The Plant Cell, Vol. 1, 1175–1183, December 1989

Two Regulatory Genes of the Maize Anthocyanin Pathway Are Homologous: Isolation of *B* Utilizing *R* Genomic Sequences

Vicki L. Chandler,[a,1] J. Pablo Radicella,[a] Timothy P. Robbins,[b] Jychian Chen,[b] and Devon Turks[a]

[a] Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403
[b] Department of Biology, Yale University, New Haven, Connecticut 06511

Genetic studies in maize have identified several regulatory genes that control the tissue-specific synthesis of the purple anthocyanin pigments during development. Two such genes, *R* and *B*, exhibit extensive allelic diversity with respect to the tissue specificity and developmental timing of anthocyanin synthesis. Previous genetic studies demonstrated that certain *B* alleles can substitute for *R* function, and in these cases only one functional allele at either locus is required for pigment synthesis in the aleurone. In addition, biochemical studies have shown that both genes act on the same biosynthetic pathway, suggesting that the genes are functionally duplicate. In this report we describe DNA hybridization experiments that demonstrate that the functionally duplicate nature of *B* and *R* is reflected in DNA sequence similarity between the two genes. We took advantage of this homology and used the *R* genomic sequences to clone *B*. Two different strategies were pursued and two genomic clones isolated, a 2.5-kilobase BglII fragment linked to the *b* allele in W23 inbred stocks and a 1.0-kilobase HindIII fragment linked to the *B* allele in CM37 stocks. Examination of several independent transposable element insertion mutations in *B* and revertant derivatives demonstrated that our clones recognize the functional *B* gene. Genomic clones representing the entire *B-Peru* allele were isolated, and a detailed restriction map was prepared. Using these clones we have identified a 2.2-kilobase mRNA in husks from plants containing either *B-I* or *B-Peru* alleles, but no *B* mRNA was detected in plants containing a *b* allele. The transcript is at least 100 times more abundant in strongly pigmented *B-I* husks than in weakly pigmented *B-Peru* husk tissue. Expression of functional *B* alleles in husk tissue correlates with the coordinate increase in mRNA levels of two structural genes of the pathway, *A1* and *Bz1*, consistent with the postulated role of *B* as a regulatory gene.

INTRODUCTION

The synthesis of anthocyanin pigments in maize plant tissues or seeds requires the products of numerous genes, both structural and regulatory (Styles and Ceska, 1977; Coe, Hoisington, and Neuffer, 1988). Mutational analyses have identified numerous regulatory genes that influence the production of anthocyanin pigments. Several are pleiotropic, affecting seed development as well as pigment synthesis, but four loci, *R*, *B*, *C1*, and *Pl*, are specific for anthocyanin synthesis. These four loci coordinately regulate the activities of at least three of the structural genes in the pathway (Dooner, 1983; Cone, Burr, and Burr, 1986): *C2*, which encodes chalcone synthase (Wienand et al., 1986); *A1*, which encodes dihydroquercetin reductase (Schwarz-Sommer et al., 1987); and *Bz1*, which encodes UDPglucose: flavonoid 3-*O*-glucosyltransferase (UFGT) (Dooner and Nelson, 1977).

The *R* locus (on chromosome 10) regulates the formation and distribution of anthocyanin pigments in certain tissues of the plant and seed. The *B* locus (on chromosome 2) controls anthocyanin formation in plant tissues independent of *R*, and a few *B* alleles also control anthocyanin formation in the seed (Styles, Ceska, and Seah, 1973). Experiments have demonstrated that both genes act on the same biosynthetic pathway, in that a functional *R* or *B* allele is required for the induction of the structural enzyme encoded by the *Bz1* gene (Dooner and Nelson, 1977, 1979; Dooner, 1983; Gerats et al., 1984), and the same anthocyanins are produced (Styles et al., 1973). In the seed, a functional *R* or *B* allele is not sufficient for activation of the anthocyanin pathway; the product of the *C1* gene is also necessary. In the absence of a functional *C1* or *R* allele, no transcripts from *Bz1*, *C2*, or *A1* are detected in kernels (Cone et al., 1986; Ludwig et al., 1989). *C1* is not needed for anthocyanin synthesis in plant tissues. The regulatory gene, *Pl*, influences plant color and was recently cloned using *C1* genomic sequences as a hybridization

[1] To whom correspondence should be addressed.

probe (K. Cone, personal communication), demonstrating that those genes share a high degree of sequence similarity and are homologous as proposed from genetic analyses (Coe, 1985). Although *Pl* is not absolutely required for pigment expression in plant tissues, as *pl* plants still make pigment when a functional *B* or *R* allele is present, *Pl* intensifies the amount of pigment produced and decreases the requirement for direct sunlight (Emerson, 1921; for review, see Briggs, 1966).

Genetic and molecular studies have demonstrated that some *R* alleles are complex. In those alleles tissue-specific control is mediated through compound elements; in *R-r* one component specifies aleurone color and another component specifies color in anthers and several other plant tissues (Stadler and Nuffer, 1953; Dooner and Kermicle, 1971). The two components required for expression in the plant (P) or seed (S) can be independently mutated and separated by recombination (Stadler and Nuffer, 1953). Recent molecular analysis of the compound *R-r* allele and mutant derivatives confirms that there are several cross-hybridizing restriction fragments containing regions required for function in the various tissues (Dellaporta et al., 1988).

In some tissues certain *B* and *R* alleles act as duplicate genes; for example, *B-Peru* can substitute for *R* function in the seed, and only one functional allele at either locus is required for pigment synthesis (Styles et al., 1973). In other tissues only the *B* or *R* locus is active. Table 1 lists the phenotype of plants with different *R* and *B* allelic combinations. For instance, a subset of *R* alleles will cause pigmentation of the anthers, but no *B* alleles are known that function in this tissue (Styles et al., 1973; Coe, 1979). In addition to the tissue-specific regulation, certain *B* and *R* alleles appear to trigger anthocyanin biosynthesis at defined developmental times in several tissues (Styles et al., 1973).

Given the duplicate function and proposed homology (Coe, 1985) of several *R* and *B* alleles, we hypothesized that the genes might have a high degree of DNA sequence similarity. Accordingly, *R* genomic sequences were used as a hybridization probe to identify restriction fragments linked to the *B* locus. These fragments were then cloned and used to characterize the structure and transcription of several *B* alleles. The role of *B* in the regulation of the expression of the structural genes, *A1* and *Bz1*, was also examined.

RESULTS

Cloning *B* Using *R* Genomic Sequences

We reasoned that the functionally duplicate nature of certain *B* and *R* alleles may reflect DNA sequence similarity between the two genes that might be detectable using DNA gel blot hybridization. To investigate this we have used cloned *R* sequences as hybridization probes to screen for restriction fragment length polymorphisms in DNA samples from various *B* alleles using a variety of hybridization conditions. Two different strategies were successful.

First, a HindIII digest of DNA samples prepared from recombinant inbred individuals (Burr et al., 1988) segregating for two different *B* alleles were examined on DNA gel blots hybridized with the pR-nj:1 clone (Dellaporta et al., 1988). As shown in Figure 1A, weakly hybridizing fragments of 1.3 kb and 1.0 kb segregate with the *B* alleles of T232 and CM37 plants, respectively, in recombinant inbreds. The *R* allele is constant and generates two strongly hybridizing fragments observed in all plants. Second, plants segregating the *b* and *B-Peru* alleles were examined using a subclone of the seed component (S) of *R-r*, which is functionally duplicate for *B-Peru* function in the seed (Styles et al., 1973). An example of one DNA gel blot showing a BglII restriction fragment polymorphism in plants containing these *B* alleles is shown in Figure 1B. The null *R* allele (*r-g*) is constant in all of the *B* stocks and generates a strongly hybridizing 4.0-kb BglII fragment. The *b* stock contains a weakly hybridizing 2.5-kb BglII fragment (lane a) that is not in the *B-Peru* stock (lane b). Instead, a large BglII fragment of about 13 kb is observed in the *B-Peru* stock. This approximately 13-kb BglII fragment is missing in a *B-Peru* mutant (*b-Perum5*; Walbot, Briggs, and Chandler, 1986; Chandler, Belcher, and Turks, 1988a) that contains a transposable element insertion in the

Table 1. Anthocyanin Phenotype of Plants with Various *R* and *B* Allelic Combinations

	Alleles				
Tissues	*b r-g*	*B-Peru r-g*	*B-I r-g*	*b R-r*	*b R-nj*
Scutellum of embryo	Colorless	Purple	Colorless		Purple
Aleurone	Colorless	Purple	Colorless	Purple	Purple crown
Seedling auricle	Green	Green	Red	Red	Green
Coleoptile	Green	Medium red	Red	Red	Red
Husks, culm, sheath, tassel branch	Green	Medium red	Purple	Green	Green
Anthers	Green	Green	Green	Purple	

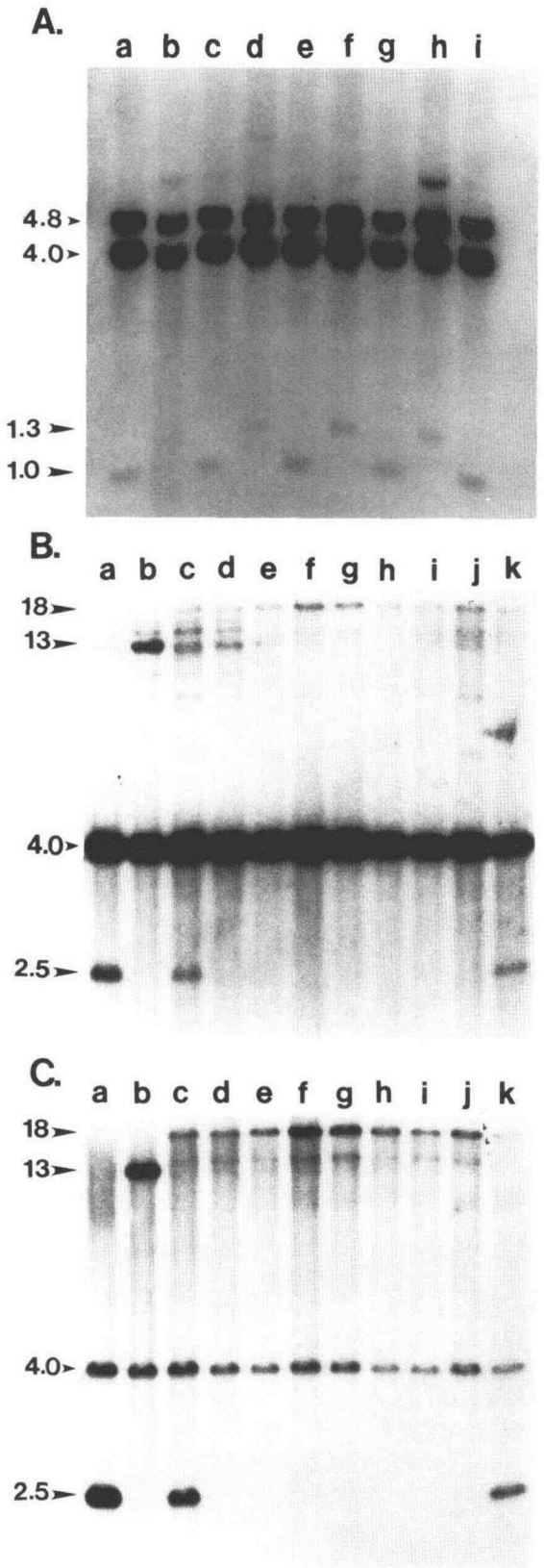

Figure 1. DNA Gel Blots of DNA from *B* and *R* Stocks Hybridized with the *R* Probe.

B-Peru gene, and an approximately 18-kb BglII restriction fragment is observed (lane c). (The *b-Perum5* individual shown in lane c is heterozygous for the *b* allele and contains the 2.5-kb BglII fragment.) This is the pattern that would be expected if the fragments hybridizing to the *R* probe were linked to the various *B* alleles.

The larger BglII fragments observed in the *B-Peru* stock and the insertion mutant were difficult to detect consistently on DNA gel blots, presumably due to the weak signal and poor transfer of large fragments. We also frequently observed additional restriction fragments hybridizing to the *R* probe in this region of the gel, masking the fragments we were trying to follow. Therefore, we addressed whether the 2.5-kb BglII fragment segregated with the *b* allele. We prepared DNA from plants with the genotype of *b/b*, *b-Perum5/b*, or *b-Perum5/b-Perum5*. The 10 individuals that were *b/b* or *b-Perum5/b* all contained the 2.5-kb BglII fragment, and none of the 27 individuals homozygous for *b-Perum5* contained the 2.5-kb BglII fragment, demonstrating that the 2.5-kb BglII fragment is linked to the *b* allele. Examples of several individuals are shown in Figure 1B, lanes c to k.

The 2.5-kb BglII and the 1.0-kb HindIII fragments were cloned from plants containing the *b* and *B*-CM37 alleles, respectively, as described in Methods. To confirm that the desired fragments were cloned, the 2.5-kb BglII "*b*" and 1.0-kb HindIII "*B*-CM37" fragments were subcloned into plasmid vectors and used as hybridization probes on DNA gel blots. Both fragments contained some repeated sequences, and further subcloning was required to obtain a

(A) Recombinant inbreds segregating *B* and *b* alleles. DNA samples (about 5 μg) were digested with HindIII, electrophoresed on 0.8% agarose, transferred to Nytran (Schleicher & Schuell), and hybridized with the 700-bp pR-nj:1 insert (Dellaporta et al., 1988). The recombinant inbred lines were chosen to be invariant for an RFLP tightly linked to *R*, but alternating for the *B* allele of CM37 and the *b* allele of T232. The small arrows indicate the invariant *R* fragments of the CM37 parent migrating at 4.8 kb and 4.0 kb. The large arrows indicate two fragments of 1.3 kb and 1.0 kb that segregate with the *b* allele of T232 and the *B* allele of CM37, respectively. The 1.0-kb *B* fragment of CM37 was cloned as described in the text and Methods. Lanes a to i contain DNA samples from different recombinant inbred maize lines containing the *B* alleles indicated: a, *B*; b, *b*; c, *B*; d, *b*; e, *B*; f, *b*; g, *B*; h, *b*; i, *B*.

(B) Segregation of *b*, *B-Peru*, and mutant *B-Peru* alleles. DNA samples (about 4 μg) were digested with BglII, electrophoresed on 0.5% agarose, transferred to nylon membrane (MSI), and hybridized with a 1.9-kb subclone of the (S) component of *R-r* (Dellaporta et al., 1988). The small arrows indicate the invariant 4.0-kb *r-g* fragment, and the large arrows indicate the fragments segregating with the various *B* alleles. The sizes are in kilobases. Lanes a to k, DNA from maize plants containing the designated *B* alleles (all stocks contain the *r-g* allele): a, *b*; b, *B-Peru*; c, *b-Perum5/b*; d–j, *b-Perum5* homozygotes; k, *b-Perum5/b*.

(C) Hybridization with putative *b* sequences. Blot shown in **(B)**, rehybridized with the 550-bp subclone of the 2.5-kb BglII fragment, cloned as described in the text and Methods (see map in Figure 3).

relatively unique sequence (data not shown). An approximately 550-bp BamHI/HindIII subclone of the *b* allele was relatively unique, as it strongly recognized the 2.5-kb *b*, the 13-kb *B-Peru*, and the approximately 18-kb *b-Perum5* BglII fragments, and weakly hybridized with the 4.0-kb BglII *r-g* fragment (Figure 1C). Under stringent wash conditions the 1.0-kb HindIII fragment of CM37 also hybridized strongly to *B* fragments, but weakly to *R* fragments (data not shown). These results demonstrated that the desired fragments had been cloned.

Cloned Sequences Recognize Functional *B* Gene

The approximately 550-bp BamHI/HindIII subclone from *b* was then used as a hybridization probe to test whether it recognized the functional *B* gene. Several independent transposable element insertion mutations into the *B-Peru* allele and revertant derivatives of each have been isolated (Walbot et al., 1986; Chandler et al., 1988a; V. Chandler, unpublished data). These independent insertion mutants and revertant derivatives have been compared with the progenitor *B-Peru* allele utilizing DNA gel blots hybridized with the putative "*b*" sequences. An example of one DNA gel blot on DNA from two mutant alleles and revertants of each is shown in Figure 2. Each DNA sample was digested with BclI, which produced a 4-kb fragment in both the *b* and the progenitor *B-Peru* alleles. Each of the mutants (*b-Perum5* and *b-Perum216*) contained restriction fragment differences relative to the wild-type *B-Peru* allele, suggesting that each mutant contains an alteration within this 4-kb fragment. The "new" restriction fragments are indicated by arrows on the autoradiograph shown in Figure 2, lanes c and e. Most important, in the revertants (lanes d and f), the restriction fragments characteristic of the insertion alleles are missing, and only the progenitor 4-kb fragment is observed. These results demonstrate definitively that the sequences we have cloned recognize the functional *B-Peru* gene.

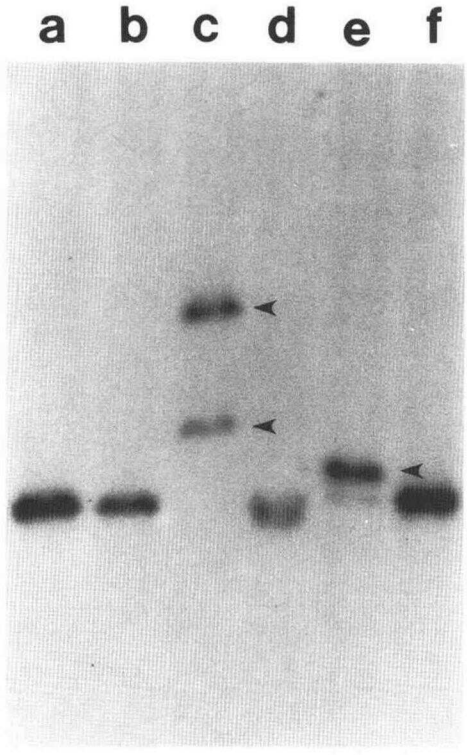

Figure 2. DNA Gel Blot of DNA from *B-Peru* and Mutant Derivatives.

DNA samples (about 4 μg) were digested with BclI, electrophoresed in 0.5% agarose, transferred to nylon membrane (MSI), and hybridized with the 550-bp *b* fragment. Lanes a to f, DNA from maize plants containing *r-g* and the designated *B* alleles: a, *b*; b, *B-Peru*; c, *b-Perum5*/*b-Perum5*; d, *b*/*Perum5-revertant*/*b*; e, *b-Perum216*/*b-Perum216*; f, *b-Perum216-revertant*/*b*. The *b-Perum5* and *b-Perum216* alleles are independent transposable element insertions into the *B-Peru* gene (Walbot et al., 1986; Chandler et al., 1988a). The arrows indicate the altered restriction fragments in the mutant plants.

Cloning, Restriction Mapping, and Transcript Analysis of *B-Peru*

The *b* sequences were then used to clone the progenitor *B-Peru* allele and the *B-I* genomic sequences. As our insertion mutants were in *B-Peru*, we have initially focused on this allele. The restriction map of *B-Peru* showing the

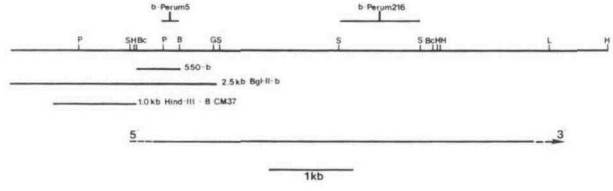

Figure 3. Restriction Map of *B-Peru* Allele.

The restriction map was generated from a combination of genomic DNA gel blots and digestions of cloned DNA sequences. Abbreviations: P, PstI; G, BglII; B, BamHI; H, HindIII; Bc, BclI; L, SalI; S, SacI. The original 2.5-kb BglII (*b*, W23) and 1.0-kb HindIII (*B*, CM37) clones map to the *B-Peru* allele as shown. The fragment indicated above the BglII clone is the 550-bp HindIII/BamHI subclone used as a hybridization probe in Figure 1C and Figure 2. The approximate location of the approximately 2.2-kb mRNA in *B-Peru* and *B-I* containing stocks (experiments are described in Methods) is also diagrammed. The dashed lines at both ends of the transcript indicate that we have not determined the precise 5′ and 3′ termini. The two mutant alleles (*b-Perum5*, *b-Perum216*) contain insertions in the *B-Peru* gene as indicated on the map. The insertion site in *b-Perum216* is within the indicated SacI restriction fragment as determined by genomic restriction mapping. The insertion site in *b-Perum5* is based on restriction digests of cloned sequences and is more accurate (within about 50 bp).

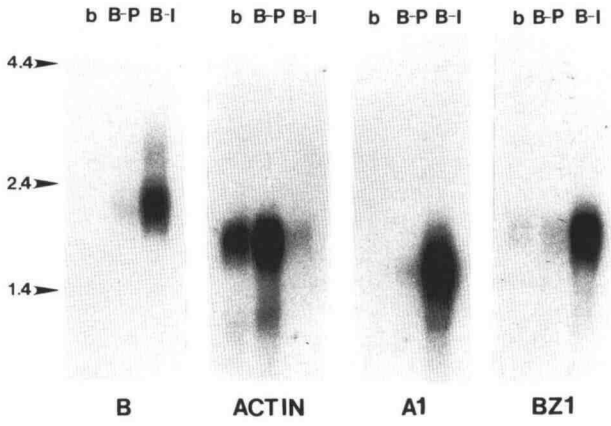

Figure 4. RNA Gel Blot Analysis of mRNA from Plants Containing *B-I*, *B-Peru*, and *b* Alleles.

Poly (A)$^+$ RNA from husks containing the indicated *B* alleles was analyzed by electrophoresis and blot hybridization as described in Methods. The amount of RNA in each lane was about 5 μg from plants containing the *b* or *B-Peru* alleles and about 0.5 μg from plants containing the *B-I* allele. All three stocks contained the null *r-g* allele. From left to right the blot shown is hybridized with the indicated probe: B, the 550-bp HindIII/BamHI fragment of *B-Peru* (Figure 3); the maize actin clone (Meagher et al., 1983); the 1.5-kb AvaI fragment of *A1* (Schwarz-Sommer et al., 1987); and the 2.1-kb PstI fragment of *Bz1* (Furtek et al., 1988). The same blot was used sequentially with all four probes. The order of hybridization was carried out with the weakest signal first: *B*, followed by *A1*, *Bz1*, and then actin.

approximate site of element insertion in the two *B-Peru* mutant alleles is shown in Figure 3. The 2.5-kb *b* clone and 1.0-kb HindIII *B*-CM37 clone are shown on the map relative to the *B-Peru* allele.

The transcribed region of *B-Peru* has been roughly positioned by hybridizing nonoverlapping genomic restriction fragments of the *B-Peru* allele to mRNA prepared from either husk or aleurone tissue from plants containing the *B-Peru r-g* or *B-I r-g* alleles. The probes that detected a 2.2-kb mRNA in both stocks spanned approximately 3.5 kb of the genomic sequences (see Methods for details). The direction of transcription was determined using ribonucleotide probes as described in Methods. Our results are summarized in Figure 3. We indicate the ends of the transcribed region with dashed lines, as we have not yet mapped the precise 5′ and 3′ termini. The size of the transcription unit relative to the 2.2-kb mature mRNA indicates there is one or more intron(s).

Levels of *B*, *Bz1*, and *A1* mRNA in Plants Containing Different *B* Alleles

RNA gel blot analysis of poly(A)$^+$ RNA extracted from husk tissue from plants containing *b*, *B-I*, or *B-Peru* alleles is shown in Figure 4. The various tissues that are pigmented in plants containing the *B-I* or *B-Peru* alleles, and the null *r-g* allele, are described in Methods. For the purpose of this experiment it is important to note that the husks of *B-I* plants are intensely pigmented, whereas the husks of *B-Peru* plants have an irregular distribution of pigment, resulting in regions with no observable pigment. No pigment is observed in *b r-g* plants. In this experiment, about 5 μg of poly(A)$^+$ RNA from *b* (b), about 5 μg of poly(A)$^+$ RNA from *B-Peru* (B-P), and about 0.5 μg of poly(A)$^+$ RNA from *B-I* plants (B-I) were examined. The transcripts appear qualitatively the same in both *B-Peru* and *B-I*, but are quantitatively very different (Figure 4). The transcripts are at least 100-fold more abundant in *B-I* husks. No transcript is detectable in poly(A)$^+$ RNA from *b* husks (Figure 4). All three plants (*b*, *B-Peru*, and *B-I*) contain the same null *r-g* allele, demonstrating that the 2.2-kb transcript observed in *B-Peru* and *B-I* plants is from *B* not *R*. Moreover, the *R* transcript is 2.5 kb (Ludwig et al., 1989), which would have migrated above the 2.2-kb *B* transcript if present and detectable with *B* probes. The differences among these alleles in *B* transcript level roughly correlate with differences in the level of anthocyanin pigment measured in husks (J.P. Radicella, unpublished data). The *B-Peru* probe was removed from the filter, and the filter was rehybridized with a maize actin clone (Meagher et al., 1983) to control for poly(A)$^+$ purification and loading differences among the three preparations (Figure 4). This control demonstrates that the amount of mRNA loaded in the b lane is about 10-fold more and the amount in the B-Peru lane is about 40-fold more, relative to the amount in the B-I lane.

Previous work demonstrated that the expression of *R* was required in the aleurone tissue for the accumulation of transcripts of *C2* and *A1* (Ludwig et al., 1989), and the expression of *C1* was required for the accumulation of transcripts of *Bz1* and *A1* (Cone et al., 1986), three genes encoding anthocyanin biosynthetic enzymes. In addition, the induction of *Bz1* enzyme activity was shown to require a functional *B* or *R* allele in leaf sheaths or aleurones, respectively (Dooner and Nelson, 1979; Gerats et al., 1984). To test whether *B* gene expression influenced the transcription of *Bz1* and *A1* in the husk tissue, the blot previously hybridized with *B* sequences (Figure 4) was rehybridized with *Bz1* and *A1* sequences. As shown in Figure 4, the *A1* transcript is detected in husks from *B-I* and *B-Peru* plants, but not in *b* plants that fail to produce detectable *B* mRNA or anthocyanin pigment. In this experiment, we would have detected *A1* mRNA in *b* plants if it had accumulated to 10-fold to 20-fold below that in *B-Peru*. Low levels of *Bz1* mRNA are detected in both *b* and *B-Peru* husks, whereas *B-I* husks have dramatically increased levels of *Bz1* mRNA. Except for the basal levels of *Bz1* transcript in *b* husks, the amounts of the two structural mRNAs are approximately proportional to the amount of *B* mRNA in the same tissue. (Compare lanes B-P and B-I in Figure 4, probes B, A1, and Bz1.)

DISCUSSION

Genetic studies of the genes required for anthocyanin biosynthesis in maize have demonstrated that several alleles of two regulatory genes, *R* and *B*, are functionally duplicate (Styles et al., 1973). We have demonstrated that this duplicate function is reflected in DNA sequence similarity, and have used *R* genomic clones to isolate *B* genomic sequences. Although DNA sequencing of the *B* alleles has not yet been completed, the hybridization conditions used suggest the extent of sequence identity between *B* and *R* is about 85% to 90%. The sequence similarity appears to extend throughout the transcribed region, as all the *B-Peru* probes hybridize with *R* (V.L. Chandler, J.P. Radicella, and D. Turks, unpublished data). Restriction mapping and partial DNA sequence analysis demonstrate that different *B* alleles appear to share greater sequence similarity with each other than with *R* (V.L. Chandler, J.P. Radicella, and D. Turks, unpublished data).

The genetic data, combined with the sequence similarity, demonstrate that the *R* and *B* genes are homologous. This raises several evolutionary questions, such as what is the ancestral *B* or *R* allele, and which was the original gene. *B* may have arisen from *R* or vice versa via any one of several means. Two simple models are considered. First, there could have been a duplication and translocation event followed by subsequent divergence of the genes. Alternatively, the chromosomes on which *B* and *R* reside might be homologous chromosomes that came together in the formation of an allotetraploid ancestral maize. Restriction fragment length polymorphism (RFLP) analysis of the maize genome identified three clones that map to chromosome 2S (where *B* resides) that are also located on chromosome 10L (where *R* resides) (Helentjaris, Weber, and Wright, 1988), demonstrating that the two chromosomes contain a duplicated region larger than the *B* and *R* genes. However, this finding does not distinguish between the two models stated above (see Helentjaris et al., 1988, for discussion).

More than 100 different *R* alleles and 30 different *B* alleles have been described that vary with respect to the timing and tissue distribution of anthocyanin biosynthesis (for review, see Coe et al., 1988). The tissue specificity observed with the different alleles of *R* and *B* may be caused by differential expression of those alleles in various tissues, different activities of the proteins, or a combination of both. Defining the DNA sequences required for the expression of the various alleles and comparing the amino acid sequences of the proteins encoded by the different alleles in combination with mutational analysis and functional assays should distinguish among these various models.

R and *B* are two of several regulatory genes necessary for the accumulation of anthocyanin pigments. *R* and *C1* are required for the accumulation of transcripts from several structural genes in the aleurone tissue of the seed (Cone et al., 1986; Ludwig et al., 1989), *B* is required for the presence of *Bz1* enzyme activity in leaf sheaths (Gerats et al., 1984), and *R* is required in aleurones (Dooner and Nelson, 1979). We have extended these studies by demonstrating the requirement of a functional *B* allele for the induction of *A1* and *Bz1* transcripts in the husk tissue of the mature plant. The amount of *A1* transcript is greatly increased in the presence of a functional *B* allele, but, as no *A1* mRNA was detected in the absence of a functional *B* allele, it was impossible to estimate the level of induction. Enzymatic activity measurements (Dooner and Nelson, 1979) reveal that, in plants with nonfunctional *C1* or *R* alleles, low levels of UFGT activity (the *Bz1* gene product) are detected throughout development, and only mutation of *Bz1* results in complete elimination of the enzyme activity. These results are in agreement with our detection of low levels of *Bz1* mRNA in husks from *b* plants (Figure 4). We offer two hypotheses to explain the fact that the amount of the *Bz1* transcript present in *B-Peru* husks is not significantly different from the amount present in *b* husks. First, in *B-Peru* husks, pigment is not uniformly distributed and, thus, *B-Peru* mRNA may not be expressed in all the epidermal cells of the husk. We have used whole husks for our assays, and the residual level of *Bz1* mRNA in many of the cells may be masking the induction of *Bz1* mRNA in the few cells that are pigmented. In situ hybridization experiments should establish whether the level of *B* and *Bz1* mRNA is uniform or variable throughout the husk epidermal layer. Another possibility is that UFGT, which catalyzes one of the last steps in anthocyanin synthesis, is not limiting for anthocyanin synthesis in the weakly pigmented *B-Peru* plants and the amount of *Bz1* mRNA observed is sufficient for the low level of pigment that accumulates. With this hypothesis we propose that, in plants containing the *b* allele, which produce approximately the same amount of *Bz1* mRNA as in *B-Peru* plants, the failure to induce another structural gene (e.g., *A1*) accounts for the lack of pigment accumulation.

Taken together, the results suggest that the regulatory genes *B* and *R* function by activating the transcription of the genes encoding the biosynthetic enzymes. Now that several of the regulatory genes and biosynthetic genes are cloned, the tools are in hand to investigate whether the activation is a direct or indirect interaction of the *B* and *R* proteins with the structural genes. Recently, the sequence of a cDNA from an *R* allele has been obtained (Ludwig et al., 1989). The amino acid sequence deduced from the cDNA revealed both a sequence similar to the *myc* homology region found in several DNA binding proteins in mammals and *Drosophila* and a highly negatively charged region characteristic of known transcriptional activators. Similarly, the sequence analysis of *C1* revealed homology to the *myb* proto-oncogene proteins and a structural similarity to transcriptional activators (Paz-Ares et al., 1987). Although the functional significance of these regions of *R*

and *C1* has not yet been defined, it is tempting to speculate that the *R* and *B* proteins, together with *C1* or *Pl*, are mediating their effects by directly regulating the transcription of the structural genes.

METHODS

Plant Stocks

The recombinant inbred lines were obtained from B. Burr, Brookhaven National Laboratory (Burr et al., 1988). The recombinant inbreds were produced by inbreeding the progeny of an F_2 population derived from two highly diverged inbred lines, T232 and CM37. T232 contained a *b* allele and CM37 a *B* allele. The recombinant inbred lines were chosen to be invariant for an RFLP tightly linked to *R* (17.07; B. Burr, personal communication), but carrying either the *B* allele of CM37 (9, 15, 18, 21, 36) or the *b* allele of T232 (12, 16, 19, 20). The stocks containing the *b*, *B-Peru*, and *B-I* alleles were obtained from E.H. Coe, Jr. and M.G. Neuffer, University of Missouri. The *B-Peru* allele was in the W22 background and the *b* and *B-I* alleles were in the W23 or K55 backgrounds. All three stocks had the null *r-g:Stadler* allele of *R* and carried the dominant alleles for the other genes required for anthocyanin biosynthesis (*A1*, *A2*, *C1*, *C2*, *Pr*, *Pl*, *Bz1*, and *Bz2*).

DNA Samples

Maize DNA was isolated from young leaves or immature ears (Dellaporta, Wood, and Hicks, 1983; Dellaporta et al., 1988). A 5.0-kb clone of the seed component of *R-r:standard* (pS) and the overlapping 700-bp pR-nj:1 clone were obtained from S. Dellaporta (Dellaporta et al., 1988). The 5.0-kb HindIII fragment in pS contained repeated DNA sequences, requiring the subcloning of a 1.9-kb HincII/HindIII fragment that was relatively unique (pS-1.9). The maize actin clone (pMAC1) was obtained from R. Meagher (Meagher et al., 1983), *Bz1* (pMBzP17) from D. Furtek and O. Nelson (Furtek et al., 1988), and *A1* (pALC2) from Z. Schwarz-Sommer (Schwarz-Sommer et al., 1987).

DNA Gel Blot Analysis

The DNA gel blot in Figure 1A was performed as described in Dellaporta et al. (1988) except that Nytran (Schleicher & Schuell) was used. The hybridization conditions were: 6 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 10% dextran sulfate, 0.5% SDS, 0.5 mg/mL heparin at 65°C. After hybridization with the pR-nj:1 probe, the blot was washed in 1 × SSC, 0.5% SDS at 65°C. DNA gel blots in Figures 1B and 1C and Figure 2 were performed as described in Chandler, Talbert, and Raymond (1988b) except that MSI nylon (Fisher) was used. All hybridizations were carried out as described (Chandler et al., 1988b) in 50% formamide at 42°C. The wash conditions varied, depending on the probe used: blots hybridized with the seed component of *R* were washed at 58°C in 0.2 × SSC and 0.1% SDS; blots hybridized with the approximately 550-bp *b* fragment were washed at 58°C in 0.1 × SSC and 0.1% SDS. The probes were labeled using random hexamer priming (Feinberg and Vogelstein, 1983).

Isolation of *B* Genomic Sequences

Two different restriction fragments representing part of two *B* alleles were cloned initially. The 2.5-kb BglII fragment from the W23 *b* stock was purified by size-fractionating the DNA in low-melting-point agarose gels. The size fractionation resulted in approximately 200-fold purification of the fragment (there is very little maize DNA in this region of the gel) and the clean separation of the putative "b" sequences from the *R* gene and other cross-hybridizing fragments. The fractionated DNA was ligated to a λ vector that will carry 0-kb to 12-kb BglII fragments (Bv2, obtained from Noreen Murray, University of Edinburgh), and the resulting phage were screened with the 1.9-kb subclone of the S component of *R-r* (pS-1.9) using the conditions established with the DNA gel blots (Figure 1B). Two hybridizing plaques were obtained in the approximately 17,000 screened. Both contained a 2.5-kb BglII fragment that hybridized with pS-1.9. A 550-bp BamHI/HindIII restriction fragment that did not contain repetitive sequences was subcloned into pUC19 (Yanisch-Perron, Vieira, and Messing, 1985).

The 1.0-kb HindIII fragment from the CM37 stock was cloned in a similar manner from size-fractionated DNA using a λ insertion vector that will accept 0-kb to 11-kb HindIII fragments (NM1149, obtained from Noreen Murray). Three hybridizing plaques were obtained from the approximately 10^6 phage screened. All three contained the 1.0-kb HindIII fragment (detected with the pR-nj:1 probe), which was subcloned into pBS (Stratagene).

Transcript Analyses

Total RNA was prepared from husks (harvested from flowering plants) by the guanidinium thiocyanate method (Chirgwin et al., 1979) after grinding the tissue in liquid nitrogen. Poly(A)$^+$ RNA was selected by batch absorption on oligo(dT)-cellulose (Maniatis, Fritsch, and Sambrook, 1982). Aleurone poly(A)$^+$ RNA was prepared as described in Cone et al. (1986). Electrophoresis of poly(A)$^+$ RNA on formaldehyde gels and transfer to nitrocellulose were according to Ausubel et al. (1987). Molecular weight markers were from Bethesda Research Laboratories. Hybridizations were performed as described in Chandler et al. (1988b) in 50% formamide including 5% (w/v) dextran sulfate at 42°C.

The *B-Peru* probes used for mapping the transcription unit were subcloned into pTZ18 or pTZ19 vectors (United States Biochemical), and purified restriction fragments were labeled using random hexamer primers (Feinberg and Vogelstein, 1983). Five nonoverlapping probes were used (restriction sites reading left to right in Figure 3): PstI (P) to HindIII (H), HindIII (H) to BamHI (B), BamHI (B) to HindIII (H), HindIII (H) to SalI (L), and SalI (L) to HindIII (H). RNA gel blot analyses of RNA from *B-Peru* and *B-I* husks identified a 2.2-kb mRNA that hybridized with the three central fragments extending from the first HindIII site through to the SalI site (Figure 3). No hybridization was detected with mRNA from *B-Peru* or *B-I* husks with either of the other two probes. For the determination of the direction of transcription, sense and antisense RNA probes were used. An approximately 300-bp PstI/HindIII genomic fragment was purified and cloned into the pTZ19 and pTZ18 vectors.

The plasmids were linearized and transcribed in vitro with T7 polymerase according to Melton et al. (1984). The labeled RNAs (6×10^6 cpm) were used as probes on RNA gel blots.

ACKNOWLEDGMENTS

We are grateful to Stephen Dellaporta for providing the *R* clones, and to Ed H. Coe, Jr., M. Gerry Neuffer, and Ben Burr for providing the maize stocks, all of which made this study possible. We also thank Doug Furtek, Zsuzsanna Schwarz-Sommer, and Richard Meagher for providing the *Bz1*, *A1*, and maize actin clones, respectively. We appreciate the photographic and typing skills of Harry Howard and Jean Parker, respectively, and the critical comments on the manuscript by our colleagues Kristine Hardeman, Linda Harris, and Garth Patterson. This research was supported by National Institutes of Health grant GM39571 and a Searle Scholars Award to V.L.C., and Department of Energy grant DE-FG02-86ER13627 to Stephen Dellaporta providing postdoctoral support for T.P.R. and J.C.

Received August 17, 1989; revised September 25, 1989.

REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.S., Smith, J.A., and Struhl, K. (1987). Analysis of RNA by Northern hybridization. In Current Protocols in Molecular Biology. (New York: Green/Wiley), pp. 4.9.1–4.9.7.

Briggs, R.W. (1966). Recognition and classification of some genetic traits in maize. J. Hered. **57,** 35–42.

Burr, B., Burr, F.A., Thompson, K.H., Albertsen, M.C., and Stuber, C.W. (1988). Gene mapping with recombinant inbreds in maize. Genetics **118,** 519–526.

Chandler, V.L., Belcher, S., and Turks, D. (1988a). Genetic properties of four *Mutator*-induced alleles of *B-Peru*. Maize Genet. Coop. Newslett. **62,** 56–58.

Chandler, V.L., Talbert, L.E., and Raymond, F. (1988b). Sequence, genomic distribution and DNA modification of a *Mu1* element from non-mutator maize stocks. Genetics **119,** 951–958.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18,** 5294–5299.

Coe, E.H., Jr. (1979). Specification of the anthocyanin biosynthetic function by *B* and *R* in maize. Maydica **XXIV,** 49–58.

Coe, E.H., Jr. (1985). Phenotypes in corn: Control of pathways by alleles, time and place. In Plant Genetics, UCLA Symposia on Molecular and Cellular Biology, M. Freeling, ed (New York: Alan R. Liss), Vol. 35, pp. 509–521.

Coe, E.H., Jr., Hoisington, D.A., and Neuffer, M.G. (1988). The genetics of corn. In Corn and Corn Improvements, G.F. Sprague and J. Dudley, eds (Madison, WI: American Society of Agronomy), pp. 81–258.

Cone, K.C., Burr, F.A., and Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc. Natl. Acad. Sci. USA **83,** 9631–9635.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA mini preparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

Dellaporta, S., Greenblatt, I., Kermicle, J., Hicks, J.B., and Wessler, S. (1988). Molecular cloning of the maize *R-nj* allele by transposon-tagging with *Ac*. In Chromosome Structure and Function: Impact of New Concepts, 18th Stadler Genetics Symposium, J.P. Gustafson and R. Appels, eds (New York: Plenum Press), pp. 263–282.

Dooner, H.K. (1983). Coordinate regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet. **189,** 136–141.

Dooner, H.K., and Kermicle, J.L. (1971). Structure of the *R-r* tandem duplication in maize. Genetics **67,** 427–436.

Dooner, H.K., and Nelson, O.E., Jr. (1977). Genetic control of UDPglucose:flavonol 3-*O*-glucosyl transferase in the endosperm of maize. Biochem. Genet. **15,** 509–519.

Dooner, H.K., and Nelson, O.E., Jr. (1979). Interaction among *C*, *R* and *Vp* in the control of the *Bz* glucosyltransferase during endosperm development in maize. Genetics **91,** 309–315.

Emerson, R.A. (1921). The genetic relations of plant colors in maize. Cornell Univ. Agr. Exp. Sta. **39,** 3–156.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction fragments to high specific activities. Anal. Biochem. **132,** 6–13.

Furtek, D., Schiefelbein, J.W., Johnston, F., and Nelson, O.E., Jr. (1988). Sequence comparisons of three wild-type *Bronze-1* alleles from *Zea mays*. Plant Mol. Biol. **11,** 473–482.

Gerats, A.G., Bussard, J., Coe, E.H., Jr., and Larson, R. (1984). Influence of *B* and *R* on UDPG:flavonoid-3-*O*-glucosyltransferase in *Zea mays* L. Biochem. Genet. **22,** 1161–1169.

Helentjaris, T., Weber, D., and Wright, S. (1988). Identification of the genomic locations of duplicate nucleotide sequences in maize by analysis of restriction fragment length polymorphisms. Genetics **118,** 353–363.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production encodes a protein similar to transcriptional activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), p. 198.

Meagher, R.B., Hightower, R.C., Shah, D.M., and Mozer, T. (1983). Plant actin is encoded by diverse multigene families. In Advances in Gene Technology: Molecular Genetics of Plants and Animals, K. Downey, R. Voellmey, F. Ahmad, and J. Shultz, eds (New York: Academic Press), pp. 171–187.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. **12,** 7035–7056.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P., and Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. **6,** 3553–3558.

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P., and Saedler, H. (1987). Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. EMBO J. **6,** 287–294.

Stadler, L.J., and Nuffer, M.G. (1953). Problems of gene structure II. Separation of *R-r* elements (S) and (P) by unequal crossing over. Science **117,** 471–472.

Styles, E.D., and Ceska, O. (1977). The genetic control of flavonoid synthesis in maize. Can. J. Genet. Cytol. **19,** 289–302.

Styles, E.D., Ceska, O., and Seah, K.T. (1973). Developmental differences in action of *R* and *B* alleles in maize. Can. J. Genet. Cytol. **15,** 59–72.

Walbot, V., Briggs, C.P., and Chandler, V. (1986). Properties of mutable alleles recovered from mutator stocks of *Zea mays* L. In Genetics, Development, and Evolution, J.P. Gustafson, G.L. Stebbins, and F.J. Ayala, eds (New York: Plenum Publishing), pp. 115–142.

Wienand, U., Weydemann, U., Niesbach-Kloesgen, U., Peterson, P.A., and Saedler, H. (1986). Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol. Gen. Genet. **203,** 202–207.

Yanisch-Perron, D., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13 mp18 and pUC19. Gene **33,** 103–119.