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Genetics

A *Ds* insertion alters the nuclear localization of the maize transcriptional activator R

(transposable elements/anthocyanin/helix–loop–helix/*zea mays*)

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ABSTRACT **The *R-sc* gene of maize is a member of the *R* gene family of transcriptional activators that regulate anthocyanin biosynthesis. A derivative of *R-sc*, *r-m9* conditions a reduced level of aleurone pigmentation due to the presence of a 2.1-kb *Ds* insertion near the 3′ end of the coding region. Excision of *Ds* from *r-m9* leaves a 7-bp insertion in the darker but still mutant *v24* derivative. Both the 7-bp insertion in *v24* and the 2.1-kb *Ds* in *r-m9* are predicted to truncate their respective R proteins proximal to the carboxyl terminus, which was shown previously to contain one of three nuclear localization sequences. We find that the reduced expression of *r-m9* and *v24* are not due to mRNA or protein instability, but most likely reflect the inefficient localization of truncated R proteins to the nucleus. To our knowledge this is the first example of a transposable element insertion that alters gene expression by affecting nuclear localization. In addition, our data indicate that the carboxyl terminus of the R protein is far more important than previously suspected and illustrates the utility of natural mutations for defining functional domains in proteins.**

The *R* and *B* genes of maize comprise a small gene family that regulates the deposition of anthocyanin pigment in plant tissues. R and B proteins, which share >80% amino acid identity, contain the basic helix–loop–helix domain and are believed to activate transcription of several structural genes in the anthocyanin biosynthetic pathway including *a1*, *bz1*, *bz2*, and *c2* (1–4). Introduction of deleted B proteins into maize aleurone tissue by particle bombardment led to the identification of domains required for activation of the *bz1* promoter (5). Similarly, bombardment of onion cells with chimeric proteins containing the reporter gene GUS fused to parts of the R protein served to define three nuclear localization sequences (NLSs) (designated NLS-A, NLS-M, and NLS-C) (6).

An alternative approach that uses the maize transposable elements *Ac* and *Ds* to create numerous mutant alleles of a single gene has been proposed as a means to dissect functional domains in proteins. This protocol, called cyclic mutagenesis, exploits the propensity of *Ac* and *Ds* elements to transpose to linked sites. The cycle begins with an *Ac* or *Ds* allele of the gene of interest. A revertant is then identified that gives rise to a high frequency of reinsertion events. These so-called second cycle mutants have been recovered from the *P* (250 *Ac* alleles) (7), *R-nj* (26 *Ac* alleles) (8), and *waxy* (30 *Ds* alleles) genes (9, 10). Similarly, Kermicle *et al*. (11) isolated 43 insertions of the *Ds* element into an *R* gene, *R-sc*, which conditions deep purple kernels.

Ds-containing alleles are particularly valuable for several reasons. First, since *Ds* elements cannot transpose when the autonomous *Ac* element is not in the genome, *Ds* mutants can be analyzed as stable alleles. Second, *Ac*-mediated transposition of *Ds* is usually accompanied by the insertion of a few base pairs at the site of *Ds* excision. These so-called transposon footprints have been shown to produce new, more subtle mutations affecting either enzymatic activity (12) or gene regulation (13). Finally, unlike transgenic plants or transient assay systems, *Ds* mutants and their progenitor and derivative alleles can be assayed in their correct chromosomal context in near isogenic lines that only differ at the *Ds* insertion site. Thus, mutant mRNA, protein, and phenotype can be compared directly with normal gene expression.

In a previous study (14), the approximate positions of the *Ds* insertions in most of the 43 derivatives of *R-sc* were determined. One allele, *r-m9* was of particular interest to us because it had a significant amount of aleurone pigmentation despite the presence of a 2.1-kb *Ds* element in a coding exon. *Ds* elements are known to behave as introns (15, 16), and this was initially believed to be the reason for the residual expression of *r-m9*. However, evidence is presented in this study that the leaky expression of *r-m9* and its stable derivative *v24*, results, in part, from inefficient nuclear localization of truncated R proteins. Aside from illustrating a novel means by which transposable elements can alter gene expression, this study demonstrates the utility of *Ds* alleles for dissecting the functional domains of proteins.

MATERIALS AND METHODS

Plant Stocks. All of the strains used in this study were in the W22 genetic background and were all homozygous for the designated alleles. Isolation of the *r-m9* allele from the *R-sc:124* progenitor has been described (11, 17, 18). *r-g:stadler* produces no anthocyanin deposition in seed or plant; *r-r:n46* pigments only plant parts. *P-vv* conditions red-striped (variegated) pericarp and cob. *v24* was isolated from *r-m9* following pollination of *r-m9/r-g*; *P-wr/P-vv* plants by *r-r:n46* (which served as a pollen contamination marker). One fully colored kernel, selected from approximately 2000 progeny kernels showing the *P-vv* phenotype, was grown and self-pollinated and the resulting ear segregated for the dark pale aleurone color of the *v24* allele.

Anthocyanin Extraction and Quantification. Anthocyanins were extracted from mature kernels by the method of Rabino and Mancinelli (19) and quantified by measuring absorbance at 530 nm. Data presented in the text is the mean of three independent experiments.

DNA Extraction and PCR Amplification. Maize genomic DNA was isolated as described (20) from 2-week-old plantlets. The *Ds* insertion site in *r-m9* was determined by PCR amplification of genomic DNA using the *R* gene-specific primer 1

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Abbreviations: NLS, nuclear localization sequence; DAP, days after pollination.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U58193 (*r-m9*) and U58194 (*v24*)]

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[5′-CGATAGCCTACCTCAAGGAG-3′, positions 1596–1615 of the *Lc* sequence (1)] and primer 2 near the 5′ end of the *Ds* element (5′-GGAATTCGAAATCGATCGGG-3′) (see Fig. 3*A* for primer positions). Primer 3 near the 3′ end of the *Ds* element (5′-GGAATTCGTTTCCGTCCCGC-3′) and an *R* gene-specific primer 4 [5′-CTTGATGGCGTCGAACACTC-3′, positions 1902–1921 of the *Lc* sequence (1)] were used to amplify the other junction fragment of *Ds* and *R* sequences. *R* gene-specific primers 1 and 4 were used to amplify *v24* genomic DNA. PCR conditions were as described (21). PCR products were cloned and sequenced using the dideoxy method (22).

RNA Isolation and Northern Blot Analysis. Total RNA was extracted as described (23). Poly(A)$^+$ RNA was purified using PolyATract mRNA Isolation Systems (Promega) according to the manufacturer. Northern blots were done as described (1). The *a1* probe is a *Kpn*I/*Pst*I fragment of a genomic clone (24), and the *c2* probe is a 1.5-kb *Eco*RI fragment of *c2* cDNA (25). The *R* probe is a 0.8-kb *Pst*I fragment of *Lc* cDNA (1). Probes were labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP using the random primer method (26). The maize actin clone (*pMAc1*) is as described (27). The hybridization signal was quantified using a PhosphorImager (Molecular Dynamics). For each sample, the signal was quantified by measuring the intensity of probe signal and dividing by the intensity of the actin control.

3′ Rapid Amplification of cDNA Ends (RACE) Procedure. 3′ RACE (GIBCO/BRL) was used as described (28) with modifications. Thirty micrograms of total RNA from kernels 25 days after pollination (DAP) was used for first-strand cDNA synthesis. For reverse transcription, 0.5 μg of an oligo-dT adapter primer (5′-GACTCGAGTCGACATGCT$_{17}$-3′) was used. The *R* gene-specific primer 1 and the adapter primer without 17 thymidine residues were used to amplify the target cDNAs. Amplification profiles were as described (21). PCR products were cloned and sequenced.

Protein Extraction and Western Blot Analysis. Crude proteins were extracted as described (29) and quantified using the protein assay (Bio-Rad) according to the manufacturer. Crude protein extracts were fractionated on an SDS/8% polyacrylamide gel and immunoblotted as described (30). Affinity-purified polyclonal Sn antibody [*Sn*, a member of the *R* gene family, is virtually identical to *Lc* (31)] was used at a dilution of 1:1000. 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 salt (Promega) were used to detect antibody binding. *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 (32) with the following modifications: Frozen sections (10 μm) of immature kernels harvested 25 DAP were placed on slides pretreated with gelatin. The specific antibody and preimmune rabbit sera were used at a dilution of 1:100. Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used 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′-phenylindole dihydroxychloride and observing them using the confocal microscope as a standard epifluorescence microscope.

RESULTS

Quantifying *r-m9* and *v24* expression. The *R-sc* allele conditions dark purple pigmentation in the aleurone and embryo of the mature seed. In the absence of an active *Ac*, the *r-m9* allele displays a stable mutant phenotype of uniform pale aleurone color (Fig. 1). In the presence of *Ac*, the *r-m9* allele is unstable with kernels containing darker pigmented spots on the pale background. *v24* is a stable derivative of *r-m9* that appears more pigmented than *r-m9* but paler than *R-sc* (Fig. 1).

Because *R* encodes a transcriptional activator rather than an enzyme, two indirect methods were used to assess *r-m9* and *v24* expression. First, anthocyanin pigments were extracted and quantified from mutant and wild-type aleurones. *r-m9* and *v24* were found to have 5.3% and 36.3% of wild-type anthocyanin levels, respectively (data not shown). Second, the effectiveness of mutant R proteins as transcriptional activators was determined by quantifying the steady state transcripts encoded by two structural genes in the anthocyanin biosynthetic pathway (*a1* and *c2*) previously shown to be under *R* control. Probes specific for *a1* and *c2* were hybridized with poly(A)$^+$ mRNA from kernels containing *R-sc*, *r-m9*, and *v24* and the relative intensity of the hybridization signals was calculated (Fig. 2). Following normalization for loading differences, it was found that the level of *a1* and *c2* transcripts in the *v24* background was $\approx$30–40% of wild-type levels, whereas the *r-m9* background conditioned only $\approx$1% of wild-type transcript levels.

The Lesions in *r-m9* and *v24*. Previous Southern blot analysis provided an approximate position for the 2.1-kb *Ds* element of *r-m9* near the carboxyl terminus of the *R* coding region (14). The precise insertion site was identified by using PCR to amplify the *Ds* termini and the flanking *R* region and sequenc-

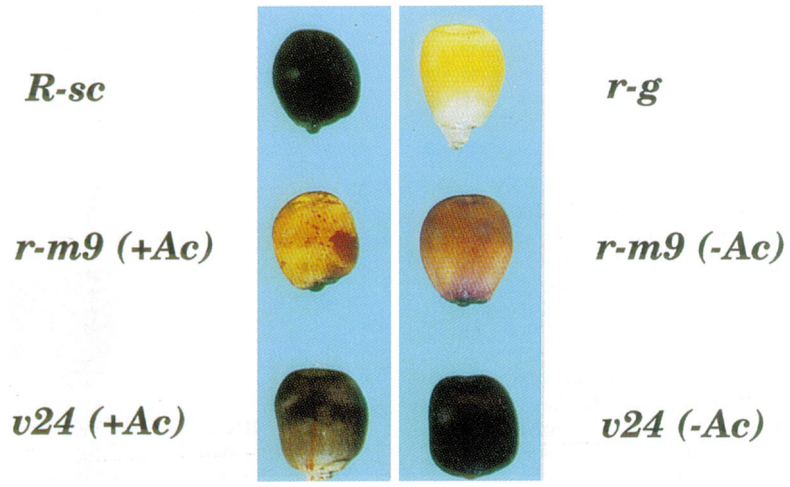

FIG. 1. Kernel phenotypes of *R-sc*, *r-m9*, and *v24* in the presence and absence of *Ac*. The *r-g* allele conditions no anthocyanin deposition. Pericarps have been removed from all kernels except for *v24 (+Ac)*.

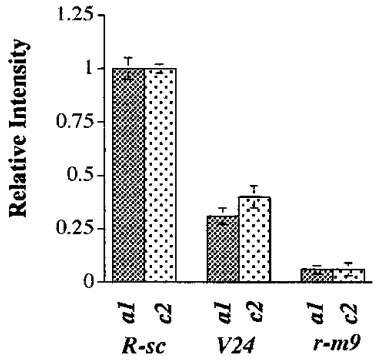

FIG. 2. Quantitation of RNA in strains with the *R-sc*, *v24*, or *r-m9* alleles. RNA blots containing 5 μg of poly(A)$^+$ RNA from immature (25 DAP) kernels were first hybridized with the *a1* probe, then stripped and rehybridized with the *c2* probe. The intensity of hybridization signals were quantified on a PhosphorImager and normalized to the value for *R-sc*. The values shown are an average of two experiments.

ing the products (*Materials and Methods*). The *Ds* element is inserted 186 nt (62 codons) upstream of the translation stop codon (Fig. 3*A*). The sequence of the 8-bp target site and the inverted repeat termini of the *Ds* element are shown in Fig. 3*B*. The site of *Ds* excision in *v24* was also deduced by using *R*-specific primers to amplify a region containing the insertion

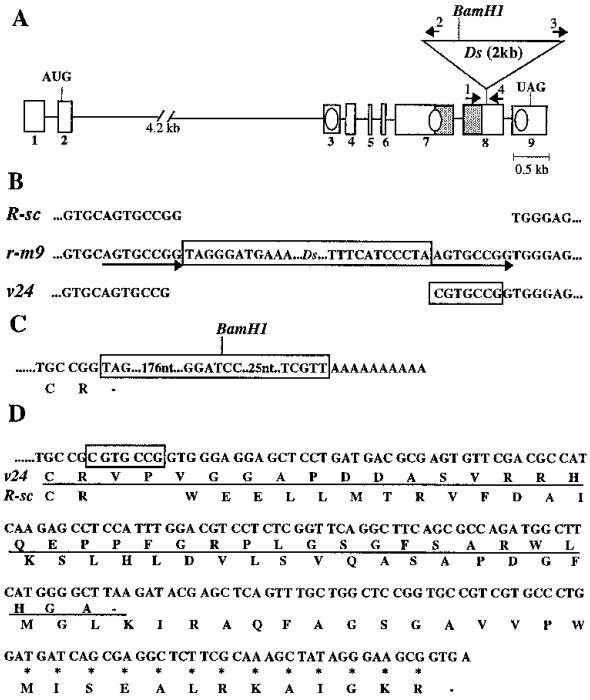

FIG. 3. Physical characterization of the *R-sc*, *r-m9*, and *v24* alleles and their predicted protein products. (*A*) The position and orientation of the *Ds* insertion (with respect to the sole *Bam*HI site) in the *r-m9* allele. The exons (numbered open boxes), introns (connecting lines), translation start and stop codons, NLSs (open circles), and helix–loop–helix domain (stippled boxes) in the progenitor *R-sc* gene are indicated. Horizontal arrows (nos. 1–4) indicate the location of oligonucleotide primers within *R* and *Ds* sequences. (*B*) DNA sequences of the *Ds* insertion site in the three alleles. Horizontal arrows are under the 8-bp host duplication flanking *Ds*. The termini of the 2.1-kb *Ds* element in *r-m9* and the 7-bp footprint in *v24* are boxed. (*C*) The 3′ end of the *r-m9* transcript and the deduced amino acid sequence (shown in one-letter code). *Ds* sequences are boxed. (*D*) Deduced amino acid sequences of the v24 (underlined) and R-sc proteins downstream from the 7-bp footprint (boxed) in *v24*. The amino acids that comprise NLS-C in the R-sc protein are shown with asterisks.

site. The sequenced product revealed a 7-bp insertion (transposon footprint) at the site of *Ds* excision (Fig. 3*B*). If *r-m9* and *v24* were transcribed and translated, both would encode truncated R proteins missing 62 amino acids of the carboxyl terminus. For *r-m9*, the first three nt of the *Ds* terminus, TAG, would introduce an in-frame but premature stop codon (Fig. 3*C*). For *v24*, the 7-bp footprint adds two codons and alters the reading frame at the insertion site resulting in the replacement of the terminal 62 codons with 34 other codons prior to encountering a stop codon in exon 8 sequences (Fig. 3*D*).

Transcripts from the *r-m9* and *v24* Genes. To determine the molecular basis for the reduced expression of *r-m9* and *v24*, mutant mRNAs were characterized. When poly(A)$^+$ RNA isolated from aleurones of immature (25 DAP) kernels was probed with the *Lc* (*R*) cDNA (1), approximately wild-type levels of transcript were revealed by visual inspection of the *r-m9* and *v24* hybridization signals (Fig. 4). When these signals were normalized to the actin control it was found that RNA levels were indistinguishable in the three strains (data not shown). These data suggest that instability of mutant transcripts cannot explain the leaky phenotypes of these alleles.

The *r-m9* allele encodes a transcript that is shorter than the wild-type 2.5-kb transcript. This could reflect splicing of *Ds* sequences from pre-mRNA, a phenomena observed for other *Ds* alleles (13, 33, 34). However, the fact that the *r-m9* transcript did not hybridize with an *R* probe derived from sequences downstream of the *Ds* element indicated that transcription terminated within the *Ds* element (data not shown). The precise site of polyadenylylation within *Ds* sequences was determined by using the RACE procedure to amplify the 3′ end of the *r-m9* transcript(s). In this way polyadenylylation was found to occur near the *Bam*HI site of the *Ds* element, 216 nt from the element terminus (Fig. 3*C*).

R Proteins Encoded by *r-m9* and *v24*. *r-m9* and *v24* strains contain approximately wild-type levels of *R* transcripts that are predicted to encode proteins missing the carboxyl-terminal 62 amino acids. The inability of these mutant R proteins to efficiently activate the anthocyanin pathway could result from protein instability and/or reduced activity. The issue of protein stability was addressed using Western blots to analyze the steady state levels of R protein in extracts from *r-m9*, *v24*, and *R-sc* aleurones (Fig. 5). The polyclonal R antibody recognizes a protein in the *R-sc* extract that comigrates with the *in vitro*-synthesized R control protein (Fig. 5, compare lane 1 and

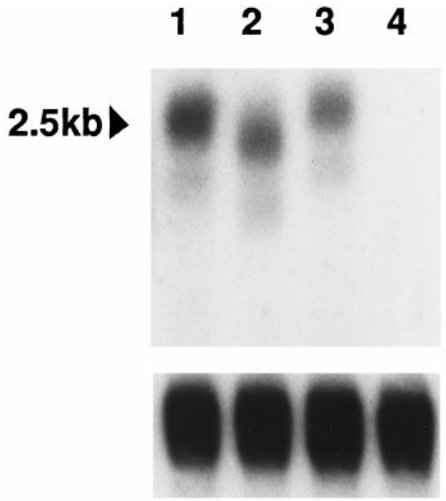

FIG. 4. RNA blot analysis of strains containing different *R* alleles. Samples (5 μg) of poly(A)$^+$ RNA isolated from immature kernels (25 DAP) containing *R-sc* (lane 1), *r-m9* (lane 2), *v24* (lane 3), and *r-g* (lane 4) were electrophoresed, transferred to Magnagraph membrane, and probed with *Lc* cDNA (*top*) and then stripped and probed with maize actin (*bottom*).

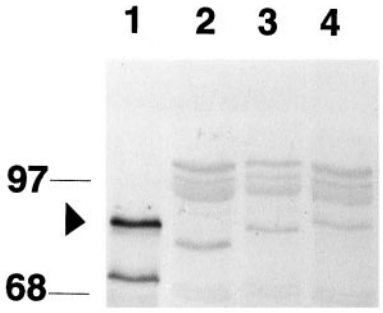

FIG. 5. Western blot analysis of wild-type and mutant R proteins. Samples of approximately 25 μg protein extracted from dissected aleurones (25 DAP) containing *r-m9* (lane 2), *v24* (lane 3), and *R-sc* (lane 4). Lane 1 contains *in vitro* translated R protein. Arrowhead represents the full-length R protein; the smaller band is not reproducible and is probably a degradation product. The mobility of protein markers is indicated at the left in kDa.

lane 4). Both *v24* and *r-m9* contain smaller proteins as predicted by the deduced amino acid sequences of these mutant genes (Fig. 3*D*). In addition, no R-specific protein was detected in the *r-g* extract consistent with the absence of steady-state R mRNA in this strain (see Fig. 4, lane 4; data not shown). All aleurones, irrespective of genotype, contain high molecular weight proteins that react with immune sera (Fig. 5, lanes 2–4). These proteins correspond to the most abundant proteins as determined by staining with Ponceau S. Taken together, these data show that R protein levels in wild-type and mutant extracts are approximately the same indicating that protein instability cannot account for the reduction in *R* activity in the mutant strains.

Immunolocalization of R-sc, r-m9, and v24 Proteins. The truncated R proteins of *r-m9* and *v24* lack the carboxyl-terminal 62 amino acids, shown previously to contain one of the three NLSs (designated NLS-C) (6). Failure to efficiently localize to the nucleus could explain why these proteins are impaired in their ability to activate the transcription of the *a1* and *c2* genes. To test this hypothesis, immunolocalization experiments were performed. The wild-type R protein was localized in the nuclei of aleurone cells of 25 DAP *R-sc* kernels, whereas the truncated R proteins of *r-m9* and *v24* were localized in both nucleus and cytoplasm (Fig. 6 *A–C*). Control sections incubated with preimmune serum did not show specific staining (Fig. 6*D*). These data suggest that inefficient nuclear localization of the r-m9 and v24 proteins due to the deletion of NLS-C is at least part of the reason for their reduced ability to activate the structural genes in the anthocyanin pathway.

DISCUSSION

The *r-m9* allele contains a 2.1-kb *Ds* insertion in exon 8 of the *R* gene. This lesion results in the production of a chimeric transcript that encodes a truncated R protein with no additional foreign amino acids at the carboxyl terminus. Despite producing wild-type levels of mRNA and a truncated R protein, *r-m9* expression is dramatically reduced as measured by both anthocyanin levels and its ability to transactivate the *c2* and *a1* genes. Failure of r-m9 protein to efficiently localize to the nucleus provides a molecular explanation for its reduced expression and is compatible with the finding that the 62 amino acids missing from the r-m9 protein include a region shown previously to contain one of three NLSs (6). This represents the first reported example of a transposable element insertion that alters the intracellular localization of a protein.

The *v24* allele has sustained a 7-bp transposon footprint at the site of *Ds* excision. This footprint is predicted to alter the coding region such that the v24 protein is missing the same 62

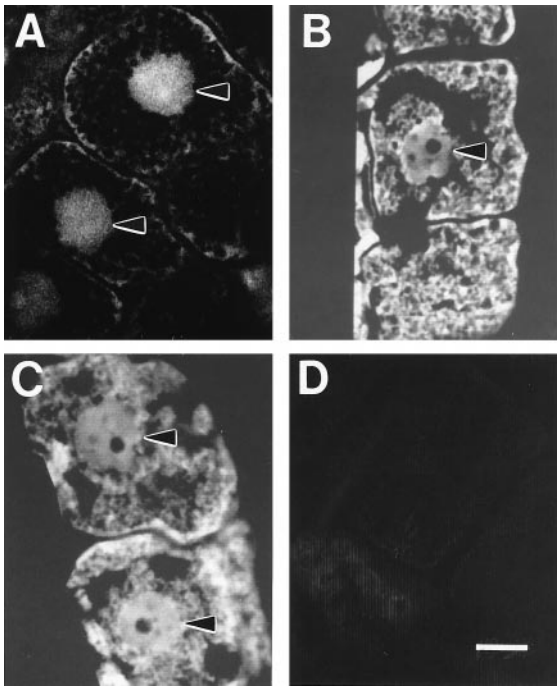

FIG. 6. Immunolocalization of the R proteins in maize aleurone cells. Sections containing aleurones from *R-sc* (*A*), *r-m9* (*B*), and *v24* (*C*) kernels were stained with affinity-purified polyclonal Sr antibody. (*D*) Sections containing aleurones from *R-sc* were stained with preimmune sera. All sections were simultaneously stained with the nuclei-specific dye 4′,6′ diamidino-2′-phenylindole dihydroxychloride to verify the nuclei indicated by an arrowhead (data not shown). (Bar = 10 μm.)

amino acids as the r-m9 protein but, unlike r-m9, should contain 36 foreign amino acids at its carboxyl terminus. Consistent with this is the finding that the v24 protein migrates slightly faster than the R-sc protein but more slowly than the r-m9 protein (Fig. 5). Although v24 is also altered in nuclear localization, it is much more effective at activating the anthocyanin pathway. Because we do not see a significant difference in nuclear localization between the v24 and r-m9 proteins, this cannot be a major contributing factor to its increased expression. These results indicate that the 36 amino acids that distinguish v24 from r-m9 must be serving a function, in addition to nuclear localization, that is normally fulfilled by the carboxyl-terminal 62 amino acids; perhaps by ensuring correct protein folding.

Two other lines of evidence suggest that the terminal 62 amino acids have an additional function, aside from nuclear localization. First, all 62 amino acids, not just the 13 amino acids that comprise NLS-C, are highly conserved when the R proteins of the distantly related grasses rice and maize are compared (71% identity, 94% similarity in this region compared with 52% identity and 66% similarity overall) (35). Second, a significant fraction of r-m9 protein is localized in the nucleus, indicating that inefficient localization cannot account for all of the almost 100-fold reduction in *r-m9* expression relative to the *R-sc* progenitor.

The data presented here serve to both extend and contradict the results of prior studies of the functional domains in R and B proteins. In addition to identifying NLS-A, -M, and -C, Shieh *et al.* (6) found that R proteins with only two of three NLSs (either A and M or M and C) could efficiently redirect the chimeric R:GUS reporter protein to the nucleus after bombardment into onion cells. However, the fact that a NLS-A:GUS fusion protein localized to both the cytoplasm and nucleus coupled with the lack of NLS-A amino acid conservation in *R*-homologs from maize, antirrhinum, and rice, led

Shieh *et al.* (6) to suggest that NLS-A may not be important and that the native R protein may use only NLS-M and NLS-C. That the r-m9 and v24 proteins, which contain NLS-A and NLS-M but not NLS-C, are not efficiently localized to the nucleus provides experimental evidence for this notion.

In contrast, our results are not consistent with the findings of Goff *et al.* (5) who showed that deletion of the carboxyl-terminal 11 amino acids (including most of NLS-C) of the *B* gene (an *R* homolog) had no effect on its ability to transactivate a *Bz1/Luc* reporter gene when co-bombarded into maize aleurone cells. Larger deletions of up to 150 amino acids of the carboxyl terminus displayed almost 50% of wild-type activity in this assay system. These deletion constructs did not include any foreign amino acids at the carboxyl terminus so they are formally equivalent to the r-m9 protein.

These different conclusions regarding the importance of the carboxyl terminus of the R protein illustrates the value of assaying the expression of mutants in their correct chromosomal context. Unlike *in vitro*-generated mutations, which must be assayed in heterologous systems (e.g., onion cells), in bombarded tissues (e.g., aleurone cells), or in transgenic plants, the expression of *Ds* alleles and their derivatives can be quantified without the complications that plague heterologous or transgenic systems such as gene dosage, position effect, or gene silencing. Most importantly, natural phenotypes, which are the basis of selection, can be assayed rather than reporter gene activity. The results of the previous studies suggesting that NLS-C was dispensable made it difficult to understand why this region has been conserved in distantly related flowering plants (36). Our data suggest that this region is far more important to the R protein than previously suspected (5) and serves to illustrate the role that *Ds* alleles can play in defining functional domains in proteins. Furthermore, this role need not be restricted to maize. *Ds* has proven to be an excellent mutagen in other plants including Arabidopsis, tobacco, and tomato (for review see ref. 37), where it has also been shown to have a strong preference for transposition to linked sites (38–40). Thus, the use of cyclic mutagenesis in other plant species should be a feasible protocol for the isolation of multiple alleles for functional analysis.

We thank Jerry Kermicle for providing *r-m9* and *R-sc* alleles, Stephen L. Dellaporta (Yale University) for the Sn antibody, Udo Wienand for the *a1* and *c2* clones, and Rich Meagher for the maize actin clone. We are grateful to Mark Farmer for his technical advice. We also thank Kelly Dawe, Lane Arthur, and Shawn E. White for critical reading of the manuscript. This study was supported by a grant from the Department of Energy to S.R.W.

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