraction and blot analysis: Protein extracts were isolated from aleurone tissue (25 DAP) as described (Radicella *et al.* 1992), quantified using the Bradford Protein Assay (Bio-Rad, Richmond, CA) according to the manufacturer, fractionated on an 8% SDS-polyacrylamide gel, and immunoblotted as described (Ausubel *et al.* 1987). Affinity-purified polyclonal Sn antibody [Sn protein is 97% identical to the R-sc protein (Consonni *et al.* 1992)] was used at 5.12 μg/ml. Alkaline phosphatase conjugated to goat anti-rabbit IgG (Promega) was used as the secondary antibody at a dilution of 1:2000, and the color reagents nitroblue tetrazolium and 5-bromo-4 chloro-3-indolylphosphate *p*-toluidine (Promega) were used to detect antibody binding.

As a positive control, *R* transcripts were synthesized from the *Lc* cDNA in the pGEM-7Z(+) expression vector and translated in rabbit reticulocyte lysates using the protocol provided by the manufacturer (Promega).

Immunolocalization: Immunocytochemistry was performed as described (Varagona *et al.* 1991) with the following modifications: frozen sections (10 μm) of immature kernels harvested 25 DAP were placed on slides pretreated with gelatin. Specific antibody and preimmune rabbit sera were used at 51.2 μg/ml. Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) served as the secondary antibody at a dilution of 1:100. Sections were viewed under a confocal laser microscope (Bio-Rad MRC 600 with Ar/Kr laser) with a 647-nm excitation filter. The location of the nuclei was verified by staining the same sections with 4′, 6′ diamidino-2′-phenylindoledihydroxychloride (DAPI) and viewing them using the confocal microscope as a standard epifluorescence microscope.

Plasmids: Construction of the following plasmids has been described previously: wild-type *Lc* expression vector (pPHI443,

Ludwig *et al.* 1990); wild-type *B* and a deletion derivative B-HLHdel (Goff *et al.* 1992); *C1* (Goff *et al.* 1990); Bz1Luc, a luciferase reporter fused to the *Bz1* promoter (Klein *et al.* 1989); and Adh1CAT [also called pAI1CN (Callis *et al.* 1987)], the *Adh1* promoter/CAT transformation control.

Lc-D12 was constructed by mutating the wild-type *Lc* cDNA in pPHI443 with the mutagenic oligonucleotide 5′-GCACCCT TCTCTGAAGCTCCTTGAGGTAGGGCTGGTAGGCTATCG TTTCGGCGAG-3′. Site-directed mutagenesis was carried out using PCR. In the same way, B-D12 and B-D6 were derived from the wild-type B expression vector using the mutagenic oligonucleotides 5′-GTACCCTTCGTTGAAGCTCCTTTAGG TAGGGCTGATAGGCTATCGTTTCGGCGAG-3′ and 5′-CGT TGAAGCTCCTTTAGATAGGGCAGGCTATCGTTTCGGC GAG-3′, respectively.

Particle bombardment: Maize suspension cells used for bombardment were prepared as described previously (Goff *et al.* 1992). Plasmid DNAs were precipitated onto 1.0-μm gold particles (60 mg/ml; Klein *et al.* 1989) and then delivered into maize cells with the Biolistic PDS-1000 (DuPont, Wilmington, DE). Each plate of cells was cobombarded with 0.2 μg of an Lc/B construct, 0.6 μg of p35SC1, 0.6 μg of Bz1Luc, and 0.6 μg of pAdh1CAT. After bombardment, the cells were incubated at 28° for 48 hr before enzyme assays.

Enzyme assays: Bombarded maize cells were ground in 350 μl of 100 mm KPO_4 (pH 7.80) and 1 mm DTT at 4°. After centrifugation, 25 and 10 μl of the supernatant were assayed for CAT and luciferase activities, respectively. CAT activity was expressed as the ethyl acetate-soluble CPM count (Sleigh 1986). Luciferase was assayed as described previously (Callis *et al.* 1987) using a model 3010 luminometer (Analytic Scientific Instruments). Luciferase activity was expressed as the number of light units detected in the first 10 sec of reaction at room temperature. Luciferase levels were adjusted by the CAT activity and expressed as the ratio of luciferase/CAT activities. Relative luciferase levels were calculated by dividing the average luciferase/CAT ratio for each construct by that of the wild-type (*Lc* or *B*) construct.

RESULTS

Phenotypes of *r-m1* and its derivatives (*D1–D12*): The progenitor *R-sc* allele conditions deep purple pigmentation in the aleurone of the mature seed (Figure 1). The *r-m1* allele was derived from *R-sc* after insertion of a *Ds* element. In the absence of the autonomous *Ac* element, *r-m1* displays pale aleurone pigmentation that is restricted to the crown of the kernel (Figure 1). In the presence of *Ac*, a few darkly pigmented spots can be seen on this pale background (data not shown). *D1–D12* are 12 germinal derivatives of *r-m1* that are both somatically and germinally stable when *Ac* is also in the genome. Of these, *D1–D5* display a deep purple revertant phenotype, *D6–D9* display a null colorless phenotype, and *D10–D12* display pale aleurone pigmentation that is restricted to the crown (Figure 1, arrowheads). The pigmentation conditioned by *D12* is reproducibly paler than that of *D10* and *D11*.

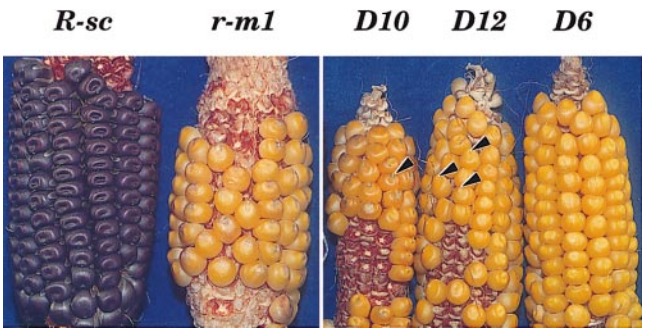

Figure 1.—Phenotypes of *R-sc*, *r-m1*, *D6*, *D10*, and *D12*. *D1–D5* are identical to *R-sc*, *D11* is identical to *D10*, and *D7–D9* are identical to *D6*. Pigmented areas in the pale derivative *D10* and *D12* are restricted to the crown (arrowheads).

To compare the expression of these alleles, anthocyanins were extracted from the aleurones of *R-sc*, *r-m1*, and the 12 derivative strains and quantified (Table 1). Kernels with a revertant phenotype (*D1–D5*) contained wild-type amounts of anthocyanin, while *r-m1* and its derivatives had 50- to 100-fold lower levels. Consistent with the visible phenotype, *D12* was found to have less anthocyanin than *D10* and *D11*. No anthocyanin was detected in the colorless *D6–D9* kernels.

***r-m1* contains a *Ds1* element:** From a previous study, it was known that *r-m1* had an insertion near the bHLH domain (Alleman and Kermicle 1993). The precise insertion site and sequence of the *Ds* element were determined after the amplification of this region by PCR and sequencing of the resultant product (see Figure 2A for the position of primers). Comparison of the *r-m1* sequence in this region with the *R-sc* sequence (M. Alleman and J. Kermicle, unpublished data) indicated that *r-m1* differed by the insertion of 404 bp into helix 2 of the bHLH motif (Figure 2, B and C). This insertion includes a 396-bp *Ds* element and an 8-bp direct repeat of host sequence generated upon element insertion (Figure 2C and data not shown). The *Ds* element shares >95% sequence identity with the *Ds1* class previously isolated from several *Ds*-induced alleles, including *adh1-Fm335*, *wx-m1*, and *bz-wm* (data not shown and Sutton *et al.* 1984; Wessler *et al.* 1986; Sullivan *et al.* 1989).

***r-m1*-encoded transcripts and proteins:** If transcribed and translated, the *r-m1* allele would encode a truncated protein as a result of the introduction of a premature stop codon 29 nt downstream from the 5′ terminus of the *Ds1* element. Although such a mutant protein could account for the residual activity of this allele, we considered the possibility that *r-m1* might encode an additional transcript because of the splicing of most of the *Ds1* sequences from pre-mRNA. Although the splicing of *Ds1* sequences has been shown to occur for the *Ds1*-induced alleles *adh1-Fm335* (Dennis *et al.* 1988) and *wx-m1* (Wessler 1991), in both cases the *Ds1* element was in the orientation opposite to that of *r-m1*. However, evidence for the splicing of *Ds1* sequences from pre-mRNA was obtained from RNA blot analysis of poly(A)$^+$ RNA isolated from *R-sc* and *r-m1*(*-Ac*) kernels (Figure 3). As can be seen, *r-m1* encodes at least two size classes of mRNA, one of wild-type size and one shorter. A larger transcript containing all of *Ds1* was not detected. Quanti-

TABLE 1

Anthocyanin content in *r-m1* and derivative alleles

Allele[a]	A_{530}[b]	Wild type[c] (%)
R-sc	1.64 ± 0.09	100
D1	1.63 ± 0.08	100
r-m1	0.07 ± 0.01	2
D6	0.04 ± 0.01	0
D10	0.06 ± 0.01	1
D12	0.05 ± 0.01	0.6
r-g	0.04 ± 0.01	0

[a] *D2–D5* are identical to *D1*, *D11* is identical to *D10*, and values for *D7–D9* are indistinguishable from *D6*.

[b] Data shown are mean ± SE calculated from three independent aleurone extractions and measurements.

[c] All values are normalized to *R-sc* (see materials and methods).

fication of the *r-m1* transcripts indicates approximately wild-type levels of accumulation (data not shown).

Mutant transcripts were further characterized using the 3′ RACE protocol with a poly(A) primer and an *R*-specific primer from upstream of the site of *Ds1* insertion. Two products were obtained: one of approximately wild-type size and the other shorter by ~700 bp. Sequencing of the longer 3′ RACE product revealed that this transcript differed from the wild-type transcript by the addition of 21 nt: 14 nt from the 5′ terminus of the *Ds1* element and 7 nt from the direct repeat downstream of the 3′ terminus of *Ds1* (Figure 2D). The new intron would be 383 nt long and flanked by the canonical GT and AG dinucleotides at the donor and acceptor splice sites, respectively. The sequence of the shorter 3′ RACE product reveals a new polyadenylation site in *Ds1* sequences, 116 nt downstream from the 5′ terminus of the element (Figure 2E).

Western blot analysis of aleurone extracts probed with R antibody identifies two proteins that could be the products of the two *r-m1* transcripts. The truncated transcript is predicted to encode a protein with 152 amino acids missing from the C terminus and 9 amino acids added from *Ds1* sequences. A smaller protein is, in fact, detected (see Figure 5). The larger *r-m1* transcript contains an in-frame insertion of 21 nt and could potentially encode a mutant protein with 7 additional amino acids

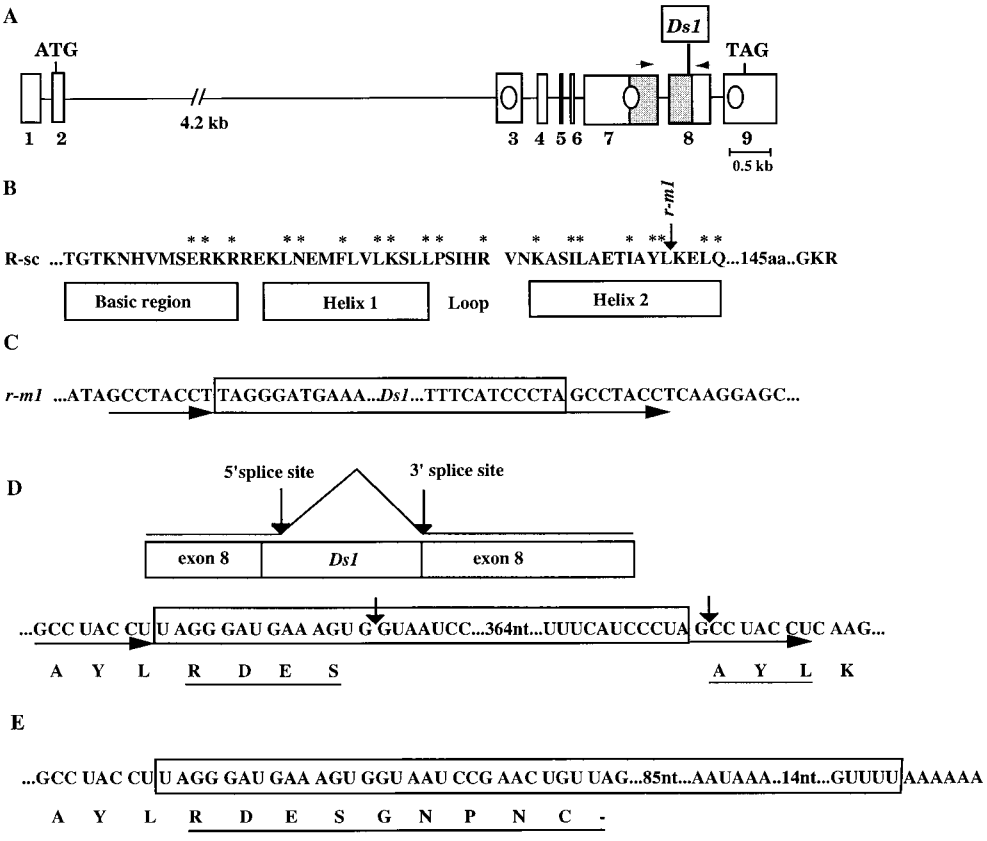

Figure 2.—*r-m1* gene structure and transcripts. (A) The position of the *Ds* insertion in the *r-m1* allele with respect to the exons (numbered open boxes) and introns (connecting lines) of the *R-sc* gene. The translation start and stop codons of *R-sc* and the approximate positions of NLSs (open circles; Shieh *et al.* 1993) are indicated. The positions of oligonucleotide primers used for PCR amplification (horizontal arrows) are also shown. The bHLH domain is represented by the shaded boxes in exons 7 and 8. (B) The *Ds* insertion site in helix 2. Amino acids are shown in one-letter code. Highly conserved amino acids in the bHLH domains of plants and animals are indicated by the asterisks (Littlewood and Evan 1995). The positions of the basic region, putative amphipathic helices 1 and 2, and the loop are shown below. (C) Sequence of the *Ds1* termini and flanking DNA in the *r-m1* allele. Horizontal arrows underscore the 8-bp host duplication. The *Ds* sequence data have been submitted to the GenBank database under accession number AF010445. (D) The deduced 5′ donor and 3′ acceptor splice sites in *r-m1* pre-mRNA. Shown is a schematic of the positions of the splice sites (top), where diagonal lines represent the intron. The precise 5′ and 3′ splice sites in the DNA sequence are also shown along with the predicted amino acid sequence of the spliced transcript. The seven-amino-acid insertion resulting from *Ds* splicing is underlined. (E) The polyadenylation site within the *Ds1* element of *r-m1*. *Ds1* sequences at the 3′ end of the *r-m1* transcript and deduced amino acid sequences are boxed. Additional amino acids encoded by *Ds1* sequences are underlined.

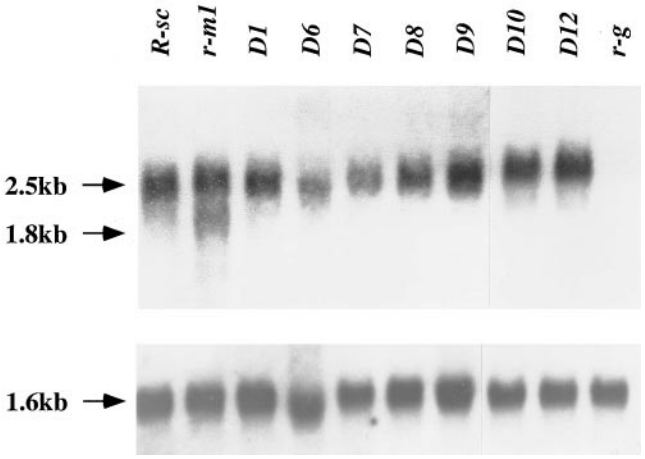

Figure 3.—RNA blot analysis of transcripts encoded by *r-m1* and its derivatives. Five micrograms of poly(A)$^+$ RNA isolated from the kernels of *R-sc*, *r-m1*, *D1*, *D6–D9*, *D10*, *D12*, and *r-g* were probed with *Lc* cDNA (top). To confirm equal loading in each lane, *Lc* probe was removed and the same filter was reprobed with the maize actin gene (bottom). *D2–D5* were found to be identical to *D1*, and *D11* was identical to *D10* (data not shown).

in the bHLH domain. A protein of wild-type size is also detected in *r-m1* aleurones.

The *Ds1* insertion site in *D1–D12*: DNA sequences at the *Ds* insertion site of the stable derivative alleles *D1–D12* were determined after PCR amplification of this region with *R*-specific primers (see Figure 2A for the position of primers). *D1–D5* were indistinguishable from wild type in this region, indicating either wild-type contamination or excision of all the *Ds1* element and one copy of the 8-bp repeat. The possibility of contamination was ruled out because all the derivative lines contained additional markers present in the *r-m1* progenitor (see materials and methods). *D6–D9* are phenotypically null and were found to contain frameshifting insertions of 5, 7, 7, and 8 bp, respectively (Figure 4). The pale derivatives *D10* and *D11* contained identical insertions of 6 bp, whereas the very pale *D12* harbored a 9-bp insertion (Figure 4).

The derivative alleles encode stable mRNA and protein: *D1–D12* encode transcripts of wild-type size. When the intensity of the hybridization signal in each sample was normalized to the actin control, it was found that RNA levels did not differ significantly in any sample (Figure 3 and data not shown). In contrast, no *R* mRNA is detected in the aleurone tissue of a strain harboring the null *r-g* allele (Figure 3). This suggests that the reduced activity of *D6–D12* is not caused by mRNA instability.

To address the related issue of mutant protein stability, relative levels of R protein in *D6–D12* were determined by protein blot analysis. As expected, the revertants *D1–D5* produced wild-type R proteins (Figure 5; data not shown). For each of the null alleles (*D6–D9*), an R-specific antibody recognizes truncated proteins (Figure 5). Finally, the pale and very pale derivatives, *D10* and *D12*, respectively, each produced wild-type levels of wild-type-sized proteins (Figure 5).

Transcript levels of structural genes in mutant *R* backgrounds: The presence of wild-type levels of mutant R proteins in *r-m1* and *D6–D12* suggests that these proteins are defective in their ability to activate the structural genes in the anthocyanin pathway. To address this question directly, the steady-state levels of transcripts encoded by two of the structural genes, *a1* and *c2*, were examined by RNA blots in strains harboring the *r-m1* allele and its derivatives (Figure 6). The results of this analysis clearly show that *a1* mRNA is virtually undetect-

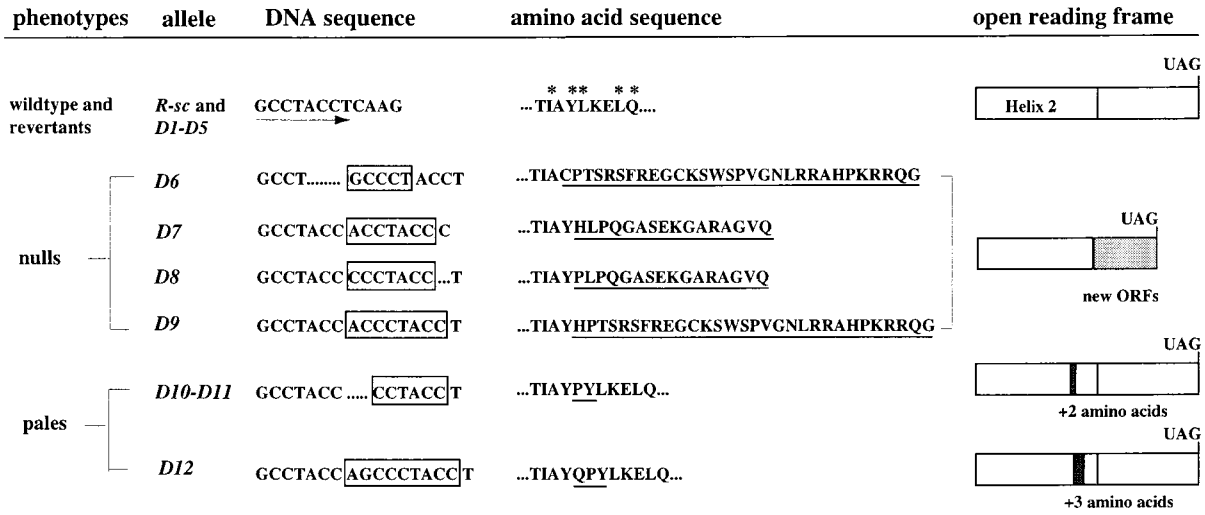

Figure 4.—Nucleotide and deduced amino acid sequences of *D1–D12* at the *Ds1* insertion site. The 8-bp duplication generated upon *Ds1* insertion is indicated by a horizontal arrow. Conserved amino acids in helix 2 are indicated by asterisks. Insertions caused by *Ds1* excision in *D6–D12* are boxed, and the derived amino acids are underlined.

able in the *R* mutant strains, while the level of *c2* is reduced to the levels seen in the null *r-g* background. The presence of some *c2* mRNA in the mutant and *r-g* backgrounds is probably caused by the expression of the second *c2* gene in the maize genome that is not induced by the R protein (Franken *et al.* 1991). Thus, reduced levels of *a1* and *c2* mRNA correlate with reduced levels of pigmentation in *r-m1* and *D6–D12*, indicating that the mutant R proteins are defective in their ability to activate transcription of the *a1* and *c2* genes. Similar results were obtained for the mRNA encoded by a third structural gene, *a2* (data not shown).

Subcellular localization of R proteins: On the basis of the data presented thus far, two scenarios could explain the failure of the mutant R proteins to activate the structural genes in the anthocyanin pathway. The amino acid additions to the bHLH domain may prevent a protein-protein interaction that is required to localize the R protein to the nucleus. Alternatively, the R proteins may be localized to the nucleus efficiently, but transactivation is impaired because the bHLH insertions interfere with protein-protein interactions. To test these two possibilities, immunolocalization experiments were performed. As shown in Figure 7, the wild-type R protein was localized to the nuclei of aleurone cells of 25 DAP *R-sc* kernels, as were the R proteins encoded by the *D10* and *D12* derivatives. In contrast, the proteins encoded by *r-m1* and *D6–D9* were localized to both the nucleus and the cytoplasm (Figure 7 and data not shown). A control section from the null *r-g* allele showed no specific staining, nor did a *R-sc* section stained with preimmune sera.

Activity of the mutant R protein in transient transformation assays: The data presented thus far suggest that an intact bHLH domain is required for R protein to function as a transcriptional activator. This conclusion differs from the conclusions of two previous studies, which showed that deletion of the entire bHLH domain of the *R* homologue *B* still gives rise to about half of the wild-type activity in transient transformation assays (Goff *et al.* 1992; Tuerck and Fromm 1994). Three scenarios could explain these apparently conflicting conclusions. Unlike the *in vivo* situation described here for *r-m1* and its derivatives, the B protein was overexpressed in transient transformation assays. It is possible that bHLH interactions are required to enhance the interaction between B/R and C1 proteins at the normally low physiological concentration, but not in the presence of a huge excess of B/R protein. Alternatively, deletion of the bHLH domain may alter the structure of B/R such that the need for bHLH interactions to activate transcription of the downstream structural genes is bypassed. Finally, although B and R proteins are homologues that are also functionally equivalent, it is possible that the bHLH domain is dispensable in the former but not in the latter.

To distinguish between these three possibilities, the D12 mutation was first incorporated into wild-type *R* (*Lc*) cDNA and assayed after bombardment into maize suspension cells (Figure 8). In this assay, the luciferase reporter gene fused to the *Bz1* promoter (Bz1Luc) was cobombarded so that luciferase levels provided a sensitive measure of *R* activity (Figure 8A). As shown in Figure 8B, the D12 mutation led to a 10-fold reduction in the ability of the R protein to activate transcription of the *Bz1* promoter. This result indicates that the D12 lesion dramatically reduces R activity both *in vivo* and in this transient assay system, and that overexpression of D12 protein does not significantly increase its activity.

To determine whether the nucleotide insertions characterized in this study had the same effect on the function of the B protein, the D12 and D6 lesions were incorporated into wild-type B cDNA in the constructs B-D12 and B-D6. Recall that the D6 mutant is phenotypically null because of a frameshifting insertion that leads to the synthesis of a truncated protein (Figures 1 and

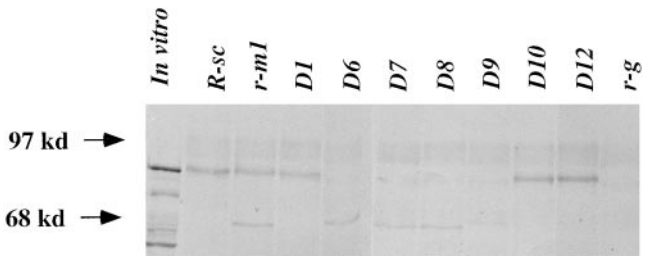

Figure 5.—Protein blot analysis of aleurone extracts from *r-m1* and its derivatives. Approximately 25 μg of protein extracted from dissected aleurone tissue (25 DAP) was resolved on an 8% SDS-polyacrylamide gel and probed with R-specific antibody. *In vitro*-translated R protein is also shown (larger bond).

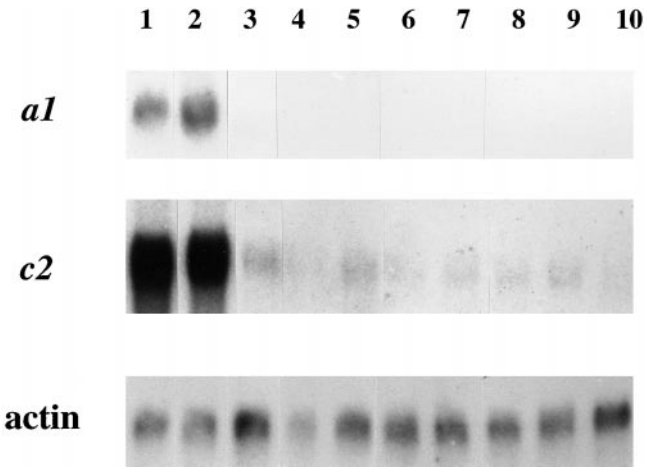

Figure 6.—RNA blots of *a1* and *c2* mRNA in different *R* backgrounds. Blots containing 5 μg poly(A)$^+$ RNA per lane were hybridized with *a1* and *c2* probes. The filter was reprobed with the maize actin gene. Lane 1, *R*-sc; lane 2, *D1*; lane 3, *r-m1*; lane 4, *D6*; lane 5, *D7*; lane 6, *D8*; lane 7, *D9*; lane 8, *D10*; lane 9, *D12*; lane 10, *r-g*.

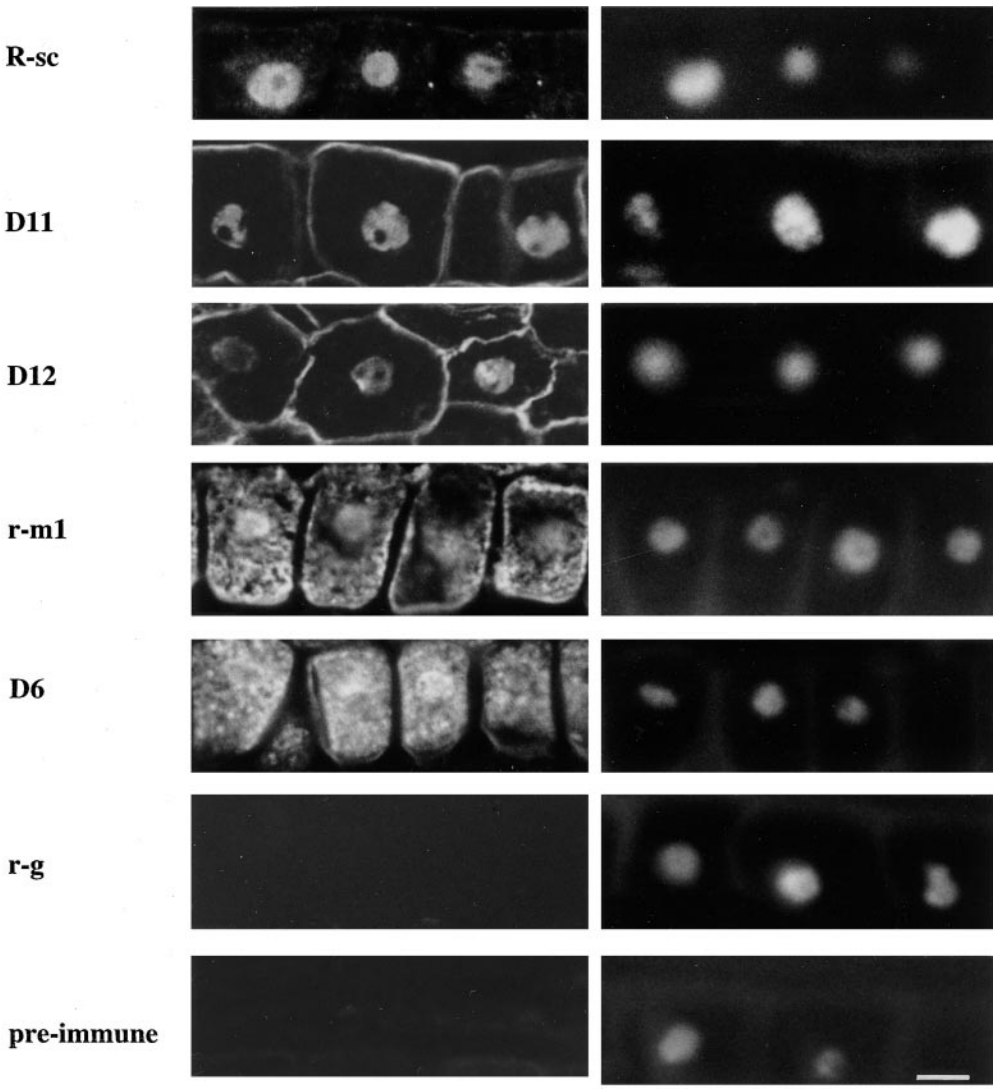

Figure 7.—Immunolocalization of mutant and nonmutant R proteins in aleurone cells. Sections were simultaneously stained with affinity-purified polyclonal Sn (R) antibody (left) and with DAPI to determine the location of the nuclei (right). Bar, 10 μm. The distribution of the R proteins designated D1–D5 was identical to the R-sc protein, and the distribution of D10 was identical to D11. The distribution of D7–D9 was indistinguishable from D6 (data not shown). Approximately half of the sections examined from strains containing the *R-sc*, *D11*, and *D12* alleles displayed labeling of the cell walls (data not shown; see the *R-sc* section in Liu *et al.* 1996).

5). Both mutant constructs encoded <5% of wild-type *B* activity (Figure 8C). In contrast, deletion of the entire bHLH domain in b-HLHdel resulted in ~50% of wild-type activity (Figure 8C), in agreement with previously published results (Goff *et al.* 1992). Taken together, these data indicate that small insertions in helix 2 of the bHLH domain have a more profound effect on *R* or *B* activity than deletion of the entire domain.

DISCUSSION

The *r-m1* allele of maize contains a 396-bp *Ds1* element in helix 2 of the bHLH domain of the R protein. Twelve stable derivative alleles, representing four phenotypic classes—null, extremely pale, very pale, and revertant—were found to result from different *Ds* excision events. The molecular characterization of *r-m1* and its derivatives provides several lines of evidence that the bHLH region is required for *R* to function as a transcriptional activator *in vivo*. These reasons are discussed in detail below. However, our conclusions differ dramatically from those obtained using transient transformation assays of constructs deleted for the bHLH domain (Goff *et al.* 1992). In this assay, system bombardment of the full-length *B* cDNA led to a 500-fold increase in the activity of the Bz1Luc reporter gene, whereas a truncated *B* cDNA, encoding only the N-terminal 245 amino acids, activates transcription by almost 250-fold. This truncated gene lacked not only the bHLH domain, but also two of the three previously identified NLSs (Shieh *et al.* 1993).

Our study provides several lines of evidence that the bHLH domain and NLS-C are required for *R* function *in vivo*. First, all five revertant derivatives of *r-m1* contain the wild-type bHLH sequence, indicating that only perfect excision of *Ds* and all of the 8-bp direct repeat are compatible with full expression. We are confident that these are bona fide revertants and not wild-type contaminants because of the presence of additional genetic markers that were included to guard against such a possibility (see materials and methods). Restoration of the wild-type sequence upon *Ds* excision is usually a

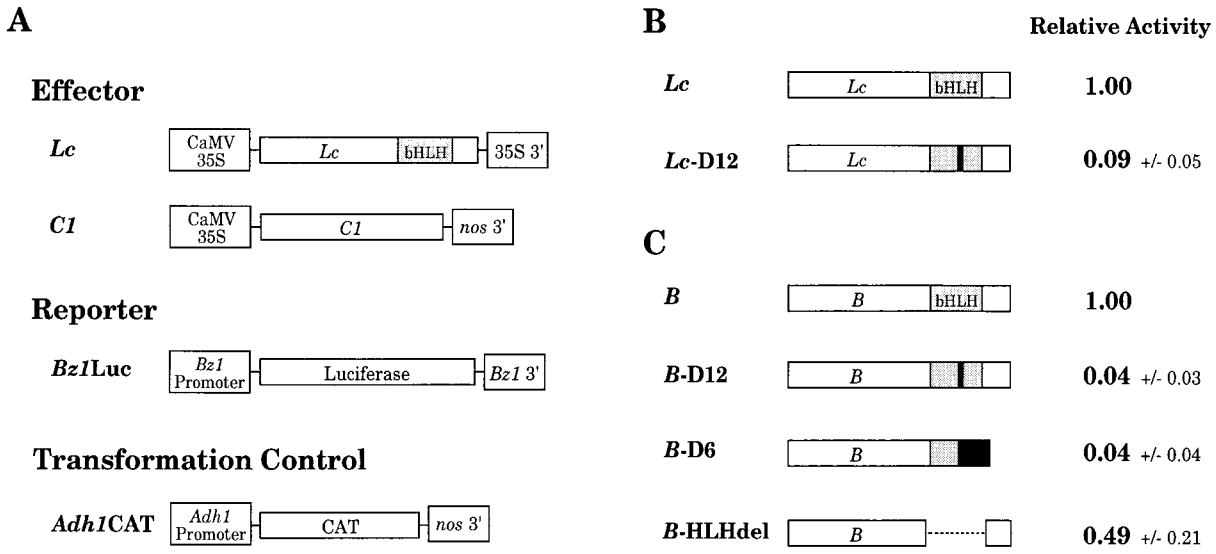

Figure 8.—Functional analysis of the D12 and D6 lesions by transient transformation assays. (A) Constructs used in particle bombardments. CaMV 35S and 35S 3′ represent the 35S promoter and 3′ terminator from the cauliflower mosaic virus, and nos 3′ represents the 3′ terminator from the nopaline synthase gene of *Agrobacterium tumefacians.* The luciferase gene in Bz1Luc is between the maize *Bz1* promoter and 3′ terminator (Bz1 3′). (B) Activity of D12 relative to wild-type *Lc.* Luciferase activity has been normalized to the expression level of a cobombarded CAT gene (see materials and methods). The relative activity of *Lc* is set at 1.00. Each value represents the average of eight independent assays. A gray box indicates the bHLH domain, and the three-amino-acid insertion is represented by a black bar (same below). (C) Effect of the D12 and D6 mutations on *B* function. The relative activity of wild-type *B* is set at 1.00. The deleted region in B-HLHdel is represented by a dotted line. D6 is a frameshift mutation that alters and truncates downstream amino acids (black box).

rare event; however, it occurs more frequently with the *Ds1* class of element found in the *r-m1* allele (Dennis *et al.* 1986).

The strongest evidence for the importance of the bHLH domain comes from the analysis of alleles with small insertions. Despite producing almost wild-type levels of mutant R protein, the activities of the D10/11 and D12 proteins are reduced from 50- to 100-fold, as measured by anthocyanin content and the steady-state level of structural gene transcripts. Furthermore, the small but reproducible differences in the activities encoded by these alleles, where *r-m1*>*D10/11*>*D12*, correlate with the extent of the helix 2 disruption; *i.e.*, *D12*, with its three-amino-acid insertion, is less active than *D10*, with its two-amino-acid insertion. That both of the insertions in these derivatives contain a proline residue may explain why D10 and D12 are less active than the full-length r-m1 protein, which does not contain a proline among its seven extra amino acids. Interestingly, insertion of a proline residue into helix 2 of the bHLH domain of the mouse homologue of the Twist protein prevented bHLH-mediated dimerization with E proteins (Spicer *et al.* 1996).

These data imply that bHLH-mediated protein-protein interactions are required for activation of the anthocyanin pathway. This conclusion is supported by the fact that the bHLH domain is highly conserved in functional *R* homologues isolated from the distantly related grass, rice (*Oryza sativa*; Hu *et al.* 1996), and from the dicotyledonous plants Antirrhinum and petunia (Goodrich *et al.* 1992; Quattrocchio 1994). Conservation of amino acid sequence over this time frame usually identifies an important functional domain.

The presence of the D10 and D12 proteins in the nucleus of aleurone cells indicates a requirement for bHLH-mediated protein-protein interactions in the transcriptional activation of at least three of the structural genes (*a1*, *a2*, and *c2*) in the anthocyanin pathway. R proteins might form homodimers or they might interact with an as yet unidentified HLH-containing coregulator. The product of the duplicate maize genes *C1*/*Pl* has long been known to encode a coregulator of the anthocyanin pathway. However, the C1 protein contains a *myb* domain, not a bHLH domain (Cone *et al.* 1986; Paz-Ares *et al.* 1986). Furthermore, the C1 protein has been shown to interact with the first 250 amino acids of the B protein, which lies upstream of the bHLH domain (Goff *et al.* 1992; Sainz *et al.* 1997b). That extensive genetic screens for mutants defective in pigment production have not identified another bHLH-containing regulatory protein does not rule out the possibility that such a protein exists. The putative R partner may be functionally redundant, *i.e.*, a member of a gene family, or it may be constitutively expressed and required for other essential processes. Similar bHLH-mediated heterodimeric interactions between tissue-specific and constitutive partners predominate in animal systems. For example, the ubiquitous E12 tran-

scription factor typically forms heterodimers with lineage-specific bHLH proteins such as MyoD (French *et al.* 1991).

In a previous study (Liu *et al.* 1996), we demonstrated that deletion of NLS-C (Figure 2C) correlated with inefficient nuclear localization *in vivo*. Specifically, it was shown that the *Ds*-induced *r-m9* allele encoded wild-type levels of a protein that was distributed throughout aleurone cells and lacked 62 amino acids from the C terminus. Data presented in this study provide additional evidence that NLS-C is required for efficient nuclear localization *in vivo*. First, localization of the R proteins encoded by *r-m1* to both the nucleus and the cytoplasm (Figure 7) can be explained by recalling that *r-m1* encodes both a protein with 7 amino acids inserted in the bHLH domain and a truncated protein missing the carboxy 152 amino acids (Figure 2, D and E). The former is probably nuclear localized, while the latter is not. In addition, the truncated proteins encoded by the null derivatives *D6–9* also fail to efficiently localize to the nucleus (Figure 7). Like the r-m9 protein, D6-9 and the truncated r-m1 proteins contain NLS-A and NLS-M, but not NLS-C (Figure 2A).

As discussed above, the results of transient transformation assays indicate that a B protein deleted for the bHLH domain was still capable of significant (50%) activation of the *bz1* promoter (Goff *et al.* 1992; Sainz *et al.* 1997b). Similarly, the *a1* promoter was activated by a B protein lacking the bHLH domain to 70% of the level observed with intact B protein (Tuerck and Fromm 1994). In our current study, a *B* construct without the bHLH domain was also able to activate transcription of the Bz1Luc reporter to 50% of the level of the wild-type *B* cDNA (Figure 8B). However, in this same transient assay system, the D12 mutation, when introduced into both the B and R cDNAs, reduced their expression >10-fold when they are compared to their wild-type counterparts. These data clearly indicate that the activity difference observed between deletion of the bHLH domain and a small insertion in this domain does not result from the assay system or from the use of B *vs.* R proteins. Rather, deletion of the bHLH domain is not functionally equivalent to small insertions into helix 2.

Although it is not known at this time why these lesions should have such different consequences on R/B function, we believe that the previously held view that the bHLH domain is dispensable is in error. The strict conservation of the bHLH in distantly related flowering plants, coupled with our analysis of *r-m1* and its derivatives, provides strong evidence that an intact domain is required for transcriptional activation *in vivo*. Perhaps deletion of the bHLH domain, but not small insertions in helix 2, bypasses a requirement for bHLH-mediated protein-protein interactions in transcriptional activation. This could be accomplished, for example, by changing the conformation in such a way that the deleted protein no longer requires dimeric interactions to function as a coregulator with the C1 protein.

Regulatory gene evolution is believed to play a significant role in organismal diversification (Dickinson 1991; Doebley 1993). It has been suggested that transposable elements have contributed to macroevolution by inserting into promoters and introducing new regulatory motifs (McDonald 1993). In contrast, the analysis of *r-m1* and its derivatives illustrates how a single transposable element can modify the structure of a regulatory protein in a variety of ways. First, insertion of *Ds1* into *R-sc* produces an allele that can now encode two stable proteins: one is truncated and probably cytoplasmically localized, while the other is full length because of the splicing of most of the element sequences from pre-mRNA. Second, excision of the *Ds1* element produces new *R* alleles that encode either truncated, cytoplasmically localized proteins or full-length, nuclear-localized proteins with insertions in the bHLH domain. Although R is a dispensable protein, one can imagine how similar modifications in integral regulatory proteins might have profound effects on developmental decisions, especially in cases where the proteins are encoded by gene families like *R*.

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