Allelic diversity of the maize B regulatory gene: different leader and promoter sequences of two B alleles determine distinct tissue specificities of anthocyanin production

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The B gene encodes a transcription factor of the basic helix-loop-helix class, which controls the synthesis of the anthocyanin pigments in maize. This gene, as well as the highly homologous R gene family, displays extensive allelic variation in that different alleles cause distinct distributions of anthocyanin pigments in different tissues and at different developmental times. The analysis of the expression of two B alleles, with distinct tissue-specific patterns of anthocyanin synthesis in plant and seed tissues, demonstrates that the amount of B transcripts correlates with the accumulation of anthocyanins in the various tissues. The comparison of the genomic clones for the two alleles reveals high sequence identity in the coding and 3'-flanking regions (98% and ~90%, respectively). In contrast, the most 5' region of their mRNAs and the 5'-flanking sequences share no significant sequence identity. This result suggests that the alleles diverged from each other by complex genome rearrangements rather than by simple base pair substitutions. We have used the high velocity microprojectile transformation assay to demonstrate that the differential expression of the two alleles in the seed is determined by their 5' variant sequences. Thus, the variation in tissue-specific anthocyanin synthesis in plants with these different B alleles is controlled at the level of B gene expression.

[Key Words: Anthocyanin; basic helix-loop-helix regulatory protein; allelic diversity]

Received July 6, 1992; revised version accepted August 24, 1992.

The regulation of the genes required for the synthesis of the purple anthocyanin pigments in maize provides an excellent system for investigating the control of gene expression in higher organisms. Genetic analyses have identified numerous genes required for the synthesis of anthocyanins in maize (Coe et al. 1988). Among these are four regulatory loci, b, r, pl, and cl, which specifically control the transcription of genes encoding the biosynthetic enzymes (Dooner 1983, Goff et al. 1990, 1991, 1992, Ludwig et al. 1990). These four genes encode two sets of functionally equivalent regulatory proteins. The C1 and PI gene products share sequence identity with DNA-binding domains from the MYB oncogene family of proteins (Paz-Ares et al. 1987). C1 is required for the synthesis of anthocyanins in the aleurone and embryo, whereas the homologous PI gene product (Cone and Burr 1988) is required for pigment production in most of the plant body [for review, see Coe 1985]. The r and b genes encode proteins with homology to the basic helix-loop-helix motif in the MYC, MYO-D, and E12 proteins [Ludwig et al. 1989; Perrot and Cone 1989; Radicella et al. 1991]. Numerous alleles of the b and r loci have been isolated from different geographic locations. These alleles show great diversity in the tissue specificity and developmental timing of anthocyanin synthesis [Styles et al. 1973]. Most B alleles specify pigment production in some part of the leaves, sheaths, or tassel, whereas many R alleles pigment the aleurones, anthers, or coleoptiles [Styles et al. 1973]. In some tissues certain B and R alleles can function as duplicate genes, with either locus sufficient for pigmentation.

Genetic and molecular studies have revealed that the r gene is complex. Historically, naturally occurring variants of the r gene have been called alleles, yet this is a misnomer for many alleles, as they contain multiple genes. For example, the standard R-r allele, which pigments plant and seed tissues, contains two components (P and S, for plant and seed coloration, respectively). Each component can be independently mutated and separated by recombination (Stadler and Nuffer 1953). Molecular analysis of R-r shows that the multiple components correlate with the presence of distinct cross-hy-
bridizing DNA restriction fragments [Dellaporta et al. 1988; Robbins et al. 1991]. Thus, each component represents a separate gene that functions in different tissues. Additional members of the \( r \) gene family, such as \( Lc \) and \( Sn \), with distinct patterns of anthocyanin synthesis, are located 2 map units from \( r \) [Dooner and Kermicle 1976; Gavazzi et al. 1990]. In contrast, the \( b \) locus appears to be simple. The \( B\)-\( Peru \) allele, like the \( R-r \) complex, stimulates the pathway in both seed and plant tissues. However, several independent transposable element insertions into the \( B\)-\( Peru \) locus were isolated, and each affected pigment synthesis in all of the tissues normally pigmented by this allele [Patterson et al. 1991]. These insertions all map within a 5-kbp genomic region, and \( B \) probes strongly hybridize with only the region of the genome that spans the \( B\)-\( Peru \)-coding region [Chandler et al. 1989; Patterson et al. 1991]. These results suggest that the tissue-specific regulation conferred by \( B\)-\( Peru \) is likely to be mediated by tissue-specific controlling sequences of a single coding region rather than separate genes expressed in different tissues.

The high degree of amino acid similarity among the putative gene products of the \( B \) and \( R \) alleles [Ludwig et al. 1989; Perrot and Cone 1989; Radicella et al. 1991] suggests that the proteins produced by the different genes and alleles might be functionally equivalent. In support of this view, when the cDNAs from \( Lc \), \( B-I \), and \( B\)-\( Peru \) were fused to a constitutive promoter, CaMV-35S, and introduced into various maize tissues, the constructs were able to induce the anthocyanin biosynthetic pathway in almost every tissue tested, including several in which the alleles normally do not function [Goff et al. 1990; Ludwig et al. 1990]. These experiments support the hypothesis that the various \( B \) and \( R \) proteins are functionally equivalent and that the allelic diversity resides in the differential expression of the \( b \) and \( r \) genes.

To investigate this hypothesis further, we have analyzed the expression and structure of two \( B \) alleles with very different patterns of anthocyanin synthesis. In addition, we have used transient transformation assays to localize the genomic sequences responsible for the control of \( B\)-\( Peru \) expression in the aleurone.

**Results**

**Phenotypes of \( B-I \) and \( B\)-\( Peru \) alleles**

The \( B-I \) and \( B\)-\( Peru \) alleles give very distinct tissue distributions of anthocyanins in the epidermal layer. Plants with the \( B-I \) allele have dark purple husks, tassels, culms, and sheaths [Fig. 1A]. The leaves of \( B-I \) plants also accumulate anthocyanin pigments, but to a lesser extent. The \( B\)-\( Peru \) plants are mostly green until almost fully mature, at which time anthocyanins accumulate in several organs [Fig. 1B]. The tassels of \( B\)-\( Peru \) plants are uniformly pigmented, although the amount of pigment is less than in tassels of \( B-I \) plants. A variable and irregular anthocyanin pigmentation can be observed in the husks and sheaths of \( B\)-\( Peru \) plants. These tissues are mostly green, with small nonclonal sectors of medium pigmentation. Anthocyanins also accumulate in the culms of \( B\)-\( Peru \) plants just below the nodes.

Another striking difference in anthocyanin deposition between these two alleles occurs in the kernels. Figure 1C shows ears from plants carrying the \( B-I \) or \( B\)-\( Peru \) alleles. \( B\)-\( Peru \) causes intense and uniform anthocyanin accumulation in the aleurones and weaker accumulation in the embryo and scutellum, but no pigment accumulates in the outer seed coat, the pericarp [Fig. 1C, bottom ear]. In contrast, \( B-I \) seeds do not accumulate anthocyanins in the embryo, scutellum, or aleurone. The pigment observed at the base of the \( B-I \) kernels [Fig. 1C, top ear] is in the pericarp.

**Expression patterns of the \( B \) alleles**

To investigate whether the different patterns of anthocyanin accumulation correlate with the amount of \( B \) mRNA present, RNA was isolated from different tissues of plants carrying the \( B-I \) or \( B\)-\( Peru \) alleles. Three tissues, tassel, sheath, and aleurone, showing distinct differences between the two alleles, were analyzed. The expression pattern was assessed by Northern blot analysis of poly(A)⁺ RNA from plants of the same age with either the \( B-I \) or \( B\)-\( Peru \) allele [Fig. 2A]. The predominant transcript encoded by each allele in all tissues is the same size and is ~2.0 kb in length.

The steady-state levels of \( B \) mRNA vary dramatically from tissue to tissue and between alleles. To compensate for this, different amounts of RNA from the different tissues were loaded in each lane. The differences in the amounts of RNA loaded in each lane are indicated by probing the same blot with the maize actin gene [Fig. 2B]. The levels of \( B \) transcript are relatively high in \( B\)-\( Peru \) aleurones relative to \( B\)-\( Peru \) tassels and sheaths, where they are ~4- and 50-fold lower, respectively. When comparing the transcript levels between alleles, \( B \) mRNA was ~5- and 100-fold higher in \( B-I \) tassels and sheaths relative to \( B\)-\( Peru \) tassels and sheaths. The levels of \( B \) mRNA observed in the various tissues correlated well with the distribution of anthocyanin pigments in those tissues.

**Analysis of the \( B-I \) and \( B\)-\( Peru \) genomic sequences**

As a first step toward identifying the sequences that determine the different expression patterns of the \( B \) alleles, the genomic sequences for \( B-I \) and \( B\)-\( Peru \) were isolated and compared. The cloned 10.5-kbp \( B\)-\( Peru \) sequences extend ~2 kbp upstream and 3.5 kbp downstream of the transcribed region [Chandler et al. 1989]. Using \( B\)-\( Peru \) genomic sequences as probes, two overlapping fragments of genomic DNA containing the \( B-I \) gene were isolated [Materials and methods]. The reconstructed \( B-I \) genomic clone is ~14 kb long and spans ~0.9 and 9 kb upstream and downstream of the transcribed region, respectively. A combination of restriction mapping, DNA sequencing, RNase and S1 protection assays, and rapid
Figure 1. Plant and kernel phenotypes of B-I and B-Peru. (A) Tassel (left) and husk, leaves, and culm (right) from plants carrying the B-I allele. (B) Tassel (left) and husk, leaves, and culm (right) from plants carrying the B-Peru allele. (C) Mature ears from B-I (top) and B-Peru (bottom) plants.
amplification of cDNA ends (RACE) was used to determine and compare the structures of the B-Peru and B-I genomic sequences and transcripts.

Throughout the transcribed regions, the restriction maps for both alleles are identical [Fig. 3]. In contrast, the upstream sequences have completely different restriction maps. To compare the alleles more precisely, the B-Peru and B-I cDNAs, ~6.0 kbp of the B-Peru genomic region and the most 5' ~2.0 kbp of the B-I genomic region have been sequenced. Comparison of the cDNA sequences for both alleles revealed that the B-I and B-Peru alleles are 98% identical in their coding regions and the exon/intron junctions are identical for exons 2–9. The ATG at the beginning of the long open reading frame in the cDNAs is in exon 2. Figure 4 shows the alignment of the B-I and B-Peru genomic sequences in their 5' regions. A sharp boundary can be drawn at nucleotide 532 of the B-I sequence in Figure 4. This boundary is indicated by a vertical line in Figures 3 and 4. No significant sequence identity could be found in the sequences upstream of that point.

In contrast to the different 5'-flanking sequences, the alleles show significant sequence similarity within the 3 kbp downstream of the polyadenylation site. Although these regions have not been sequenced, Southern blot analysis of the 3'-flanking region of the B-I clone was performed using the most 3' SacI–BamHI fragment [Fig. 3] of B-Peru as a probe. With the hybridization conditions used, we estimate a minimum of 85–90% identity between the alleles within the fragments between the polyadenylation site and the most 3' HindIII site in the B-I allele indicated in Figure 3 (Materials and methods).

The B-I and B-Peru transcription units

The alignment of the B-Peru genomic and cDNA sequences [Radicella et al. 1991] defined the exon/intron organization of this allele in the coding region spanning exons 2–9 [Fig. 3]. Comparison of the B-I cDNA sequence [D. Brown and V. Chandler, unpubl.] with the B-Peru cDNA indicated that the B-I allele has the same structure as B-Peru throughout exons 2–8 [Fig. 3]. However, the poly(A) + tails are added at slightly different positions, causing the B-Peru exon 9 to be 18 nucleotides shorter at the 3' end relative to the B-I exon 9. The isolated cDNA clones extended through exon 2, but on the basis of their size and confirmed by RNase protection assays, they were missing all or most of exon 1. There-
fore, several experiments were performed to determine the structure of the 5' ends of the B mRNA.

RNase protection assays were performed on RNA isolated from either B-Peru or B-I plants. Labeled antisense RNA probes spanning the genomic sequences from SacI in the first intron to PsI in the second exon (Fig. 3) protected a RNA species of ~60 bases for both alleles, confirming that both B-Peru and B-I transcripts include a similar exon 2 carrying the putative ATG initiation codon. This was substantiated further by the cDNA sequences (Radicella et al. 1991; D. Brown and V. Chanler, unpubl.). However, when probes that extended into the divergent 5' regions of the alleles were used, different sizes of RNA species were protected by RNA from each allele. The asterisk (*) indicates identities between B-I and B-Peru. Gaps represent gaps introduced to align the sequences. Exons 1 and 2 are boxed. (O) The most 5' ends of the mature transcripts. Putative TATA boxes for both alleles are indicated by a thick underline, and the ATGs upstream of the long open reading frame in the first exon of B-I are indicated by a thin underline. The long open reading frame begins in exon 2 as indicated.

**Figure 4.** DNA sequences from the 5' regions of the B-I [BI] and B-Peru [BP] alleles. The nucleotide sequences from B-I are shown on the top lines, with the differences in the B-Peru sequence indicated on the bottom lines. The allele (*). The most 5' ends of the mature transcripts. Putative TATA boxes for both alleles are indicated by a thick underline, and the ATGs upstream of the long open reading frame in the first exon of B-I are indicated by a thin underline. The long open reading frame begins in exon 2 as indicated.

The presence of a 99-bp exon 1 in B-I mRNA, spanning the point where the B-I and B-Peru sequences diverge (Fig. 5A). RNase protection assays using polymerase chain reaction [PCR] clones fusing exon 1 with exon 2 confirmed that the major splicing event observed in steady-state RNA fused the 99-base exon 1 to exon 2. Minor protected fragments that corresponded to intron 1 remaining unspliced were also observed (data not shown). The 5' ends of the PCR-derived clones are indicated by arrows in Figure 5A. The most 5' end of the B-I transcripts, as defined by the RACE clones and indicated by an asterisk in Figure 5A, was confirmed by S1 and RNase protection assays (data not shown). Thus, this 5' end may represent the start of transcription. However, the sequences immediately upstream of this 5' end are similar to a consensus 3' splice site. To determine whether transcription initiated upstream of this region, ~1.5 kbp of B-I sequence upstream of this site was used for RNase protection experiments. No additional exons were detected (data not shown). Thus, we have no evidence of tran-
Figure 5. RACE mapping of exons 1 and 2 for B-I [A] and B-Peru [B]. The arrows show the most 5' B nucleotide occurring in the clones obtained by the RACE procedure. The numbers beneath each arrow indicate the number of clones that were obtained that ended at that nucleotide. The asterisk (*) indicates the most 5' end identified. In all but one case, when the clones extended into exon 1, exon 1 was fused to exon 2. In the one exception, exon 1 from B-Peru was fused to the underlined sequences adjacent to exon 2 [B]. Exons 1 and 2 are boxed.

scripts initiating upstream of the most 5' end that we have mapped. The identified exons add up to the size predicted from Northern blot analysis, suggesting that if a more 5' exon is missing, it is quite small.

Using procedures similar to those described for B-I, several 5' RACE-derived B-Peru clones were isolated and sequenced. The analysis of the clones from the amplification of the 5' region of the aleurone mRNA from B-Peru plants established the presence of a 25-bp exon 1. The 3' exon/intron junction for this first exon is identical to the splice site of the first B-I exon. Of the 18 clones sequenced, 10 carried the 25-bp exon fused to the second exon; 9 of these clones defined the putative start of transcription indicated by the asterisk in Figure 5B; the tenth clone ended 3 bp downstream of that point [Fig. 5B]; and an additional clone reflected the use of an alternative
splicing site 39 bp upstream of the normal one for exon 2, as indicated by the underlined sequences in Figure 5B. This does not create any additional ATG codons in the B-Peru leader and, therefore, should not affect the sequence of the protein encoded by this allele. For the other seven clones, the 5' end was located at various points within the intron sequences (Fig. 5B).

Our results demonstrate that the B-Peru and B-I alleles have completely different upstream sequences and produce transcripts with different 5' ends. The 99-bp exon from B-I contains two ATG codons (underlined in the B-I sequence in Fig. 4), the first of which is in-frame with the ATG in the second exon. If translation begins at the first ATG, the B-I-encoded protein would be 28 amino acids longer than the B-Peru-encoded protein. To ascertain whether the upstream ATG in exon 1 of B-I is translated, we compared the sizes of the proteins encoded by B-I clones without exon 1 to the size of the B-I protein produced in plant tissues. As it is not possible to detect B proteins in transiently transformed maize tissues, we transformed yeast with the B-I cDNA deleted for exon 1 and cloned into a yeast expression vector such that translation begins at the ATG in exon 2. As shown in Figure 6A, the B protein synthesized in yeast comigrates with the B protein isolated from husks and tassels of B-I plants. In reconstruction experiments, we were able to readily detect a 24-amino-acid difference between B proteins synthesized in yeast [Fig. 6B]. The B protein translated from the ATG in exon 2 [Fig. 6B, lane f] was compared with mutants deleted for amino acids 4–27 [Fig. 6B, lanes d,e] and amino acids 4–66 [Fig. 6B, lanes b,c]. The fact that we detect a 24-amino-acid difference between B proteins synthesized in yeast, combined with the comigration of the B protein from B-I plants with the B protein translated from the ATG in exon 2, suggests that the major B protein produced in B-I plants is translated beginning at the second ATG located in exon 2.

Protein from a negative control strain containing the expression vector with no B sequences (same strain used in A, lane b), [lanes b,c] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–66, [lanes d,e] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–27, [lanes f] yeast protein from a transformant containing the B expression plasmid, engineered such that the first ATG corresponds to the ATG in exon 2; [lane c] total protein isolated from husks of B-I plants, [lanes d,e] total protein isolated from tassels of B-I plants, [lanes f,g] total protein isolated from tassels of B plants. The recessive b allele produces no detectable B mRNA. The arrowhead indicates the B protein. All of the other protein bands are nonspecific. The large nonspecific protein band observed in tassels corresponds to the most abundant protein observed on Coomassie-stained gels. (B) Immunoblot prepared using -0.1 mg of total yeast protein in each lane. [Lane a] Protein from a negative control strain containing the expression vector with no B sequences (same strain used in A, lane a), [lanes b,c] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–66, [lanes d,e] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–27, [lanes f] yeast protein from a transformant containing the B expression plasmid, engineered such that the first ATG corresponding to the ATG in exon 2 (same strain used in A, lane b). The samples from yeast strains containing the deletion mutant derivatives contained much less B protein relative to the strain expressing the complete B protein. This might have occurred because the proteins missing the amino-terminal amino acids are less stable or because their different nontranslated leaders cause differences in expression.

Functional analysis of the B-Peru and B-I genomic sequences

When delivered to the aleurone with high velocity microprojectiles, the B-Peru genomic clone is able to induce the expression of the firefly luciferase reporter gene under the control of the structural gene promoters, Bronze1 (Bz1) or A1 [Goff et al. 1990]. If the B-Peru and B-I genomic clones are regulated in the transient transformation assays as they are in plants, our expectation would be that the B-I genomic clone would be unable to induce the Bz1 promoter in aleurone tissue. Accordingly, the cloned B-I and B-Peru genomic sequences, together with the reporter Bz1–luciferase construct, were introduced by particle gun bombardment into aleurones from the homozygous recessive double mutant [b t]. Another

Figure 6. Immunoblot analyses of B-I-encoded proteins synthesized in yeast and plants. Total proteins were isolated from yeast strains and maize plants expressing B and from control strains and plants with no B expression, and immunoblotted using B antibodies. [A] (Lane a) Yeast total protein from a negative control strain containing the expression vector with no B sequences, [lane b] yeast total protein from a strain containing the B expression plasmid, engineered such that the first ATG corresponds to the ATG in exon 2; [lane c] total protein isolated from husks of B-I plants, [lanes d,e] total protein isolated from tassels of B-I plants, [lanes f,g] total protein isolated from tassels of B plants. The recessive b allele produces no detectable B mRNA. The arrowhead indicates the B protein. All of the other protein bands are nonspecific. The large nonspecific protein band observed in tassels corresponds to the most abundant protein observed on Coomassie-stained gels. [B] Immunoblot prepared using -0.1 mg of total yeast protein in each lane. [Lane a] Protein from a negative control strain containing the expression vector with no B sequences (same strain used in A, lane a), [lanes b,c] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–66, [lanes d,e] total yeast protein from two independent transformants containing a mutated B cDNA deleted for amino acids 4–27, [lanes f] yeast protein from a transformant containing the B expression plasmid, engineered such that the first ATG corresponds to the ATG in exon 2 (same strain used in A, lane b). The samples from yeast strains containing the deletion mutant derivatives contained much less B protein relative to the strain expressing the complete B protein. This might have occurred because the proteins missing the amino-terminal amino acids are less stable or because their different nontranslated leaders cause differences in expression.
were averaged and are expressed as the average plus or minus the standard errors of the mean (S.E.M). (B) B-mediated induction of anthocyanin pathway. Aleurones were bombarded with 2 μg of B genomic DNA. Before tissue grinding, the number of pigmented cells was counted, extracts were prepared, and CAT activity was determined. The total number of pigmented cells per aleurone was then divided by the CAT activity obtained in extracts of the same aleurone. Each value plotted represents the average of at least three independent experiments (± S.E.M).

Figure 7. Functional analysis of intact and chimeric B-I and B-Peru genes. The BP::Bl construct contains the 5’ region of B-Peru fused to the coding and 3’ region of B-I. The Bl::BP construct contains the 5’ region of B-I fused to the coding and 3’ region of B-Peru. (A) Trans-activation of the BzI promoter. Increasing amounts of B genomic DNAs were delivered to b r aleurones by high velocity microprojectile bombardment along with the BzI-luciferase reporter gene and the Adh–CAT internal control constructs. Induction levels were determined by dividing the luciferase/CAT activity ratio of each bombardment by the average luciferase/CAT activity ratio obtained from bombardments using the vector DNA as a control. Independent determinations from at least six sets of bombardments were averaged and are expressed as the average plus or minus the standard errors of the mean (S.E.M). (B) B-mediated induction of anthocyanin pathway. Aleurones were bombarded with 2 μg of B genomic DNA. Before tissue grinding, the number of pigmented cells was counted, extracts were prepared, and CAT activity was determined. The total number of pigmented cells per aleurone was then divided by the CAT activity obtained in extracts of the same aleurone. Each value plotted represents the average of at least three independent experiments (± S.E.M).

Identification of cis-regulatory sequences

Sequences upstream of the point where the B-Peru and B-I alleles diverge are obvious candidates for controlling the differences in expression between B-Peru and B-I. To test this hypothesis, constructs were made in which the sequences upstream of the SacI or HindIII sites (at positions 595 and 635, respectively, in Fig. 4) were exchanged between the alleles. There are only three nucleotide substitutions between B-Peru and B-I in the 50 bp between the point of divergence and the SacI site, and no differences between the SacI and HindIII sites. Two chimeric clones were generated: Bl::BP, comprising 945 bp of B-I 5’-flanking sequences and first exon fused at the HindIII site to the B-Peru-coding and 3’-flanking regions, and BP::Bl, the reciprocal construct, comprising 2.1 kbp of B-Peru 5’-flanking sequences and first exon fused at the SacI site to the B-I coding and 3’-flanking regions. To test whether these clones were functional, they were introduced into leaves from homozygous double mutant (b r ) plants. After 48 hr, numerous purple cells were detected in the leaves transformed with either construct (data not shown), demonstrating that both chimeric clones could generate a functional B protein.

The differential tissue-specific expression of these chimeric genes was then investigated in aleurones. For that purpose, different amounts of the constructs were delivered into aleurones from the homozygous recessive double mutant (b r ) plants, together with the BzI-luciferase and Adh–CAT genes. The aleurones were examined for purple cells before grinding the tissue. Extracts were then prepared, and the luciferase and CAT activities were determined. The dose response curves for the induction of luciferase for the BP::Bl and Bl::BP constructs superimpose with the curves for the B-Peru and B-I clones, respectively (Fig. 7A). Similar results were obtained when the aleurones were analyzed for the presence of pigmented cells. The only difference between the two assays is that more DNA was required to induce the complete anthocyanin pathway. With the B-Peru sequences, significant numbers of purple cells [up to 1000 per aleurone] were detected when 1 μg or more of genomic DNA was used. In the case of B-I, almost no purple cells were detected when as much as 3 μg of genomic DNA was used. At 10-fold higher DNA concentrations the B-I clone also produced purple cells. Figure 7B shows the results of the quantitation of pigmented cells in aleurones transformed with 2 μg of either the B-I or B-Peru...
genomic clones. As found with the B-Peru genomic clone, 2 μg of the BP::BI construct elicited the appearance of a significant number of pigmented cells, reflecting the induction of the entire anthocyanin pathway [Fig. 7B]. Almost no purple cells were detected in the aleurons after delivery of 2 μg of the BI::BP construct [Fig. 7B]. The ability of the chimeric BP::BI construct to activate the pathway in b t aleurons demonstrates that the B-I-coding region can produce a B product capable of activating the anthocyanin biosynthetic pathway in aleurons. Furthermore, these results indicate that most, if not all, of the cis-acting elements that determine differential expression of these two alleles in the aleuron are present in the upstream sequences spanning the 5'-flanking region, the putative transcriptional start sites, and the first exon.

Discussion
The high degree of sequence similarity among the B and R gene products, functional assays in which constitutively expressed B and R cDNAs were shown to activate the anthocyanin pathway in b t maize tissues, and the correlation between the appearance of anthocyanin pigments in a particular tissue and the steady-state level of B or R mRNA in the same tissue support the view that the different spatial and temporal distributions of pigment are caused by differential expression of the regulatory genes [this paper; Chandler et al. 1989; Ludwig et al. 1989, 1990; Perrot and Cone 1989; Goff et al. 1990; Racicella et al. 1991; Tonelli et al. 1991].

Molecular analysis of B-I and B-Peru demonstrates that both alleles are very similar [98% identical] in the region spanning the coding sequences, with the identity preserved across all exon/intron boundaries. Several kilo-base pairs of 3'-flanking sequence are also similar between the alleles [~85–90% identity]. In contrast, the 5'-flanking sequences and transcription initiation sites are essentially unrelated, with no significant sequence identity within the 1.5 kbp sequenced. Transient transformation assays with the genomic clones were able to mimic the seed expression patterns of the B-Peru and B-I alleles, in that introduction of the B-Peru genomic clone activated the anthocyanin pathway, whereas introduction of similar amounts of the B-I genomic clone failed to do so. Introduction of chimeric constructs of B-Peru and B-I sequences into aleurones from homozygous recessive double mutant [b t] plants indicates that the difference in expression between the B-I and B-Peru alleles in aleurones is controlled by cis-acting sequences present in the nonidentical regions of the genes. Thus, a major component determining the differences in the patterns of pigmentation between plants carrying the B-I and B-Peru alleles is at the level of accumulation of transcripts from the B regulatory gene, and the sequences controlling this lie within the promoter or leader regions, or both.

At least two models could account for the differential expression of the alleles in the aleuron. First, it is possible that the accumulation of B transcripts is controlled at the level of transcription. In that case, the regulatory sequences might be recognized by aleurone-specific factors that act as either activators (in B-Peru) or inhibitors (in B-I) of transcription. A second possibility is that the mRNAs from the alleles have different stabilities in different tissues. The sequences exchanged to obtain the chimeric BI::BP and BP::BI constructs include the first exon, which is different between the two alleles and contains part of the nontranslated leader. It is possible that the different leaders could influence transcript stability in a tissue-specific manner. Transcription assays in nuclei isolated from multiple tissues, as well as the testing of new constructs that alter only the leaders or only the 5'-flanking sequences, will be required to distinguish between these two models.

The finding that the tissue-specific alleles have completely different 5'-flanking sequences may provide clues regarding how tissue specificity might have evolved. If differential transcription is controlled by tissue-specific enhancer sequences, then multiple base pair substitutions might be required to generate altered tissue specificity. Multiple nucleotide substitutions would be expected to be extremely rare. In contrast, altered tissue specificity could be generated in a single step by placing tissue-specific regulatory sequences next to a gene by a genome rearrangement. One speculation is that the various B alleles arose through nonhomologous recombination events, possibly mediated by transposable elements. There is ample evidence of transposable elements mediating altered patterns of expression and several reports of tissue-specific alterations (Coen et al. 1986; Sullivan et al. 1989, Kloeckener-Gruissem et al. 1992). These have occurred when elements insert into the promoter sequences and cause DNA rearrangements, often upon excision. The types of rearrangements that have been observed range from small insertions of a few base pairs to large deletions and inversions. Many of these rearrangements result in a novel sequence, either replacing or becoming adjacent to the promoter sequences. It is not yet clear what type of rearrangements might have occurred at B-Peru and B-I. As a first step toward addressing this issue, we asked whether the 5'-flanking region of B-I is in the B-Peru genome, and vice versa. A fragment from the B-I 5'-flanking region does hybridize to sequences linked to the B-Peru locus [G. Patterson and V. Chandler, unpubl.]. The B-Peru 5'-flanking sequences are represented in ~10 copies in both genomes, so we have not yet addressed whether one or more of these sequences is linked to B-I. Characterization of the sequences surrounding the B alleles might reveal whether the various alleles are related to one another by insertions, deletions, or more complicated rearrangements.

Detailed genetic experiments comparing R alleles with different tissue-specific patterns of anthocyanin synthesis have established that some R alleles are complex and that tissue-specific control is mediated through duplicated coding regions with different expression patterns. Genetic experiments have localized the tissue-specific components to the 5' ends of the alleles [Kermicle
which have different restriction maps (Robbins et al. 1991). It has been proposed that the diversity of R alleles may be a consequence of an ancestral gene duplication undergoing multiple unequal exchanges and subsequent molecular divergence (Robbins et al. 1989, 1991). The b locus is likely to have also arisen from a duplication of an r ancestral gene, or vice versa. However, the B alleles examined to date do not appear to be complex, as they each consist of a single coding region. Thus, the model of unequal exchange between duplicated coding regions is unlikely to explain the diversity of B alleles.

The ectopic expression of a regulatory gene that can occur by juxtaposition of novel DNA sequences next to that gene, such as we have observed at b, could have major evolutionary implications. It has been shown that dominant mutations in pattern formation genes cause significant morphological changes. For example, mutations affecting leaf morphology in maize (Veit et al. 1990) and embryogenesis in C. elegans (Ruvkun et al. 1991) and Drosophila (Schneuwly et al. 1987) have been described. Ruvkun and his colleagues have speculated that such dominant mutations could produce a variety of individuals with morphological differences, even in a large outbred population [Ruvkun et al. 1991]. Unlike recessive mutations, dominant mutations affect the phenotype when heterozygous. Thus, a dominant mutation from a heterozygous individual will be segregated to one-half of the progeny, all of which will show the phenotype. If the mutation is selectively advantageous, it will spread through the population much more rapidly than a recessive mutation.

Very few genes have such a large tissue-specific diversity as that observed at b and r. Maize adh1 does have several natural alleles with differences in tissue distribution. Restriction maps of six Adh1 alleles demonstrated that sites within the transcriptional unit and in nearby upstream sequences were conserved, but extensive polymorphism occurred outside this region [Johns et al. 1983]. There are also several examples of multigene families in which different genes have distinct tissue-specific expression and different 5′-flanking regulatory sequences [Fluhr and Chua 1986; Hudspeth et al. 1986; Simpson et al. 1986; Tingey et al. 1988; Sheen 1991].

One interesting example in maize is the chloroplast and cytosolic pyruvate, orthophosphate dikinase (PPDK) genes [Sheen 1991]. The maize PPDK gene encoding the chloroplast protein appears to have been created by the addition of an exon encoding the chloroplast transit peptide at a site upstream of a PPDK gene encoding the cytosolic protein. The mechanisms contributing to the evolution of these two genes are not understood.

The extensive allelic diversity at B and R may have arisen because of the easily scorable phenotype and the nonessential nature of the anthocyanin pigments. For many pathways, alterations that change the time and place of the expression of a regulatory gene would be expected to be lethal or produce deleterious dominant gain-of-function mutations. The nonessential nature of the anthocyanins may have permitted the survival of variant B alleles. In addition, the rare individuals with altered patterns of anthocyanin pigmentation would be readily identified. As many of these alleles produce strikingly beautiful plants, they may have been selected through agricultural breeding. We speculate that the processes that generated these variants at b and r may be occurring at other genes as well.

Materials and methods

Plant stocks

Stocks containing the b, B-Peru, and B-I alleles were obtained from E.H. Coe, Jr. and M.G. Neuffer (University of Missouri, Columbia). The B-Peru allele is in the W22 background, and the b and B-I alleles are in the W23 background. All three stocks had the null r-g: Stadler allele of R and carried dominant alleles for the other genes required for anthocyanin biosynthesis (A1, A2, C1, C2, Pr, P1, Bz1, Bz2, and Vp1).

DNA materials

The isolation and characterization of the B-Peru genomic and cDNA clones were described previously [Chandler et al. 1989; Radicella et al. 1991]. The B-Peru sequences were used to isolate a 1.9-kbp B-I cDNA clone by screening a Agt10 library prepared from B-I husk tissue (provided by K. Cone, University of Missouri, Columbia). The B-I genomic sequences were cloned in two fragments, a 2.5-kbp BgIII fragment containing the 5′ end of the transcribed region and upstream flanking sequences, and a 12-kbp BamHI fragment containing the remaining transcribed region and 3′-flanking sequences, using λ Bv [N. Murray, University of Edinburgh, Scotland] and the EMBL3 vector [Frischauf et al. 1983], respectively. Maize DNA fragments were subsequently subcloned into GenescrIBE vectors [U.S. Biochemical]. B-I genomic clones were reconstructed by ligating restriction fragments together using the unique SpeI site in the middle of the coding region. Both a clone beginning at the SalI site, −527 from the transcription start site and extending to the SalI site 1 kbp downstream of the poly[A] site, and a clone with −1 kbp of λ DNA at the 5′ end with the SalI site in the 3′ end with the B-I sequences beginning at the BgIII site, −831 from the transcription start site and extending to the BamHI site −9 kbp downstream of the poly[A] site, had the same activity in the transient transformation assays. The BI:BP chimeric clone contained 987 bp of B-I extending from the BgIII site in the 5′-flanking region to the HindIII site in intron 1 and −5 kbp of B-Peru extending from the HindIII site in intron 1 to the SalI site in the 3′-flanking region. The BP::B1 chimeric clones contained 2.1 kbp of B-Peru extending from the SpeI site in the 5′-flanking region to the SacI site in intron 1 and −5 kbp of B-I extending from the SacI site in intron 1 to the SalI site in the 3′-flanking region. There are no nucleotide differences between the alleles in the region between the SacI and HindIII sites in the first intron. DNA sequencing was by the chain-termination method [Sanger et al. 1977] using Sequenase (U.S. Biochemical).

The maize actin clone [pMAC1] was obtained from R. Meagher [Meagher et al. 1983]. The Bz1 promoter–luciferase plasmid was described previously [Klein et al. 1989]. The Adh promoter–CAT plasmid was described previously as plAI1CN (Callis et al. 1987).

The yeast strain used to express B is YPH499 [mata, ura3-52, lys2-801amber, ade2-101amber, trp1-Δ63, his3-Δ200, leu2-Δ1, Sikorski and Hieter 1989]. The negative control strain [Fig. 6A,B, lane a] carries vector pSGVE12, a CEN/ARS plasmid with
a his3 selectable marker and the 3-phosphoglycerate kinase (PGK) promoter and terminator. The strain expressing B protein carries plasmid pSGVII4, which is pSGVEI2 with the B-I cDNA inserted between the PGK promoter and terminator (Goff et al. 1992). The B-I cDNA was modified such that the first maize nucleotides are the ATG of exon 2, which begins the long open reading frame. The construction of the B mutant derivatives deleted for amino acids 4–27 and 4–66 was described previously (Goff et al. 1992). To express the cDNAs in yeast, the EcoRI fragments containing 17 bp of the B leader and the deleted cDNAs were cloned between the PGK promoter and terminator.

Hybridization analysis

The 12-kbp BamHI fragment from the B-I genomic clone was digested with either HindIII or HindIII and SalI together and analyzed by blot hybridization using the 3.2-kbp SacI–BamHI fragment from the 3’ region of the B-Peru genomic clone (Fig. 3) as the probe (Chandler et al. 1989). The hybridization was carried out at 35°C in 50% formamide (-12°C below Tm, assuming 50% GC content and 90% sequence identity), and the blots were washed at 50°C in 0.1× SSC (1× SSC = 0.15 m sodium chloride, 0.015 m sodium citrate at pH 7.0), 0.1% SDS. The 1.2-kbp HindIII–SalI, 0.75-kbp SalI–HindIII, 0.4-kbp HindIII–SalI, and 0.7-kbp SalI–HindIII fragments all hybridize with the B-Peru probe. The three smaller fragments all hybridized with equal intensity, whereas the larger fragment hybridized about two times better.

Because the GC content is unknown, we cannot accurately determine the percent identity between B-I and B-Peru in this region. However, given the hybridization conditions, we estimate the percent identity to be at least 85–90%. Lower percent identities would not have produced strong signals on the blots. The sequences beyond the most 3’ HindIII site of B-I did not hybridize under our conditions, suggesting that divergence occurs in the same area that restriction site differences are detected.

RNA isolation and analysis

Total RNA was prepared from tassels, sheaths, and husks by the guanidinium thiocyanate method after grinding the tissue in liquid nitrogen (Chirgwin et al. 1979). RNA from aleurones harvested ∼30 days after pollination was extracted with phenol and liquid nitrogen (Chirgwin et al. 1979). RNA from aleurones prepared as described by the manufacturer, and used to purify poly(A)+ tail added by incubating the product of the RT reaction for 10 min at 37°C with 10 units of terminal dT transferase (Bethesda Research Laboratories) in tailing buffer (BRL) and 0.4 mM dATP. Ten microliters of each of the poly(A)+-tailed cDNA products was amplified by PCR. For both RNA samples, the primers used were a B-specific primer (5’ Amp = 5’-GAAATGAGCCAGAAGAG), an adapter primer consisting of a polylinker sequence tagged with 18 dT residues complementary to the poly(A)+ tail added after the RT (Adp dt), and the adapter primer without the dT residues (Adp). PCR amplification of the cDNAs was as follows: Ten microliters of cDNA (heated for 5 min at 95°C, cooled on ice), 10 pmoles of Adp dt, 25 pmoles of Adp, 25 pmoles of 5’ Amp primers, 67 mM Tris (pH 8.3), 0.5–2.0 mM MgCl2, 10% dimethylsulfoxide (DMSO), 200 μM dNTP, 0.5 unit enhancer (Stratagene), and 0.5 unit Taq DNA polymerase (Cetus) in a final volume of 50 μl, overlaid with mineral oil. Amplification profiles were [1] 1 min at 95°C, 1.5 min at 46°C, 10 min at 72°C, [2] 40 cycles of 1 min at 95°C, 1 min at 46°C, 1.5 min at 72°C, and [3] 15 min at 72°C. PCR products hybridizing to B-specific probes were gel purified, cleaved with PstI, which cuts in the adapter primer and at the PstI site present in the second exon, and cloned into the pTZ18U vector (U.S. Biochemical). Sequences were determined by the chain-termination method (Sanger et al. 1977) using Sequenase (U.S. Biochemical).

Production of B antigen and generation of B antiserum

B antigen for production of antiserum was made in Escherichia coli strain MC1061 [Casadaban and Cohen 1980] by cloning the 1632-bp PstI–HindIII fragment and the 1532-bp BglII–HindIII fragment of the B-Peru cDNA, respectively, into the E. coli expression vectors pEXP2 and pEXP3, containing an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter [Raymond et al. 1990]. Plasmids pPSt/HBP[EXP2] and pG/HB[EXP3] produced ∼70- and ∼63-kd species, respectively, upon induction with IPTG. The fusion protein produced from plasmid pPst/HBP[EXP2] was purified from the insoluble fraction as described in Roberts et al. (1989). B-specific antibodies were prepared by injecting B antigen into New Zealand white rabbits essentially as described [Vaitukaitis 1981]. A 2-ml affinity column was constructed by linking B antigen [produced from the pG/HBP[EXP3] plasmid and purified as described in Roberts et al. (1989)] to CNBr-activated Sepharose 4B (Pharmacia), prepared as described by the manufacturer, and used to purify B-specific antibodies.

Protein extracts

Yeast protein extracts A saturated overnight culture of yeast grown in SD–His [SD = 6.7 g/liter of yeast nitrogen base (Difco), 2% glucose] was diluted 1:9 into fresh, prewarmed SD–His medium and grown for 8 hr at 30°C, with shaking (to Å00 = 0.5–1.0). Cells were spun down, washed once with ice-cold TE buffer (10 mM Tris at pH 7.5, 1 mM EDTA), and the cell pellets were weighed. Cells were resuspended in preheated (85°C) Thormer buffer (50 mM Tris at pH 6.8, 5% SDS, 8 M urea, 1 mM EDTA, 10% glycerol; 2.5 ml Thormer buffer per gram of cells), with 4% β-mercaptoethanol and 1 mM PMSF. Preheated glass beads were added [2.5 ml of Thormer buffer per gram of cells, 2.5 grams of glass beads per milliter of buffer], and cells were vortexed for 1 min, placed at 85°C for 5 min, revortexed, and centrifuged in a microcentrifuge. Approximately 100 μg of total protein was loaded per lane.

Maize protein extracts Two grams of tissue was frozen in liquid nitrogen and ground to a fine powder. The powder was mixed with 4–6 ml of Thormer buffer, preheated at 85°C, with 4% β-mercaptoethanol and 1 mM PMSF, and the suspension was homogenized further, using a polytron with husk tissue and
a mortar and pestle with tassles, before placing at 65°C for 5 min. Samples were centrifuged at 3000g for 10 min, and ~5–10 μl of each preparation was loaded per lane.

**Immunoblot analysis**

Protein extracts were electrophoresed on an 8.5% SDS–polyacrylamide gel and immunoblotted as described (Ausubel et al. 1987). The chemiluminescent reaction was then performed using Amersham ECL Western blotting reagents.

**High velocity microprojectile bombardment**

Plasmid DNA [purified by equilibrium ultracentrifugation using cesium chloride gradients] was delivered into aleurones (isolated from kernels containing the b r-g alleles) by high velocity microprojectiles using the Biolistics device as described (Klein et al. 1989). A total of 30 μg of plasmid DNA was used in each microprojectile preparation. For delivery to aleurones, various amounts of effector plasmid were used together with 10 μg of reporter plasmid [Bz1–luciferase] and 10 μg of internal control plasmid [Adh–CAT]. Vector plasmid was added to complete the 30 μg of DNA. After the bombardment, tissues were incubated for 48 hr at 24°C with illumination.

**Enzyme assays**

Anthocyanin biosynthesis in aleurones and leaves was measured by visual detection of pigmented cells using 25- to 50-fold magnification under a Zeiss dissecting microscope. Tissue extracts were prepared by grinding transformed aleurones with mortar and pestle in 1.5 ml of lysis buffer (100 mM potassium phosphate buffer at pH 7.8, 1 mM dithiothreitol) at 4°C. The extract was centrifuged for 5 min at 4°C in a microcentrifuge. Luciferase was assayed in tissue extracts as described previously (Callis et al. 1987) and is expressed as the number of light units detected in 10 sec at 25°C per 100 μl of extract. CAT activity was determined by the conversion of 3HClabeled acetyl-coenzyme A to ethyl acetate soluble counts per minute for 1 hr at 37°C and counted as described previously (Sleigh 1986). CAT activity is expressed as counts per minute generated from 25 μl of extract incubated for 1 hr at 37°C.

**Acknowledgments**

We are grateful to Steve Goff for helping us to set up the microprojectile bombardment assay in our laboratory, to Ry Meeks-Wagner for his assistance with the RACE procedure, and to Fenella Raymond and Chris Raymond for technical assistance and advice on the B antigen preparation. We also thank Fenella Raymond and Jeff Nichols for help with the sequencing of the B-Peru genomic clone; Leonard Sacco for his technical assistance; Steve Goff, Fenella Raymond, and Chris Raymond for plasmid vectors; and Garth Patterson and Kristine Hardeman for advice and comments throughout this work. We also thank Kristine Hardeman, David Hawthorne, Ken Kubo, Ry Meeks-Wagner, Garth Patterson, and Manuel Sainz for the critical reading of the manuscript. This work was supported by a National Institutes of Health grant (GM35791) and a Searle Scholar's Award to V.L.C.

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*Genes Dev.* 1992, 6:
Access the most recent version at doi:10.1101/gad.6.11.2152