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Maize Anthocyanin Regulatory Gene *pl* Is a Duplicate of *c1* That Functions in the Plant

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Genetic studies in maize have identified several regulatory genes that control the tissue-specific synthesis of purple anthocyanin pigments in the plant. *c1* regulates pigmentation in the aleurone layer of the kernel, whereas pigmentation in the vegetative and floral tissues of the plant body depends on *pl*. *c1* encodes a protein with the structural features of eukaryotic transcription factors and functions to control the accumulation of transcripts for the anthocyanin biosynthetic genes. Previous genetic and molecular observations have prompted the hypothesis that *c1* and *pl* are functionally duplicate, in that they control the same set of anthocyanin structural genes but in distinct parts of the plant. Here, we show that this proposed functional similarity is reflected by DNA sequence homology between *c1* and *pl*. Using a *c1* DNA fragment as a hybridization probe, genomic and cDNA clones for *pl* were isolated. Comparison of *pl* and *c1* cDNA sequences revealed that the genes encode proteins with 90% or more amino acid identity in the amino- and carboxyl-terminal domains that are known to be important for the regulatory function of the C1 protein. Consistent with the idea that the *pl* gene product also acts as a transcriptional activator is our finding that a functional *pl* allele is required for the transcription of at least three structural genes in the anthocyanin biosynthetic pathway.

INTRODUCTION

Anthocyanins are red and purple pigments that can be synthesized in a variety of tissues of the maize plant, including the aleurone and embryo of the kernel, the root and coleoptile of seedlings, and many mature organs of the plant body, such as stem, leaf, tassel, and anthers. The biosynthesis of anthocyanins is controlled by a number of genes that have both structural and regulatory roles (reviewed in Dooner et al., 1991). The structural genes *c2*, *a1*, *a2*, *bronze1* (*bz1*), and *bronze2* (*bz2*) encode biosynthetic enzymes in the anthocyanin pathway. Expression of the structural genes is controlled coordinately by a number of regulatory genes, *c1*, *pl*, *r*, *b* *lc*, and *sn*. Early biochemical and genetic analyses established that mutations in the regulatory genes affected the levels of enzymes encoded by the structural genes (Dooner and Nelson, 1977; Dooner, 1983).

More recent studies showing that the regulatory genes control the accumulation of structural gene mRNAs have prompted the proposal that expression of the structural genes is controlled at the level of transcription (Cone et al., 1986; Wienand et al., 1986; Paz-Ares et al., 1987; Schwarz-Sommer et al., 1987; Cone and Burr, 1988; Chandler et al., 1989; Ludwig et al., 1989; Tonelli et al., 1991). Indirect evidence supporting this idea was provided by the discovery that the regulatory genes encode proteins with the structural features of known transcription factors. The *r*, *b*, *lc*, and *sn* genes encode related proteins with an internal domain homologous to the basic-helix-loop-helix DNA binding–protein dimerization domain of Myc oncoproteins (Ludwig et al., 1989; Perrot and Cone, 1989; Radicella et al., 1991; Consonni et al., 1992). The *c1* gene encodes a protein with a basic amino-terminal domain similar to the DNA binding domain of the Myb class of oncoproteins and an acidic carboxyl-terminal domain similar to the acidic transcriptional activation domains present in many regulatory proteins (Paz-Ares et al., 1987).

Genetic and molecular analyses of *r*, *b*, *lc*, and *sn* have established that these genes comprise a multigene family (the *R/B* family) encoding functionally equivalent proteins with distinct patterns of spatial and tissue-specific expression (reviewed in Ludwig and Wessler, 1990; Consonni et al., 1993). In some tissues, certain *b* and *r* alleles can function as duplicate genes with either locus being sufficient for anthocyanin pigmentation. For example, the *R-S* gene controls pigmentation in the kernel, whereas the *B-I* gene specifies pigmentation of the plant body. By contrast, the *B-Peru* gene plays a duplicate role and leads to pigmentation in both the kernel and the plant body (Styles et al., 1973). This functional homology suggested the genes encoded by members of the *R/B* family might be similar at the molecular level. An allele of *R* was cloned by transposon tagging (Dellaporta et al., 1988), and subsequently other members of the gene family were isolated due to their

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DNA homology to the *R* clone (Chandler et al., 1989; Ludwig et al., 1989; Consonni et al., 1992). This collection of homologous genes then provided the tools to biochemically explore the functional interchangeability of the *R/B* proteins through the use of transient expression assays. When the cDNAs from *Lc* and two different *B* alleles were fused to the constitutive cauliflower mosaic virus (CaMV) 35S promoter and introduced into various maize tissues, the *R/B* genes were capable of inducing anthocyanin synthesis in virtually every tissue tested (Goff et al., 1990; Ludwig et al., 1990). These results suggest that the spatial diversity of anthocyanin pigmentation in the plant is dependent upon the pattern of tissue-specific expression of the *R/B* regulatory genes. Additional evidence confirming this interpretation has been obtained from recent experiments showing that the differential expression of the *B-I* and *B-Peru* alleles is controlled by distinct regulatory sequences flanking the genes (Radicella et al., 1992).

Functionally duplicate roles have also been proposed for the *c1* and *pl* regulatory genes, initially based on the observation that there are alleles of both *c1* and *pl* that require light to induce anthocyanin synthesis (Coe, 1985). The recessive *c1-p* allele causes colorless kernels to develop pigment during germination in the light (Chen and Coe, 1977). One class of recessive *pl* alleles conditions a "sun-red" phenotype in which only portions of the plant body that are exposed to light become pigmented (Briggs, 1966). In these alleles, light induces or increases expression of the regulatory gene, thus allowing induction of anthocyanin synthesis (Cone and Burr, 1988; Cone et al., 1993). The similar mode of regulation of these genes suggests that they might have arisen from duplication of a common light-regulated ancestor.

The functional equivalence of *c1* and *pl* is further supported by the fact that pigmentation requires interaction between a member of the *R/B* gene family and functional alleles of either the *C1* gene or the *Pl* gene (Coe et al., 1988). Anthocyanin synthesis in the kernel requires not only *R-S* but also *C1*, and pigmentation in the mature tissues of the plant requires both *B-I* and *Pl*. In transient assays, *C1* can interact equally effectively with either *B-I* or *R-S* to *trans*-activate anthocyanin synthesis in aleurones; this interaction is probably mediated by direct protein–protein interaction between the C1 and R/B proteins (Goff et al., 1990, 1991). The fact that *B-I* normally interacts in the plant with *Pl*, yet also can interact with *C1*, indicates that *Pl* and *C1* must bear some structural and/or functional similarity. This idea is reinforced by the recent analysis of an allele of *pl*, called *Pl-Bh*, that can lead to pigmentation in the kernel. *Pl-Bh* was originally described as a dominant gene (designated *Blotched* [*Bh*]), conditioning a blotchy or variegated pattern of anthocyanin synthesis in the aleurone of kernels homozygous for recessive *c1*, thus effectively substituting for *C1* in the aleurone (Rhoades, 1948). We have shown that *Bh* is an allele of *Pl* that controls anthocyanin synthesis in both the kernel and the plant body (Cocciolone and Cone, 1993). This dual tissue specificity argues that the roles played by *C1* in the kernel and *Pl* in the plant body are the same.

Given the established homology among members of the *R/B* family and the proposed duplicate function of C1 and PL, we predicted *c1* and *pl* might show a high level of DNA sequence similarity. We used a *c1* genomic fragment as a hybridization probe to identify restriction fragments that cosegregated with a particular PI phenotype. Genomic and cDNA clones corresponding to the *c1*-hybridizing sequences were isolated, and the cloned sequences were mapped to the *pl* locus by restriction fragment length polymorphism (RFLP) analysis. Transcription patterns in plants of differing *pl* genotypes were analyzed to establish the role of *pl* in controlling transcription of the anthocyanin structural genes.

RESULTS

Molecular Segregation Analysis

To explore the possibility that the proposed functional similarity between *c1* and *pl* might be mirrored at the nucleotide level, plants segregating for two different *pl* alleles were screened by hybridization with a DNA fragment from the *c1* gene. The plants chosen for this study were the recombinant inbred (RI) family CO×Tx (Burr et al., 1988; Burr and Burr, 1991). This RI family was constructed by crossing two well-characterized inbreds, CO159 and Tx303, selfing the F_1 to produce an F_2 generation, and continually selfing the progeny of individual members of the F_2 to produce a family of inbred lines. CO159 contains a nonfunctional *pl* allele (*pl-CO159*), which does not promote pigment production, and Tx303 contains a functional *pl* (*pl-Tx303*) allele, which leads to anthocyanin pigmentation. After numerous generations of selfing, each RI line should be homozygous for blocks of linked alleles derived from one of the original parents. Thus, at the *pl* locus, RIs should contain either the *pl-CO159* or *pl-Tx303*.

The CO×Tx RIs were planted in the field and scored for anthocyanin pigmentation. DNA was isolated from leaves of plants in each family. DNA samples were digested with a restriction enzyme (BglII) that did not yield polymorphism for the parental *c1* alleles. The digests were fractionated on agarose gels, blotted to nitrocellulose, and hybridized with a DNA fragment derived from the *c1* gene. Figure 1 shows the results of the molecular segregation analysis. All the lines show an invariant fragment of 4.5 kb representing the parental *c1* alleles. In addition, the CO159 parent contains a 5.1-kb fragment and the Tx303 parent contains a 3.8-kb fragment. The 3.8-kb fragment segregates with a pigmented phenotype (designated +), and the 5.1-kb fragment segregates with an unpigmented phenotype (designated −). RI lines that segregated both pigmented and unpigmented plants (designated ±) contain both the 3.8-kb and the 5.1-kb fragments. This pattern of cosegregation suggests the 3.8-kb and 5.1-kb *c1*-hybridizing fragments represent the *pl* alleles present in the Tx303 and CO159 parents, respectively.

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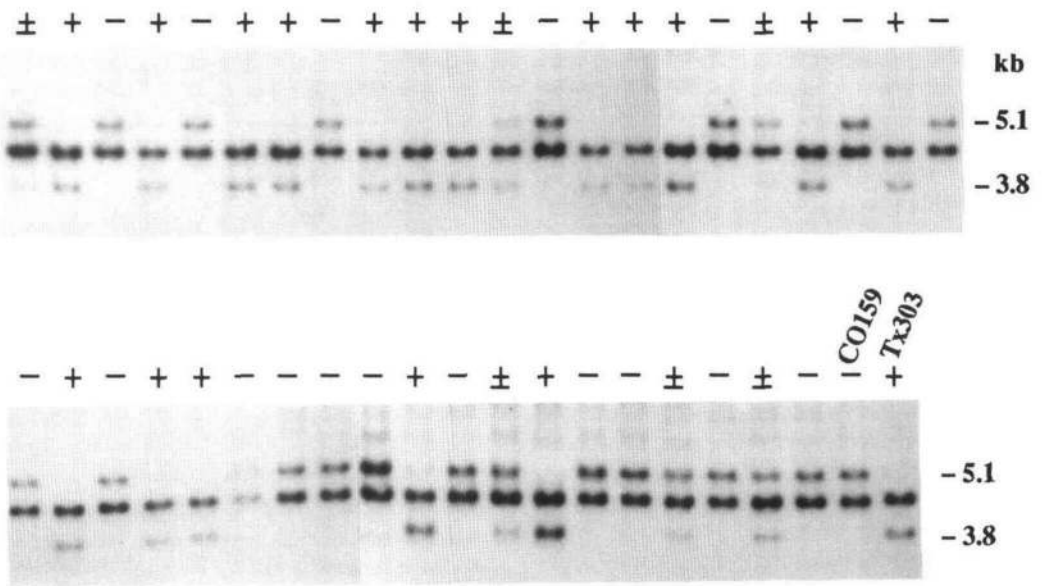

Figure 1. Cosegregation Analysis.

Plants from the RI family CO×Tx were scored for anthocyanin production. The (+) denotes lines in which all plants were pigmented, (−) denotes lines in which all plants were unpigmented, and (±) denotes lines that contain both pigmented and unpigmented plants. DNAs from each of the RI families were digested with BglII and hybridized with a *c1* DNA probe. The last two lanes in the lower panel contain DNA from the parental inbreds. Lengths (in kilobases) of *c1*-homologous bands are indicated to the right. The invariant band seen in all lanes is 4.5 kb and represents the parental *c1* alleles.

Cloning of the *c1*-Homologous Sequence from Tx303

As a preliminary step in determining whether the *c1*-homologous sequence from Tx303 was *pl*, a genomic DNA fragment containing this sequence was cloned. DNA from Tx303 was digested with BamHI, size fractionated, and cloned into λEMBL4. DNA was isolated from recombinant phage that hybridized with the *c1* probe and analyzed by restriction mapping. Figure 2 shows the restriction map of the clone. Comparison of this restriction map to the map of *c1* revealed a region of similarity of ~1.5 kb (indicated by the shaded box). Within this region, a number of restriction sites are conserved between the two genes. Outside this conserved region, however, there is little restriction site identity. Two restriction fragments (labeled A and B) flanking the region of similarity with *c1* were used as probes on DNA gel blots of Tx303 genomic DNA and were found to represent single-copy sequences. Restriction fragments flanking A and B are present multiple times in genomic DNA (data not shown). Fragment A was subcloned and used in subsequent analyses to confirm the identity of the cloned DNA as *pl*.

RFLP Mapping

pl maps genetically to position 49 on chromosome 6L. To determine if the cloned *c1*-homologous sequence maps to the same location as *pl*, we took two approaches. First, we localized the *c1*-homologous sequence to a chromosome arm using B-A translocations (Beckett, 1978; Evola et al., 1986). Second,

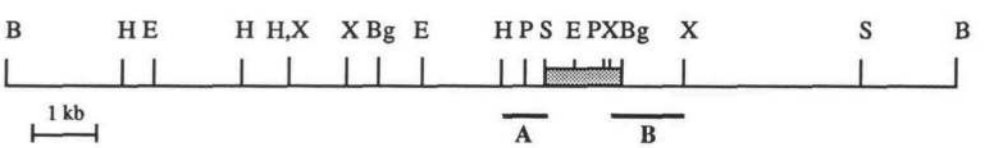

Figure 2. Restriction Map of the *pl-Tx303* Allele.

The restriction map was generated by digestion of the *pl-Tx303* λ clone with the following restriction enzymes: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI; and X, XhoI. The shaded box denotes the region of restriction site and nucleotide similarities between *c1* and *pl*. Transcription is from left to right. Fragments used as hybridization probes are given below the map. A, 0.6-kb HindIII-SalI fragment; B, 1.1-kb XhoI fragment.

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we mapped the sequence relative to other molecular markers by comparing its pattern of segregation in the CO×Tx RI family to patterns of linkage known for this family (Burr et al., 1988; Burr and Burr, 1991).

For the chromosome arm localization, we used the B-A translocation, TB-6Lc, in which the long arm of chromosome 6 has been translocated to the centromere of the supernumerary B chromosome. Details of the procedure are presented in Methods. Plants heterozygous for the translocation Tb-6Lc were crossed as pollen parents to females homozygous for the recessive seedling marker, *piebald4* (*pb4*), on the long arm of chromosome 6. *pb4* leads to the production of light green irregular banding in seedling leaves. The male plants carried dominant alleles for *pb4*. The F_1 progeny were germinated in the field, and the seedling phenotypes were scored at the four- to five-leaf stage. As expected, most of the F_1 seedlings were green, but a few were piebald. DNA was extracted from immature leaves of the parental plants and F_1 progeny, digested with a number of restriction enzymes, and hybridized with probe A derived from the Tx303 *pl*λ clone *pl-Tx303* (Figure 2). As shown in Figure 3, digestion with BamHI revealed RFLPs for the *pl* alleles in the female (*pb/pb*) and male (Tb-6Lc/+) parents. Both alleles present in the male parent are represented by a single band. The green (wild-type) progeny show two bands, one derived from the female and the other derived from the male. However, the piebald (*pb*) progeny contain only the single band derived from the female parent; the allele from the male parent is absent due to fertilization by a hypoploid nucleus. The presence of a single female-derived band in the progeny displaying the piebald phenotype implies the hybridization probe is linked to *pb4* on chromosome 6L. If the probe had been derived from some chromosome arm other than 6L, the piebald progeny would have contained bands of both maternal and paternal origin.

To more precisely locate the map position of probe A, the blots shown in Figure 1 were stripped and rehybridized with probe A. The resulting blots (data not shown) showed a pattern of segregation for the 3.8- and 5.1-kb bands identical to that obtained when the DNAs were hybridized with a *c1* probe. The 4.5-kb band was missing, demonstrating that probe A contains sequences not present in *c1*. Each RI was scored for its parental allelic composition, and the distributions of parental alleles were compared by computer with the accumulated data base for the CO×Tx family. The analysis established not only that the presence and/or absence of anthocyanin pigmentation mapped to the position of *pl* on chromosome 6L, but also that the hybridization with probe A cosegregated with *pl*. Taken together with the results of the chromosome arm localization, these results indicate that the *c1*-homologous sequence cloned from Tx303 is *pl*.

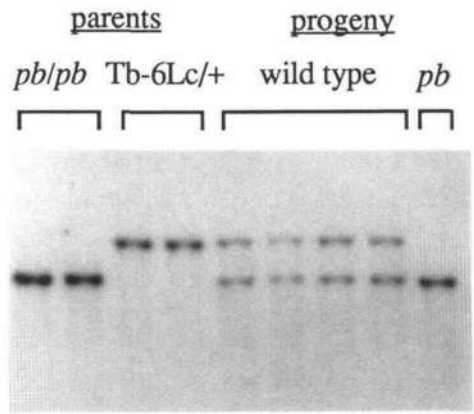

Figure 3. Chromosome Arm Localization of *pl* by RFLP Analysis.

DNAs were prepared from parental and progeny plants. Parents: female, *pb4* homozygote (*pb/pb*); male, translocation heterozygote (Tb-6Lc/+). Progeny: *pb4/pb4* × Tb-6Lc/+. The wild-type progeny seedlings were phenotypically green, and *pb* progeny seedlings were piebald. DNA samples were digested with BamHI and hybridized with probe A from the *pl-Tx303* λ clone (see Figure 2). Lengths of hybridizing bands are 19 and 16 kb.

Sequence Analysis

The *c1* probe was used to screen a cDNA library constructed with poly(A)$^+$ RNA from purple husks of plants carrying the dominant *Pl-Rhoades* (*Pl*) allele. Two cDNAs were recovered and sequenced. Figure 4 shows the nucleotide sequence and deduced amino acid sequence compared to *C1* (Paz-Ares et al., 1987). Within the protein coding regions, the nucleotide sequences are 90% identical. In the 20 bp just 5′ and 3′ of the protein coding region, the untranslated portions show ~80% identity. The remainder of the untranslated region lacks significant sequence homology.

At the amino acid level, the predicted C1 and PL proteins are 80% identical. However, the distribution of amino acid differences throughout the protein sequence is not random. Specifically, in the Myb-homologous domain (amino acids 1 to 114), the proteins are 96% identical, and in the acidic domain (amino acids 234 to 261 in C1 and 234 to 262 in PL), the proteins are 93% identical. All of the amino acid substitutions within these domains are conservative changes that would not be expected to alter protein structure or charge. These results underscore the importance of the Myb-homologous and acidic domains to the function of the proteins. By contrast, the amino acid sequences separating the Myb-homologous and acidic domains exhibit only 67% identity between C1 and PL with few of the amino acid substitutions representing conservative changes. This observation suggests that this part of the protein may be less functionally important than the flanking domains.

RNA Analysis

The expression of the *pl* gene was examined by analyzing mRNAs produced in husk tissue with different *pl* genotypes.

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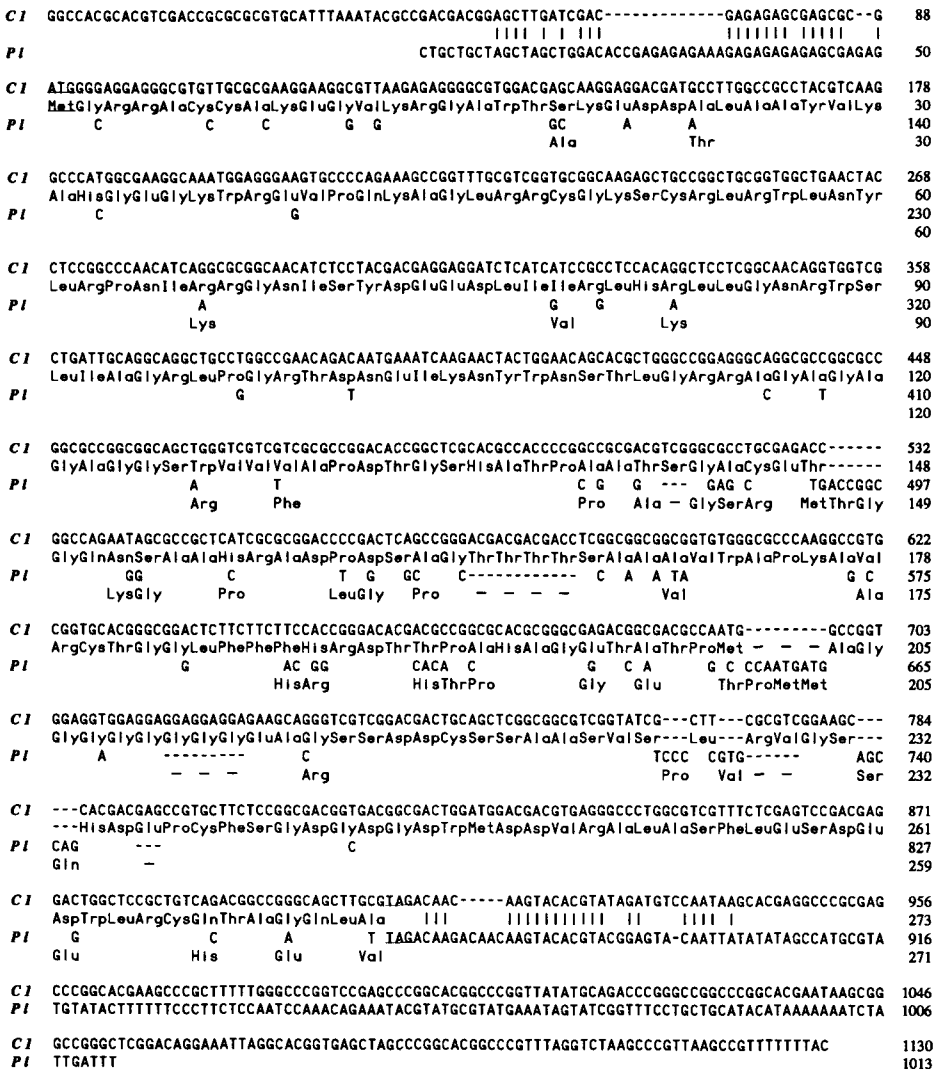

Figure 4. Comparison of Nucleotide and Deduced Amino Acid Sequences for *C1* and *Pl* cDNAs.

The DNA and amino acid sequences of the *Pl-Rhoades* (*Pl*) allele are aligned underneath the sequence of *C1* (Paz-Ares et al., 1987). Within the coding region, only nucleotides and amino acids in *Pl* that differ from those in *C1* are shown. Long and short dashes denote deletions introduced to maximize homology during alignment. Positions of start and stop codons are underlined. Nucleotide similarities outside the protein coding region are marked by vertical lines.

Poly(A)$^+$ mRNAs isolated from husks were fractionated on denaturing agarose gels, blotted to nylon membranes, and hybridized with the *c1* probe. Figure 5 shows the results of the hybridization analysis. Purple husks from plants carrying dominant *B* and *Pl* alleles contain a 1.2-kb mRNA that hybridizes strongly to the *c1* probe. The same size transcript is present in unpigmented (green) husks from plants with genotype *b, Pl.* This result indicates that *Pl* expression is not dependent on the expression of a functional *B* gene. No *pl* mRNA was detected in unpigmented (green) husks of plants carrying a recessive *pl* allele, suggesting that either this allele is not expressed or its level of expression is below the detection limits by gel blot analysis. No transcripts the length of *c1* mRNAs (1.4 and 1.6 kb) were detected in any of the lanes, even though all plants were homozygous for a functional *C1* gene and had colored kernels. This observation indicates that *c1* is not expressed in husk tissue.

In the kernel, accumulation of structural gene transcripts requires a functional *C1* allele. To determine if structural gene expression in vegetative plant tissues requires a dominant *Pl* allele, the RNA gel blots were stripped and reprobed with DNA fragments derived from the anthocyanin structural genes *A1*, *C2*, and *Bz1*. The bottom panel of Figure 5 shows the results of hybridization with an *A1* cDNA. A 1.4-kb transcript is detected

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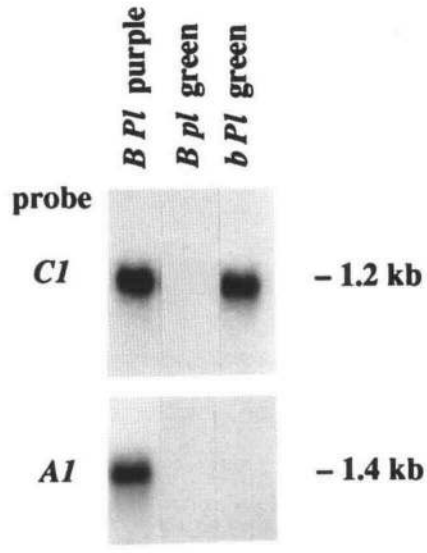

Figure 5. RNA Gel Blot Analysis.

Poly(A)$^+$ RNAs were prepared from husk tissue of plants carrying various combinations of different *b* and *pl* alleles. All plants carried a dominant *C1* allele.

(Top) Poly(A)$^+$ RNAs prepared from husks were fractionated on a denaturing gel, blotted, and hybridized with a *c1* probe.

(Bottom) The RNA gel blot was stripped and reprobed with an *A1* cDNA probe. The blot was also hybridized with a maize actin gene probe to verify equal RNA loading (data not shown).

Each lane contains 10 μg of RNA. Lengths of hybridizing transcripts (in kilobases) are indicated at right.

in purple husks with genotype *B*, *Pl* but not in green husks carrying recessive *b* or *pl* alleles. The same hybridization pattern was obtained with the *C2* and *Bz1* probes (data not shown). These results not only confirm previous genetic, biochemical, and molecular observations that anthocyanin pigmentation in vegetative tissues is controlled by the concerted action of both *B* and *Pl*, but they also show that the regulation occurs at the level of structural gene mRNA accumulation.

The level of steady state mRNA is a function of both synthesis and turnover rates. Given the homology of *b* and *pl* to genes encoding other eukaryotic proteins with known DNA binding and/or transcriptional activation roles, it is tempting to hypothesize that *b* and *pl* function as transcription factors. To test this theory, we employed a nuclear run-on transcription assay to determine if the transcription of the anthocyanin structural genes was dependent on functional *B* and *Pl* alleles. Assays were performed with nuclei isolated from pigmented husks of genotype *B*, *Pl* and nuclei from unpigmented husks of genotypes *B*, *pl* and *b*, *Pl*. As shown in Figure 6, *A1*, *Bz1*, and *C2* transcription was higher in purple husks than in green husks. This is the result predicted if *B* and *Pl* act at the level of transcription. Quantitation of structural gene transcription in purple husks and in unpigmented husks revealed that transcription was enhanced ~70-fold for *A1*, ninefold for *Bz1*, and 135-fold for *C2* in the purple husks. These results support the idea that *B* and *Pl* control anthocyanin pigmentation by regulating structural gene transcription.

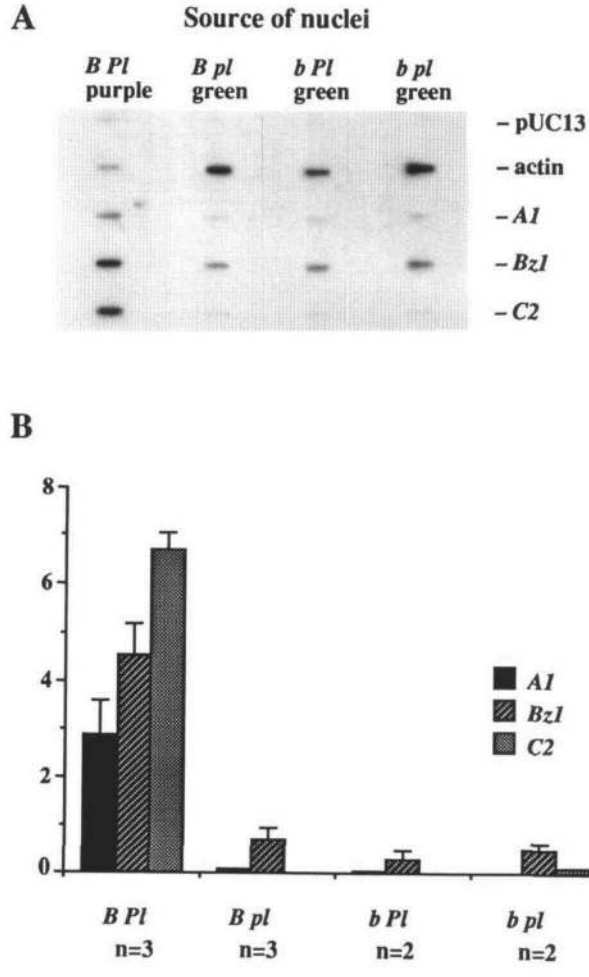

Figure 6. Nuclear Transcription Assays.

Nuclei were isolated from purple and green husks of plants with different allelic combinations of *b* and *pl*. Nuclear transcripts were labeled by incubating the nuclei with ^{32}P-UTP. RNA was purified and hybridized to single-stranded DNAs immobilized on nitrocellulose filters.

(A) Autoradiograph of hybridization signals produced by RNA from nuclei of four genotypes. pUC13 and maize actin served as controls for transcription and hybridization efficiency.

(B) Quantitation of hybridization signals. Autoradiographs were scanned with a densitometer to measure hybridization intensities. Values for hybridization to *A1*, *Bz1*, and *C2* were normalized by subtracting any background hybridization to pUC13 and dividing by the value for hybridization to actin DNA. The *n* values indicate the number of nuclei samples used to generate the mean normalized values for each genotype. Error bars represent the standard error of the mean.

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DISCUSSION

The maize anthocyanin regulatory genes *c1* and *pl* have been proposed to be functional duplicates. We have demonstrated that this duplicate function is mirrored by sequence similarity at the nucleotide and amino acid levels. Using a *c1*-specific DNA fragment as a hybridization probe, we have isolated *pl* genomic and cDNA clones. Within the protein coding regions, the two genes show 80% identity at the amino acid level. This degree of similarity is comparable to that displayed by other sets of functionally duplicate genes in maize that have been cloned recently. For example, the members of the *R/B* gene family share greater than 80% amino acid identity (Consonni et al., 1993), and the two genes encoding the β subunit of tryptophan synthase are 98% identical at the amino acid level (Wright et al., 1992).

Genetic, biochemical, and molecular studies have long supported the idea that the C1/PL and R/B proteins function in transcriptional activation of the anthocyanin structural genes. However, a strictly conservative interpretation of previous data only permits the conclusion that the regulatory genes control structural gene mRNA accumulation and, thereby, biosynthetic enzyme activity. The results of our nuclear run-on transcription assays show definitively that in husks, *Pl* and *B* regulate the structural genes at the level of transcription. The high degree of sequence conservation in the amino-terminal Myb-homologous domain and in the carboxyl-terminal acidic domain of the C1 and PL proteins underscores the importance of these domains to the transcriptional activation function of these proteins. In vivo assays of *C1* expression have shown that the acidic domain can function in transcriptional activation when fused to the DNA binding domain of the yeast transcription factor GAL4. Conversely, substituting the GAL4 acidic domain for the C1 acidic domain generates a fusion protein capable of activating the anthocyanin biosynthetic pathway (Goff et al., 1991).

The functional role of the Myb domain of C1/PL is not as clear. In other Myb-related proteins this domain functions in DNA binding (Lüscher and Eisenman, 1990). Such a role for C1 is supported by the discovery that correct regulation of the *Bz1* structural gene by C1 and B requires a sequence in the promoter with homology to the Myb consensus DNA binding site (Roth et al., 1991). Mutations in this Myb site drastically reduce *trans*-activation of the promoter (Roth et al., 1991). The Myb domain is also implicated in mediating a protein–protein interaction between C1 and B proteins (Goff et al., 1992). Such an interaction is proposed to promote a conformational change in either C1 or B leading to enhanced DNA recognition and binding ability. Studies in progress should provide further insight about detailed mechanisms of the transcriptional activation.

The sequence conservation between *c1* and *pl* suggests that the two genes arose by a gene duplication event. Gene duplications in maize are common, usually involving large chromosomal segments that are duplicated between nonhomologous chromosomes (Helentjaris et al., 1988). Perusal of the most recent maize maps reveals that the *c1* and *pl* genes lie within such a segmental duplication (Coe, 1993). Figure 7 shows partial maps of chromosomes 6L and 9S. These chromosome arms contain duplications of previously characterized genetic loci, i.e., the *pale green* (*pg*) genes and the *bz1* gene, as well as RFLP markers. For the most part, the duplicated genes are found in the same order on both chromosome arms.

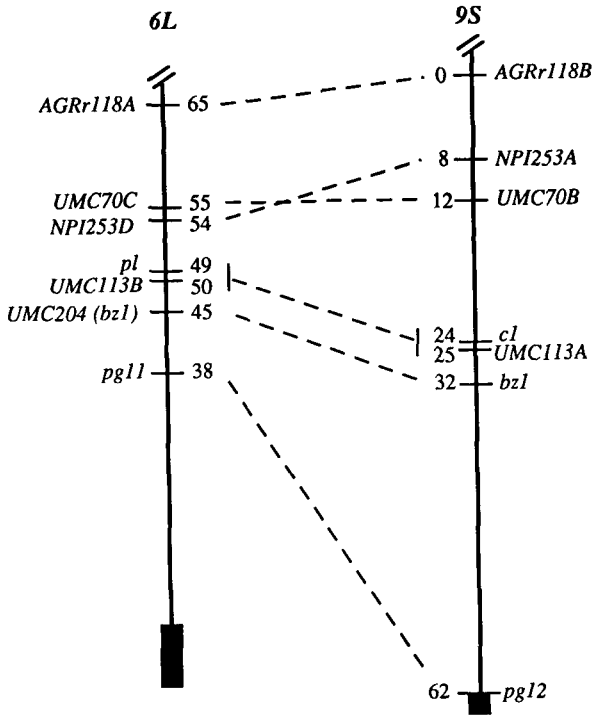

Figure 7. Maps of Chromosomes 6L and 9S Showing Locations of Duplicated Markers.

Only markers that are duplicated on both chromosome arms are indicated. Numbers represent distance in centimorgans. Centromeres are represented by filled boxes. Positions of markers were deduced from linkage maps compiled from a number of sources (Coe, 1991, 1993; Shoemaker et al., 1992).

The nature of the duplication event giving rise to *c1* and *pl* is not yet clear. Maize, with a haploid chromosome number of 10, is proposed to have originated by allotetraploid hybridization between two species with haploid chromosome numbers of 5 (Anderson, 1945; Rhoades, 1951; Celarier, 1956). It is possible that the *c1* and *pl* genes arose via duplication of a chromosomal segment as a result of allotetraploidization. One way to begin to examine this idea would be to ask whether *c1* and *pl* are found as duplicate genes in grasses closely related to maize. Our preliminary analysis along these lines indicates that sorghum and Coix each have a single gene and the teosintes have multiple genes that hybridize to probes derived from *c1* and *pl* (S. Cocciolone, J. Stone, B. Kent, and K. Cone, unpublished results). Continued study of this small gene

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family should furnish additional insights into the evolutionary events that have led to the production of functionally duplicate genes with distinct tissue specificities.

METHODS

Plant Materials

Maize stocks were obtained originally from B. McClintock and intercrossed to produce lines carrying various combinations of dominant and recessive alleles for the anthocyanin regulatory genes. All stocks were homozygous dominant for all anthocyanin structural genes. The dominant *P1* allele in the McClintock stocks is *P1-Rhoades* (abbreviated here as *P1*); the recessive *p1* allele is designated *p1-McC* (abbreviated here as *p1*).

The recombinant inbred (RI) family CO×Tx used for scoring the molecular and phenotypic segregation of *p1* was derived from an F_2 population made by crossing the inbred lines CO159 and Tx303, as previously described (Burr et al., 1988; Burr and Burr, 1991). The inbred Tx303 contains a functional allele of *p1* (designated *p1-Tx303*), whereas the inbred CO159 contains a nonfunctional *p1* allele (designated *p1-CO159*). The *p1-CO159* allele is recessive to *p1-Tx303*, and both of these alleles are recessive to *P1-Rhoades*. Both Tx303 and CO159 appear to carry the same *R* allele that, together with *p1-Tx303*, leads to pigmentation in the leaf sheaths at the base of the plant and in the prop roots of mature plants. Phenotypic segregation associated with *p1* was scored in the CO×Tx RI lines grown in the field by looking for anthocyanin pigmentation in the two lowest leaf sheaths of plants at the five-leaf stage and in the prop roots of plants at the time of flowering. Thirty-five of the RI lines produced either all pigmented or all unpigmented plants, indicating homozygosity for *p1-Tx303* or *p1-C0159*, respectively. Six of the RI lines contained both pigmented and unpigmented plants, indicating that those lines were not homozygous for the linkage groups around the parental *p1* alleles. This level of heterozygosity is not surprising, because at the time of this experiment, the RIs had only been self-pollinated for six generations. More recent scoring of this RI family, after three additional generations of selfing, shows that all but one of the RIs have reached fixation for this linkage group and are homozygous for one or the other parental *p1* alleles.

For localizing the cloned *p1* sequence to a chromosome arm, we used the B-A translocation TB-6Lc. A stock containing the B-A translocation Tb-6Lc was obtained from the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana, IL) and maintained by repeated crossing using a McClintock stock, containing all the dominant markers for kernel and plant anthocyanin pigmentation, as the recurrent male. Plants heterozygous for the translocation were identified by their high frequency of pollen abortion and were used as males in crosses to a stock homozygous recessive for the seedling mutant *piebald4* (*pb4*) located on chromosome 6L. Contaminants due to self-pollination were ruled out by scoring plant and kernel color in the progeny (the *pb4* line had unpigmented plant and kernels).

TB-6Lc contains the centromere of the supernumerary B chromosome linked to the long arm of chromsome 6. B chromosomes undergo a high frequency of nondisjunction at the second postmeiotic mitotic division during microsporogenesis (Beckett, 1978). The nuclei generated by this nondisjunction contain either two copies of the B with linked sequences (hyperploid) or no copies of the B with linked sequences (hypoploid). If the hypoploid nucleus fertilizes an embryo nucleus containing recessive markers for the chromosome arm of interest, the recessive markers will be uncovered in the hemizygous progeny. In addition, any molecular markers present on the same chromosome arm will be represented in the single copy in the resulting hemizygote.

Nucleic Acid Isolation and Hybridization Analysis

For analysis of DNA from the CO×Tx RI family, leaves were harvested from young field-grown plants. For RI lines that were heterozygous for the parental *p1* alleles, leaves were harvested from both pigmented and unpigmented plants and pooled prior to DNA isolation. DNA was isolated and purified by centrifugation in CsCl–ethidium bromide as previously described (Cone et al., 1986). Poly(A)$^+$ mRNA was prepared from husks harvested at the time of silk emergence (Cone et al., 1986; Wright et al., 1992). The methods for DNA and RNA gel blot hybridizations were as previously described (Cone et al., 1986). For DNA gels, each lane contained 1 to 2 μg of digested DNA. The hybridization probes were a 0.9-kb SalI-PstI fragment that encompassed approximately two-thirds of the coding region of the *c1* gene and a 0.6-kb SalI-HindIII fragment located just upstream of the coding region of the *p1-Tx303* gene.

Cloning and Sequence Analysis

For library construction, genomic DNA from the inbred Tx303 was digested with BamHI, size fractionated on a preparative low melting point agarose gel, purified, and cloned into the BamHI site of λEMBL4 as previously described (Cone et al., 1986). Recombinant phages were screened with the *c1* DNA fragment described above. The source of RNA for constructing cDNA libraries was purple husk tissue from a fully pigmented McClintock line carrying all the dominant genes for anthocyanin synthesis, including *P1*. Poly(A)$^+$ mRNA was isolated from husks harvested at the time of silk emergence. cDNAs were synthesized from the RNA using a commercially available kit (Bethesda Research Laboratories), methylated using EcoRI methylase, and cloned into the EcoRI site of λZAP (Stratagene) using EcoRI linkers. The resulting library, containing ~10^5 plaques, was screened by hybridization with a *c1* fragment. Two positive plaques were obtained. The cDNA sequences were recovered from the phage as plasmid subclones according to a protocol supplied by the manufacturer (Stratagene).

DNA sequences were determined by the dideoxynucleotide chain termination method using Sequenase (U.S. Biochemical). Oligonucleotide primers were obtained from the University of Missouri–Columbia DNA CORE facility. Sequence analysis and alignments were performed using software from DNASTAR (Madison, WI). The sequence of the *P1-Rhoades* allele has been submitted to GenBank as accession number L19494.

Nuclei Isolation and Run-On Transcription Assays

Nuclei were isolated from fresh husks by a modification of a previously described method (Watson and Thompson, 1986). Ears were harvested at the time of silk emergence. Outer husks, silks, and cob were removed, and the inner husks were cut into 1-in pieces. Approximately 300 g of husks were processed in batches of 50 g as follows. Husks were covered with cold ether for 3 min. The ether was drained off, and the husks were blotted briefly with paper toweling and placed into a cold Waring blender with a standard blade containing 250 mL of

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cold nuclear extraction buffer (1.0 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol). All subsequent steps were conducted at 4°C. Husks were disrupted by blending on high speed for ~5 sec until husks were coarsely shredded. Overblending resulted in poor yield of nuclei. The mixture was filtered through a double layer of cheesecloth, prewet with ice-cold working buffer ([WB] 0.5 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol), and collected in a beaker containing a magnetic stir bar. The crude extract was held on ice at this point to allow processing of remaining batches. Beakers were placed on a magnetic stirrer, and the extracts were stirred while 5 mL of 25% Triton X-100 was added dropwise. The extracts were held on ice for 5 min and then filtered through Miracloth (Calbiochem Corp., La Jolla, CA) into 250-mL centrifuge bottles.

Nuclei were pelleted by centrifugation at 2000 rpm in a rotor (model JA14; Beckman) for 10 min. The supernatant was quickly decanted, and bottles were allowed to drain briefly upside down. Each pellet was resuspended in 10 mL of WB by gentle swirling and layered on top of one Percoll step gradient formed in a 50-mL round bottom polycarbonate centrifuge tube. Percoll solutions were made from a 90% stock (containing 0.5 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0) using WB without β-mercaptoethanol as the diluent. The gradients consisted of 7.5 mL each of 90, 70, and 60% Percoll. Gradients were centrifuged at 5000 rpm in a swinging bucket rotor (model JS13.1; Beckman) for 20 min with brake set at 40%. Nuclei typically banded at the 60 to 70% interface, cellular debris banded above the nuclei, and starch pelleted. The upper portions of the gradient were removed by pipetting, and the band containing nuclei was transferred to a fresh tube. Nuclei were diluted two- to threefold with cold WB, pelleted by centrifugation at 2000 rpm in the rotor (JS13.1) for 10 min, and resuspended in 2 mL of nuclei storage buffer (20 mM Hepes-KOH, pH 7.9, 5 mM $MgCl_2$, 2 mM DTT, 50% glycerol).

An aliquot of nuclei was stained by adding one-twentieth volume of azure C (0.1% azure C [Sigma], 0.25 M sucrose, 0.02% sodium azide) and counted using a hemocytometer. Protein concentrations were determined by the Bradford method. Typical yields per gram of husk tissue were 3 to 5 × 10^5 nuclei, representing 15 to 20 μg of nuclear protein. Nuclei were stored at −80°C.

For run-on transcription assays, 5 × 10^6 nuclei were thawed on ice, pelleted by centrifugation at 3000 rpm for 10 min in a rotor (JA20), resuspended in 50 μL of nuclei storage buffer, and added to 50 μL reaction buffer (20 mM Hepes-KOH, pH 7.9, 20 mM $MgCl_2$, 200 mM $[NH_4]_2$ SO_4, 1 mM each ATP, CTP, GTP, 100 μCi α-^{32}P-UTP [800 Ci/mmol], 80 units RNasin) in a 15-mL polypropylene tube. The reactions were incubated at 30°C for 25 min, and labeled RNA was purified as described (Ausubel et al., 1989). The typical yield of labeled RNA was 5 to 20 × 10^6 cpm (Cerenkov).

Slot blots were prepared by the following method. Twenty micrograms of linearized plasmid in 100 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was denatured by adding 11 μL 1 N NaOH and incubating at room temperature for 30 min. The DNA was then neutralized by adding 900 μL of 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), and each DNA sample was split into four aliquots and immediately applied to nitrocellulose filters using a slot blot apparatus (Bethesda Research Laboratories), so that each slot contained 5 μg of DNA. Plasmids used were pUC13 (Yanisch-Perron et al., 1985); pALC2, a genomic clone of the *A1* gene (Schwarz-Sommer et al., 1987); pBF227, a genomic clone of the *Bz1* gene (Fedoroff et al., 1984); pC2, a cDNA clone for the *C2* gene (Wienand et al., 1986); and pMAc1, a genomic clone for the actin gene from maize (Shah et al., 1983). The blots were air dried, baked in vacuo at 80°C for 2 hr, cut into narrow strips, and rolled to fit into 1-dram shell vials. The strips were prehybridized, hybridized in a volume of 2 mL with 4 × 10^6 cpm of labeled RNA for 2 days in a 42°C shaker bath, and washed. Conditions for hybridization and washing were as previously described (Klessig and Berry, 1983). After washing, the blots were air dried and subjected to autoradiography using Kodak XAR5 film and DuPont Lightning-Plus intensifying screens for 1 to 3 days. Autoradiograms were scanned on a densitometer (Ultrogel II; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), and resulting values were normalized against the signals obtained from hybridization to the pUC13 negative control and to the actin control.

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