Pl-Bh, an Anthocyanin Regulatory Gene of Maize That Leads to Variegated Pigmentation

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ABSTRACT

Anthocyanins are purple pigments that can be produced in virtually all parts of the maize plant. The spatial distribution of anthocyanin synthesis is dictated by the organ-specific expression of a few regulatory genes that control the transcription of the structural genes. The regulatory genes are grouped into families based on functional identity and DNA sequence similarity. The *C1/Pl* gene family consists of *C1*, which controls pigmentation of the kernel, and *Pl*, which controls pigmentation of the vegetative and floral organs. We have determined the relationship of another gene, *Blotched* (*Bh*), to the *C1* gene family. *Bh* was originally described as a gene that conditions blotches of pigmentation in kernels homozygous for recessive *c1*, suggesting that *Bh* could functionally replace *C1* in the kernel. Our genetic and molecular analyses indicate that *Bh* is an allele of *Pl*, that we designate *Pl-Bh*. *Pl-Bh* differs from wild-type *Pl* alleles in two respects. In contrast to the uniform pigmentation observed in plants carrying *Pl*, the pattern of pigmentation in plants carrying *Pl-Bh* is variegated. *Pl-Bh* leads to variegated pigmentation in virtually all tissues of the plant, including the kernel, an organ not pigmented by other *Pl* alleles. To address the molecular basis for the unusual pattern of expression of *Pl-Bh*, we cloned and sequenced the gene. The nucleotide sequence of *Pl-Bh* showed only a single base-pair difference from that of *Pl*. However, genomic DNA sequences associated with *Pl-Bh* were found to be hypermethylated relative to the same sequences around the wild-type *Pl* allele. The methylation was inversely correlated with *Pl* mRNA levels in variegated plant tissues. Thus, we conclude that DNA methylation may play a role in regulating *Pl-Bh* expression.

ANTHOCYANINS are purple flavonoid pigments that are synthesized in the aleurone layer of the maize endosperm, in the embryo, and in many vegetative plant organs, including leaf, stem anthers, glumes of the cob and tassel, and coleoptiles. Genetic analyses have identified a number of genes required for the synthesis of anthocyanin pigments (COE, NEUFFER and HOISINGTON 1988; DOONER, ROBBINS and JORGENSEN 1991). Among these are four regulatory genes, *R*, *B*, *C1* and *Pl*, which encode proteins necessary for the transcription of a common set of structural genes encoding the biosynthetic enzymes of the anthocyanin pathway (DOONER 1983; CONE, BURR and BURR 1986; GOFF *et al.* 1990; LUDWIG *et al.* 1990; GOFF, CONE and FROMM 1991; GOFF, CONE and CHANDLER 1992). These regulatory genes have been grouped into two families, *R/B* and *C1/Pl*, based on the fact that they encode two sets of functionally equivalent proteins. The products of the *R* and *B* genes share sequence homology to the basic helix-loop-helix domain that is involved in DNA binding and protein dimerization of the *myc* class of oncoproteins (LUDWIG *et al.* 1989; PERROT and CONE 1989; RADICELLA, TURKS and CHANDLER 1991; CONSONNI *et al.* 1992). The products of the *C1* and *Pl* genes share homology to the DNA binding domain of the *myb* class of oncoproteins (PAZ-ARES *et al.* 1986; CONE and BURR 1988). Pigment production in any particular part of the plant requires the interaction of a member of the *R/B* family and a member of the *C1/Pl* family. For example, anthocyanin synthesis in the body of the plant requires the *B* and *Pl* genes, whereas anthocyanin production in the aleurone layer of the endosperm requires the seed-specific *R-S* gene and the *C1* gene. Recessive mutations in the regulatory genes generally lead to unpigmented phenotypes.

Another gene involved in anthocyanin production, *Blotched* (*Bh*), was originally described genetically as a dominant factor that conditioned a blotchy pattern of pigmentation in the aleurones of plants carrying a recessive *c1* allele (EMERSON 1921). The phenotype of blotchy kernels is characterized by pigmented cells that are usually clustered in sectors that appear to be clonal in origin (Figure 1). However, within a sector the degree of pigmentation varies on a cell-by-cell basis, ranging from very darkly pigmented to virtually unpigmented (Figure 1B). This blotchy pattern of anthocyanin synthesis is in sharp contrast to the uniform pigmentation of cells in sectors that arise from

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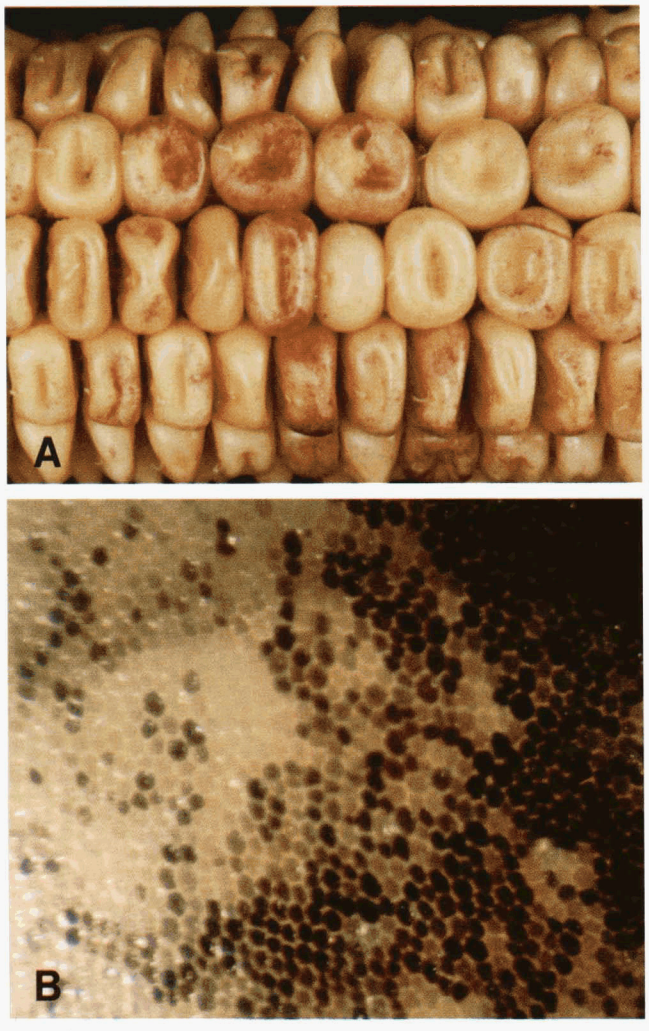

FIGURE 1.—Kernel phenotype of *Bh*. (A) Blotchy pigmentation in kernels of a plant homozygous for recessive *c1*. In the absence of *Bh*, the phenotype of these kernels would be colorless. The blotchy pigmentation shown here is limited to the aleurone layer of the endosperm. (B) Close-up of blotchy aleurone showing cell-to-cell variability in the levels of anthocyanin production.

somatic excision events typical of transposable element-induced alleles of *C1* and to the uniform pigmentation seen in all cells of the aleurone of kernels with a wild-type *C1* allele. However, based on the observation that *Bh* does lead to pigmentation in the absence of functional *C1*, the *Bh* gene was assumed to replace *C1*, in restoring anthocyanin synthesis in at least some cells of the aleurone (RHOADES 1948). This functional equivalence suggested to us that *Bh* might constitute an additional member of the *C1/Pl* regulatory gene family, albeit one with a very unusual pattern of expression.

We envisioned two hypotheses to explain the possible relationship of *Bh* to the other two members of the *C1/Pl* gene family. The first is based on genetic mapping results that placed *Bh* 1 map unit distal to *Pl* on the long arm of chromosome *6* (RHOADES 1948). This proximity suggests that *Bh* might have arisen from a tandem duplication of *Pl* to generate a functionally equivalent gene with a distinct pattern of expression and an altered organ specificity. (Other alleles of *Pl* are not expressed in the kernel.) Such an arrangement is found at the *R* locus where the seed-specific *R-S* and plant-specific *R-P* genes are situated in tandem and are separable by recombination (STADLER and NUFFER 1953). The second hypothesis is that *Bh* is an allele of *Pl* that has a different organ specificity than the wild-type allele. Again, within the *R/B* gene family, there is precedent for alleles of the same gene having different organ specificities (STYLES, CESKA and SEAH 1973). For example, most *B* alleles lead to pigmentation in the plant body; however, the *B-Peru* and *B-Bolivia* alleles lead to pigmentation in both the seed and the plant. In the case of the *B-Peru* allele, the alteration in organ-specific expression has been correlated with a complex genomic rearrangement such that the coding portion of the gene is flanked by 5′ sequences not present upstream of the wild-type *B-I* allele (RADICELLA *et al.* 1992).

In this report, we present genetic and molecular evidence to suggest that *Bh* is an allele of *Pl*, designated *Pl-Bh*, that leads to a variegated pattern of anthocyanin pigmentation in virtually all tissues of the plant, including the kernel. Our results indicate that the level of mRNA expression from *Pl-Bh* in husk tissue is lower than from the wild-type *Pl* allele, and that this lower level of expression is correlated with hypermethylation of *Pl-Bh* DNA.

MATERIALS AND METHODS

Maize stocks: Unless otherwise indicated, all stocks contained dominant markers for anthocyanin synthesis. Lines carrying *c1*, *Bh Pl* and *c1*, *Bh pl* were obtained from the Maize Genetics Cooperative Stock Center as stocks 604A and 604B, respectively. An allelism test was performed by crossing the *Bh Pl* stock by a McClintock line containing a recessive *pl* allele (*pl-McC*, abbreviated here as *pl*). The progeny of this cross were self-pollinated to generate an F_2 population. To determine if the *Bh Pl* stock carried a dominant modifier capable of altering the expression pattern of a wild-type *Pl* allele, the *Bh Pl* stock was also crossed to a McClintock line carrying the dominant *Pl* allele, *Pl-Rhoades* (abbreviated here as *Pl*). The two *Bh* stocks were also crossed to each other to verify that they carried the same *Pl* allele.

DNA isolation and gel blot analysis: Genomic DNA was isolated from young leaves by CsCl centrifugation, as described by CONE, BURR and BURR (1986). A modification of this procedure was used for rapid DNA isolation from large populations. Leaf tissue (0.3 g) was harvested from young seedlings, frozen in liquid nitrogen, and ground in a 15-ml polypropylene tube with a glass rod. The frozen powder was thawed at 42° in 0.6 ml lysis buffer containing 0.35 M NaCl, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA, 7 M urea, and 2% Sarkosyl. Samples were shaken in a 37° bath for 10 min and then extracted by adding 0.6 ml of phenol:chloroform:isoamyl alcohol (100:100:1), vortexing for 30 sec, and shaking at 37° for 10 min. The mixture was transferred to a 1.5-ml microcentrifuge tube and spun for 5 min in a microcentrifuge. A 500-μl aliquot of the aqueous

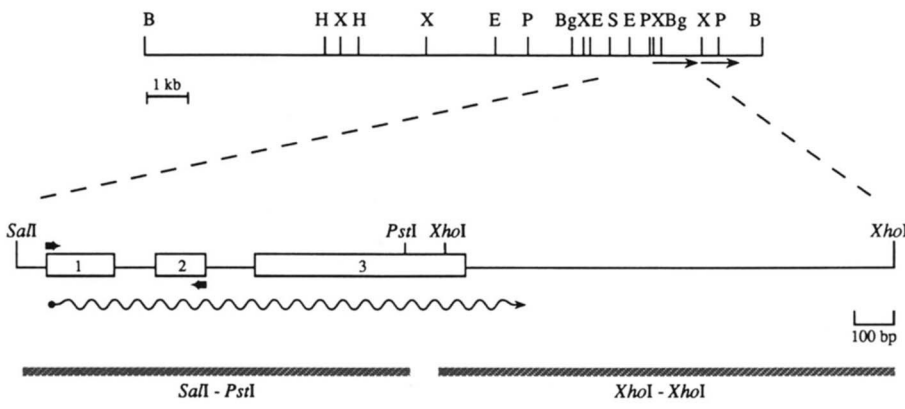

FIGURE 2.—Restriction map of *Pl*. Upper line is map of a genomic *Bam*HI fragment cloned into λ. Long arrows show location of direct duplication flanking the 3′ end of the gene. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I. The lower portion of the figure shows the organization of the *Pl* gene. Exons are depicted by open boxes. The transcribed region of the gene, as determined from cDNA analysis, is indicated by the wavy arrow. Primers used in PCR analysis are shown by small arrows. DNA fragments used as probes are shown: 900-bp *Sal*I-*Pst*I fragment is complementary to both *C1* and *Pl*; 1.1-kb *Xho*I fragment is specific for *Pl*.

phase was removed to a new tube. DNA was precipitated by adding 50 μl of 3 M sodium acetate, pH 5, and 600 μl of isopropanol and then pelleted by centrifugation for 1 min. The supernatant was discarded and the pellet was washed by adding 500 μl of 70% ethanol and centrifuging for 30 sec. The supernatant was discarded and the DNA pellet was resuspended in 100 μl 10 mM Tris-HCl, pH 8, 1 mM EDTA. DNA was stored at −20°.

Genomic DNAs were digested with restriction endonucleases according to the specifications of the manufacturer. To ensure complete digestion of DNA prepared by the rapid method described above, spermidine was added to a final concentration of 4 mM. The digests were fractionated on agarose gels, blotted to nitrocellose, and hybridized as previously described (CONE, BURR and BURR 1986). Blots were hybridized with either a 1.1-kb *Xho*I fragment *Pl*-specific fragment derived from the 3′ end of the gene or a 900-bp *Sal*I-*Pst*I fragment (Figure 2). The 900-bp fragment is complementary to both *C1* and *Pl*.

RNA isolation and gel blot analysis: Total RNA was prepared from inner husk leaves harvested at the time of silk emergence as described (WRIGHT *et al.* 1992). Aleurones and pericarps were dissected from kernels 19–22 days after pollination and frozen in liquid nitrogen. Then RNA was prepared by grinding the frozen tissue in an electric coffee mill. The frozen powder was suspended in the lysis buffer described above for DNA isolation (3.5 ml/g tissue), stirred for 10 min on a magnetic stirrer, and transferred to a 30-ml Corex tube. Debris was pelleted by centrifugation in a Beckman JA 20 rotor for 10 min at 12,000 rpm. The supernatant was transferred to a new 30-ml Corex tube and 1 g of CsCl was added for every 2.5 ml of supernatant. The CsCl was dissolved by rocking the tubes and the mixture was centrifuged for 10 min at 12,000 rpm. The supernatant was layered over 1 ml of 5.7 M CsCl, 0.1 M EDTA (pH 8) in a Beckman SW 50.1 ultraclear tube and centrifuged in a SW 50.1 rotor at 30,000 rpm for 16 hr at 15°. The RNA was recovered as previously described (MANIATIS, FRITSCH and SAMBROOK 1982). Poly(A)$^+$ mRNA was isolated using the PolyATtract mRNA isolation system (Promega) according to the specifications of the manufacturer. Poly(A)$^+$ RNA was fractionated on a denaturing gel, blotted, and hybridized as previously described (CONE, BURR and BURR 1986).

Genomic cloning of the *Pl-Bh* allele: Genomic DNA from the *Bh Pl* stock was digested with the restriction endonuclease *Bam*HI using the conditions specified by the supplier (Bethesda Research Laboratories). The digest was fractionated over a sucrose gradient as described (SAMBROOK, FRITSCH and MANIATIS 1989), with the exception that the pooled fractions were diluted to approximately 10% sucrose and then precipitated by adding sodium acetate to a final concentration of 0.3 M and 2 volumes of ethanol. The genomic DNA was cloned into the *Bam*HI site of λEMBL3. Recombinant phage were packaged, plated on the *Escherichia coli* strain ER1647 (New England Biolabs) which is deficient in methyl cytosine-specific restriction systems, and screened as described elsewhere (MANIATIS, FRITSCH and SAMBROOK 1982). Fragments of the genomic clone were subcloned into plasmid vectors for sequencing. The DNA sequence was determined by the dideoxy chain-termination method using Sequenase version 2.0 according to the specifications of the manufacturer (U.S. Biochemical Corp.). Oligonucleotide primers were obtained from the University of Missouri Molecular Biology DNA Core Facility. Sequence analysis and alignment were performed using software from DNASTAR, Inc. (Madison, WI). The nucleotide sequence of *Pl-Bh* has been submitted to GenBank as accession number L13454.

RNA assays: For qualitative detection of *Pl* mRNA from aleurones, first strand cDNA, primed by random hexamers, was synthesized from poly(A)$^+$ RNA with the SuperScript Preamplification System (Bethesda Research Laboratories) according to specifications of the supplier. The first strand cDNA was used as a template for amplification by the polymerase chain reaction (PCR). The sequences of the oligonuceotide primers were: upstream primer, 5′-ACCCTGCTGCTAGCTAGCTG-3′; downstream primer, 5′-CTGTTGCCGAGGAGCTTGTG-3′. Amplification reactions contained cDNA synthesized from 1 μg total RNA, 1 × synthesis buffer (supplied with the Preamplification System), 100 μM each deoxynucleoside triphosphate, 0.1 μM each primer, 50 μCi [α^{32}P]dATP (3000 Ci/mmol, New England Nuclear), 1.25 units Taq DNA polymerase (Promega) in a volume of 100 μl. Samples were overlaid with 100 μl mineral oil. The PCR reaction was performed by denaturation at 94° for 5 min, followed by 30 cycles of denaturation at 94° for 1 min, annealing at 55° for 1.5 min, and extension at 72° for 2 min. A final extension at 72° for 10 min completed the reaction. PCR products were fractionated on 10% polyacrylamide gels and visualized by autoradiography of the dried gels.

For estimation of *Pl* mRNA abundance in husks, we used duplex PCR in which two sets of primers were included in each reaction. The first primer set was composed of primers that span the first intron of the *Pl* gene. The sequences for the *Pl* primers are: upstream primer, 5′-CACGGCGAAGGCAAATGGAG-3′; downstream primer, 5′-CTGTTGCCGAGGAGCTTGTG-3′. The second primer set is specific for the *orange pericarp-1* (*orp1*) gene, that encodes the β subunit of tryptophan synthase (WRIGHT *et al.* 1992). The

oligonucleotide sequences for the *orp1* primers are as follows: upstream primer, 5′-AAGGACGTGCACACCGC-3′; downstream primer, 5′-CAGATACAGAACAACAACTC-3′. *orp1* was chosen as the reference (over other commonly assayed constitutive genes such as actin or tubulin), because the abundance of *orp1* mRNA in husks is comparable to that of *Pl* mRNA. To insure that amplification reactions were within linear ranges, pilot experiments were initially performed to determine the smallest amount of input RNA and the fewest number of cycles of amplification necessary to give visible products for both the constitutive *orp1* and *Pl* mRNA. Optimal results were obtained with 1 μg total RNA and 30 cycles of amplification, using the conditions given above. Aliquots of each reaction were fractionated on 5% acrylamide gels and the products were visualized by staining with ethidium bromide. Gels were photographed and the photographic negatives were scanned using an LKB Ultrogel XL II densitometer to estimate the levels of *Pl* mRNA relative to the constant *orp1* mRNA levels in each sample.

RESULTS

Evidence that *Bh* is an allele of *Pl*: We obtained two stocks carrying *Bh*, one in which *Bh* was reported to be linked to a dominant wild-type *Pl* allele (*Bh Pl*), and the other in which *Bh* was reported to be linked to a recessive *pl* allele (*Bh pl*). Both stocks had blotched kernels; however, the *Pl* designations presented a paradox, because plants grown from the two lines did not show the expected *Pl* phenotypes. Anthocyanin synthesis in the plant body requires the presence of both a *Pl* allele and a member of the *R/B* regulatory gene family. Plants that carry a dominant *B* allele and a wild-type *Pl* allele display a deep purple pigmentation in most of the vegetative parts of the plant, including leaves, culm (stem), husks, and tassel glumes. By contrast, plants carrying *B* and a recessive *pl* allele display a light-dependent pigmentation known as sun-red, in which only parts of the plant that are exposed to light synthesize anthocyanins. The *Bh pl* stock did not show the light-dependent sun-red phenotype; rather, the plants had no pigmentation in the plant body. The unpigmented phenotype could be explained if this stock contained a recessive allele elsewhere in the anthocyanin pathway. We could rule out mutations in the structural genes, since the plants had pigmentation in both the kernels and the anthers. Therefore, we concluded that the lack of pigmentation in the plant body of the *Bh pl* stock most likely was due to a recessive *b* allele. However, the phenotype of the *Bh Pl* stock was not so easily explained (Figure 3). These plants had occasional purple stripes on the culm and leaf sheath, as opposed to the uniform pigmentation normally produced in plants containing wild-type *Pl* alleles. The distribution of pigment in all parts of the plant body indicated that these plants probably contained a dominant *B* allele, but the variegated pattern of anthocyanin production was unexpected.

Bh Pl plants do not carry a dominant Pl allele: We envisioned two possible explanations for the striped phenotype of *Bh Pl* plants. First, the striping might be due to the presence of an unusual recessive mutation in either *B* or one of the anthocyanin structural genes. To test this idea, the *Bh Pl* stock was crossed to a tester line containing a recessive sun-red *pl* allele and dominant alleles for all other factors necessary for plant pigmentation. In this cross, any recessive mutation carried by the *Bh Pl* stock should be complemented by the dominant factors present in the tester, and the *pl* allele in the tester should by complemented by the *Pl* allele present in the *Bh Pl* line; thus the phenotype of the F_1 progeny should be uniformly purple. However, this was not the phenotype obtained; the progeny displayed dark purple stripes superimposed on a sun-red background. A second possible explanation for the striped phenotype is that the *Bh Pl* line carries a dominant modifier that affects the expression of the wild-type *Pl* gene and leads to striping. To test this possibility, the *Bh Pl* stock was crossed to a line carrying a wild-type *Pl* allele, with the expectation that the modifier present in the *Bh Pl* line would also modify the wild-type allele and lead to striped progeny. However, all progeny displayed the uniform purple pigmentation characteristic of wild-type plants. Taken together, the results of these two experiments suggest that the *Bh Pl* plants do not carry a dominant wild-type *Pl* allele.

Bh pl plants do not carry a recessive pl allele: Analysis of the plant phenotype of the *Bh pl* stock was complicated by the fact that this line did not appear to contain a functional *B* allele, and in the absence of *B*, it is not possible to score the *Pl* genotype in the plant. To overcome this difficulty and to assess the phenotype of the *Pl* allele present in this stock, *Bh pl* plants were crossed to the *pl* tester described above that contains all the dominant factors necessary for anthocyanin synthesis, including a dominant *B* allele. The progeny of this cross were expected to be sun-red, displaying the uniform, light-dependent pigmentation characteristic of plants with the genotype *B, pl*. However, all the progeny had purple stripes, superimposed on a sun-red background–a phenotype indistinguishable from that obtained by crossing the *Bh Pl* stock to the *pl* tester. This result supports the conclusion that the unpigmented phenotype of the *Bh pl* line is due to the presence of a recessive *b* allele. More importantly, this result indicates that the *Bh pl* line does not carry a recessive sun-red *pl* allele.

Bh Pl and Bh pl lines carry the same Pl allele: The fact that crosses of both *Bh* lines to a *pl* tester gave progeny with the same phenotype suggested that the parental lines actually contain the same *Pl* allele, that is capable of producing a striped plant phenotype. To evaluate this possibility, the two blotched lines were crossed to each other. The progeny had a phenotype that was identical to that of the *Bh Pl* parent. This result lends further support to the interpretation that the two blotched lines differ in their *B* genotype, not their *Pl* genotype. Molecular confirmation of this idea came from examining *B* and *Pl* mRNA levels in vegetative tissue from the two *Bh* lines. Poly(A)$^+$ RNA was isolated from husks, fractionated on denaturing agarose gels, and blotted to nylon membrane. Hybridization of the blot with a *Pl*-specific probe (1.1 kb *Xho*I fragment; Figure 2) detected mRNAs of 1.2 kb (Figure 4A) in both *Bh* lines. This is the size transcript detected in mRNA from purple husks of plants carrying the wild-type *Pl* allele. No *Pl* mRNA is detected in husks from plants carrying a recessive *pl* allele (data not shown). The blot was stripped and rehybridized with a *B*- specific probe. Figure 4B shows that no band was detected in mRNA from *Bh pl* husks, but a strongly hybridizing band was evident in mRNA from *Bh Pl* husks. This band corresponded to a 2.5-kb mRNA, the size expected for *B* transcripts. These findings strengthen the conclusion that the *Bh Pl* line contains a functional *B* allele, while the *Bh pl* line does not. In addition, these results indicate that *Pl*-specific mRNA is present both in striped husks from *Bh Pl* plants and non-pigmented husks from *Bh pl* plants. Together, the genetic and molecular data argue that both *Bh* lines contain the same *Pl* allele, and that this *Pl* allele leads to a striped phenotype in plants that also carry a dominant *B* allele.

FIGURE 3.—Plant phenotype of the *Bh Pl* stock. Pigmentation is blotchy in all tissues of the plant, including leaf sheath, blade, and midrib; culm; tassel.

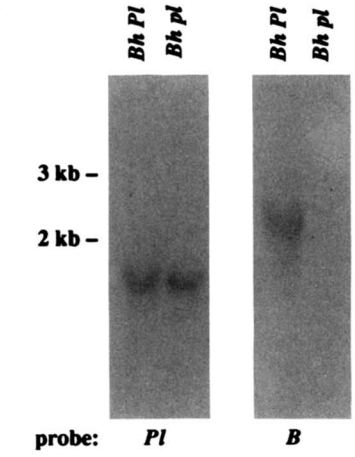

FIGURE 4.—RNA blot analysis of *Pl* and *B* mRNA levels in husk tissue from *Bh Pl* and *Bh pl* plants. Each lane contains 10 μg poly(A)$^+$ RNA isolated from husks at the time of silking. (A) Blot hybridized with the *Pl*-specific *Xho*I DNA fragment shown in Figure 2. The band seen in both lanes corresponds to a transcript of 1.2 kb. (B) The blot shown in (A) was stripped and rehybridized with a 550-bp *Hind*III-*Bam*HI fragment from the 5′ end of the *B* gene (CHANDLER *et al.* 1989). The band in the *Bh Pl* lane corresponds to a transcript of 2.5 kb. Positions of molecular weight markers are indicated to the left of the blots.

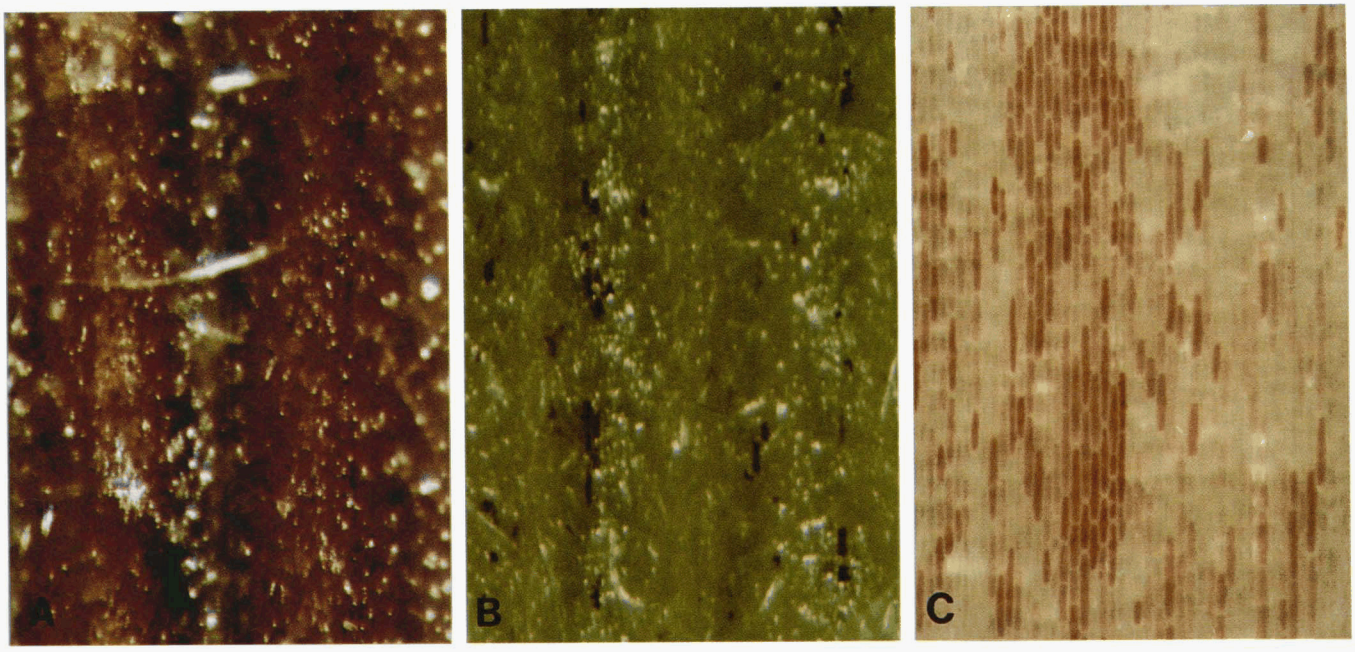

FIGURE 5.—Phenotype of *Bh* in vegetative plant organs. (A) Close-up of heavily blotched sector of leaf blade. (B) Close-up of sparsely blotched sector of leaf blade. Without magnification, this sector appears completely unpigmented. (C) Close-up of leaf midrib, showing the clonal arrangement of pigmented cells.

The phenotype of Bh plants is variegated: In observing the striped phenotype of plants with the genotype *B, Bh Pl*, we noticed that the level of anthocyanin production within the stripes is not uniform; rather, pigmentation levels vary from cell-to-cell as shown in Figure 5A. The areas between the pigmented stripes of *Bh Pl* plants are not completely unpigmented; small clusters of variably pigmented cells are scattered throughout this region (Figure 5B). In addition, the midrib (Figure 5C) of the leaf displays a variegated, or blotchy, pattern of pigmentation. This pattern of pigmentation is similar to the cell-to-cell variability seen in blotchy kernels from the same plants, and prompted us to look closely at pigmentation throughout the plant. By crossing *Bh* into genetic backgrounds differing for genes within the *R/B* gene family, we were able to score a blotchy pattern of pigmentation in virtually all organs and tissues capable of anthocyanin production in the maize plant. We observed blotchy pigmentation in leaf blade, leaf margin, midrib, sheath, husk, culm, silk hair, anther, cob glume, tassel glume, root, pericarp, aleurone and embryo. Anthocyanin synthesis in all blotchy organs shows a common pattern, *i.e.*, not every cell within a pigmented sector is pigmented to the same degree.

Another feature common to blotchy expression in all organs examined is that pigmented cells frequently occur in clusters, resembling clonal sectors. Patterns of cell division appear to dictate the spatial arrangement of pigmented cells within any organ. For example, in the aleurone where growth occurs via both anticlinal and periclinal cell divisions, the blotchy phenotype appears as clustered "spots" of pigmented cells. By contrast, in vegetative parts of the plant where cell divisions occur mainly in a longitudinal plane, blotchy expression appears as linear "stripes" of pigmented cells.

With the exception of the aleurone and the embryo, all of the organs pigmented by *Bh* are also pigmented by wild-type *Pl* alleles; thus, the organ specificity of expression of *Bh* combines that of *Pl* and its kernel-specific counterpart *C1*. This observation, together with our results suggesting that the *Bh* lines carry an unusual *Pl* allele and previous genetic mapping results placing *Bh* and *Pl* in close chromosomal proximity (RHOADES 1948) led us to speculate that *Bh* might actually be an allele of *Pl*. To evaluate this idea, we took a molecular approach.

A specific Pl allele co-segregates with the blotchy plant phenotype: To lend support to the idea that *Bh* is an allele of *Pl* which specifies an unusual phenotype, we performed a molecular segregation analysis. F_1 plants derived from the cross of *Bh Pl* plants by a *pl* tester (as described above) were self-pollinated to generate an F_2 population. Genomic DNA was prepared from individual F_2 plants grown in the field. Each plant was self-pollinated, the ears were harvested, and the presence of blotchy anthocyanin synthesis in the plant was determined by observing the glumes on the cob. Genomic DNAs were digested with a restriction enzyme that allowed the parental *Pl* alleles to be distinguished based on restriction fragment length polymorphism. Digests were fractionated on agarose gels, blotted to nitrocellulose, and hybridized with a *Pl*-specific probe.

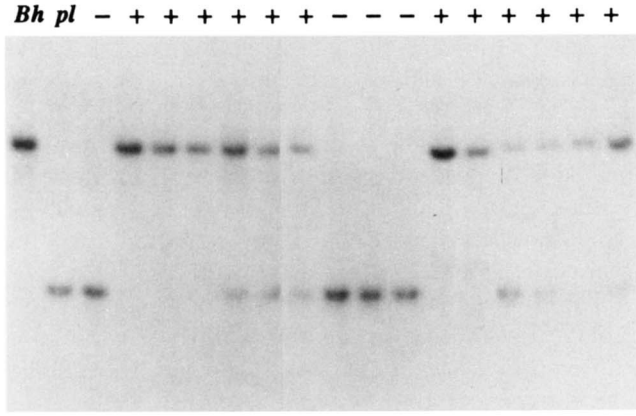

FIGURE 6.—Molecular co-segregation analysis. *Bh Pl* plants were crossed by a *pl*-tester line and the progeny were self-pollinated to generate an F_2. DNA from the parents and individual F_2 plants were digested with the restriction enzyme *Hin*dIII and hybridized with a *Pl*-specific probe. The first two lanes contain DNA from the blotched (*Bh*) and non-blotched (*pl*) parents. The remaining lanes contain DNA from the F_2 plants. The phenotypes of the F_2 plants were scored in the glumes of the cob and are indicated above the lanes; +, blotchy; −, non-blotchy.

A representative autoradiograph is show in Figure 6. Plants heterozygous or homozygous for the *Pl* allele from the blotched parent always had blotchy glumes, and plants homozygous for the *Pl* allele from the non-blotched (*pl*) parent always had colorless glumes. The co-segregation between blotchy expression in the plant and the *Pl* allele from the *Bh* parent suggests that *Bh* is either at the *Pl* locus, *i.e.*, allelic with *Pl*, or is tightly linked to *Pl*.

Bh is not a tandem duplication of Pl: In genetic mapping experiments to determine the chromosomal location of *Bh*, RHOADES (1948) detected recombination between *Bh* and *Pl* at a frequency that showed the two genes were approximately 1 map unit apart. This observation raised the possibility that *Bh* was a tandemly duplicated *Pl* gene that retains the ability to regulate anthocyanin synthesis, but has an altered pattern of expression. Such a duplication is found at the *R* locus, in which the seed-specific component *R-S* and the plant-specific component *R-P* are arranged in tandem and are separable genetically by recombination. In addition, when genomic blots are hybridized with *R*-specific probes, the two components are readily detectable as distinctly hybridizing bands. To address the possibility that *Bh* is a tandem duplication of *Pl*, genomic DNAs from both *Bh* stocks were compared by Southern hybridization to DNA from plants containing a wild-type *Pl* allele. DNA blots were first hybridized with a probe (900-bp *Sal*I-*Pst*I fragment; Figure 2) that hybridizes to both *C1* and *Pl*. With this probe, only two strongly hybridizing bands were observed in each lane, whereas with a *Pl*-specific probe derived from the 3′-untranslated region of the *Pl* gene (1.1-kb *Xho*I fragment; Figure 2), only one hybridizing band was observed in each lane (data not shown). In all cases, strongly hybridizing bands were identified as arising from either the *C1* or the *Pl* locus, based on restriction enzyme mapping of the *C1* and *Pl* alleles that have been cloned in our laboratory. Thus, we conclude that *Bh* is not a tandem duplication of *Pl*.

Pl-specific mRNA is present in kernels from Bh plants: Plants that contain the *Bh* gene show blotchy expression of anthocyanins in the kernel and the vegetative and floral parts of the plant body. Wild-type alleles of *Pl* do not lead to pigmentation in the kernel. If *Bh* is an allele of *Pl*, we would expect to detect *Pl* mRNA in blotched kernels. To test this idea, poly(A)$^+$ RNA was extracted from aleurones and the attached pericarps of kernels homozygous for *Bh*. The RNA was fractionated on a denaturing agarose gel, blotted to nylon membrane, and hybridized with a *Pl*-specific probe. After long exposure, a hybridizing band corresponding to the size of the wild-type *Pl* mRNA was seen (data not shown).

Because wild-type *Pl* is expressed in pericarp (our unpublished results), the presence of *Pl* mRNA in aleurone/pericarp peels does not provide unequivocal evidence that *Pl* is expressed in aleurone of blotchy kernels. To confirm the presence of *Pl* mRNA in blotchy aleurone, we used a more sensitive assay employing PCR. Poly(A)$^+$ mRNA, isolated from carefully dissected pericarps and aleurones from blotchy kernels, was converted to first-strand cDNA by reverse transcription. The resulting cDNA was used as a template for PCR with a set of primers that was specific for *Pl* (Figure 7A). The upstream primer is complementary to sequences in the 5′ untranslated region of *Pl*. The downstream primer is located at the 3′ end of the second exon and is complementary to sequences in both *Pl* and *C1*. Amplification from first-strand cDNA should yield a product of 316 bp. Any contaminating genomic DNA, which would include the intron, should yield a product of 426 bp. Figure 7B shows the results of the experiment. The first two lanes show the products of amplification from cloned *Pl* and *C1* genomic DNA. Amplification from a *Pl* genomic DNA yielded a product of 426 bp, the size expected from amplification across the intron of the gene. The absence of an amplification product from the *C1* genomic DNA indicates that the primers employed are specific for the *Pl* gene. In the lanes containing cDNA from blotchy pericarp and aleurone, a single 316 bp product was seen, indicating that an RNA of the size expected for *Pl* mRNA was present. In a parallel experiment, no *Pl* mRNA was detected in aleurones of kernels carrying *C1* and the wild-type *Pl* allele (data not shown). This result indicates that at least a portion of the *Pl* mRNA detected by RNA blot hybridization from *Pl-Bh* aleurone/pericarp tissue is derived from aleurone.

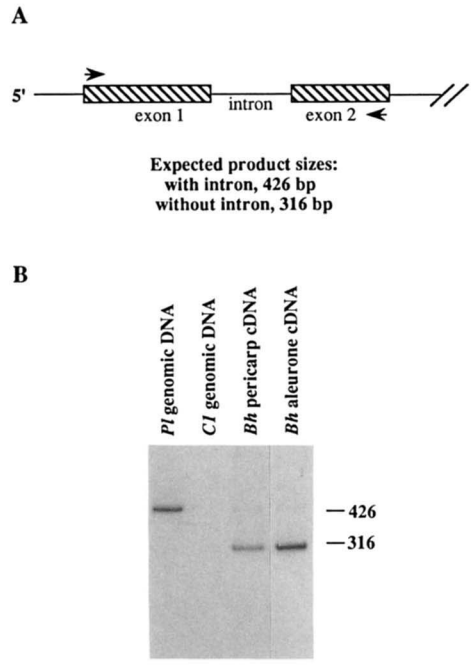

FIGURE 7.—Detection of *Pl* mRNA from blotchy kernels of plants carrying *Bh*. (A) PCR-based strategy for amplifying *Pl* cDNA. Gene organization of the 5′ portion of *Pl* is indicated. Arrows represent primers used in the amplification. The upstream primer is located in the 5′ untranslated region of the gene, and the downstream primer is located at the 3′ end of the second exon. Sizes of expected amplification products are indicated below the diagram. (B) Detection of *Pl*-specific cDNA. Amplifications were performed in the presence of a trace amount of radioactively labeled dATP to facilitate visualization of products by autoradiography. First two lanes show the results of control amplifications to demonstrate that the primers were specific for *Pl*. Reactions contained of 0.1-ng cloned genomic DNA for *Pl* and *C1*, respectively. Remaining lanes show products of amplification from cDNA synthesized from 1 μg of total RNA from either pericarps or aleurones of blotchy kernels. Sizes of amplified products in bp are indicated to the right of the autoradiograph.

Taken together, the genetic and molecular data presented here are consistent with the interpretation that *Bh* is an allele of *Pl*. We propose that *Bh* be designated *Pl-Bh*.

DNA methylation as a possible mechanism regulating the variable expression of *Pl-Bh*: Because the *Pl-Bh* allele differs from the wild-type allele in its pattern of expression and organ specificity, the expression of the two alleles must be differentially regulated. Current ideas about how gene expression is controlled in plants center around a combinatorial model (BENFEY and CHUA 1990), in which a number of modular *cis*-acting sequences can combine synergistically to direct an expression pattern not conferred by the individual modules. Based on this model, we would expect to detect specific sequence differences between the *Pl-Bh* and *Pl* alleles that could account for the different patterns of expression. The *cis*-acting regulatory regions of most plant genes tend to be located relatively close to the coding regions of genes, usually within 1–2 kb (THOMPSON and WHITE 1991). We have sequenced the 1.0-kb coding region, 1.9 kb upstream and 1.1 kb downstream of the gene. To our surprise, the only sequence difference between *Pl-Bh* and a wild-type allele is one single base-pair insertion located 632 bp downstream of the stop codon. It is possible that the sequence difference between *Pl-Bh* and wild-type *Pl* might play some role in altering the organ specificity between the two alleles. However, we were not convinced that this minor change could explain the unusual variegated pattern of expression exhibited by *Pl-Bh*. Thus, we looked for other molecular differences that might distinguish *Pl-Bh* from wild-type *Pl*.

Ideally, a mechanism explaining the variability of the blotched phenotype should allow for anthocyanin synthesis to be activated by *Pl-Bh* within some cells early in development, thereby yielding clonal sectors of pigmentation; however, it must also allow for cell-to-cell variability of gene expression, such that not all cells attain the fully activated state. The regulatory mechanism must also be heritable to explain the stable transmission of the *Pl-Bh* phenotype to progeny from generation to generation. One possible mechanism that fits these criteria is DNA methylation. Plant DNA is very heavily methylated on cytosine residues that occur in either the CpG dinucleotide or the CpXpG trinucleotide, where X = A, C or T. Such cytosine methylation has been implicated in inactivating gene expression (reviewed in SELKER 1990a,b; CHOMET 1991; HERGERSBERGER 1991; SZYF 1991). In addition, methylation patterns have been observed to change during the course of development, *e.g.*, genomic imprinting in mammals (CHAILLET *et al.* 1991). In plants, both developmental methylation changes and heritable methylation patterns have been described for transposable elements (BANKS, MASSON and FEDOROFF 1988; BANKS and FEDOROFF 1989; MARTIENSSEN *et al.* 1990). If methylation is the mechanism controlling the expression of *Pl-Bh*, we predict that the *Pl-Bh* allele would be subject to DNA methylation, and the result of this methylation would be both silencing and modulation of gene expression. Early during development the *Pl-Bh* gene would be differentially methylated and this methylation would be more or less maintained through subsequent cell division, producing clonal sectors in plant tissues of predominantly pigmented cells (unmethylated) and sectors of predominantly unpigmented cells (methylated). Within a sector, methylation would act as a modulator of *Pl-Bh* expression if the level of *Pl-Bh* expression were inversely proportional to the number or distribution of methylated sites. Methylation might also serve as a mechanism for activating *Pl-Bh* expression in the kernel if the methylated sequences prevented the binding of some factor that normally func-

tions to repress *Pl* expression in the kernel. Cell-to-cell differences in the methylation pattern could be explained through the failure to maintain methylation or through the occurrence of *de novo* methylation.

Genomic sequences near Pl and Pl-Bh are differentially methylated: Our first insight that *Pl-Bh* DNA is methylated came from our difficulty in cloning the gene. After several futile efforts to clone *Pl-Bh* by standard methods used to clone other *Pl* alleles in our laboratory, we succeeded by plating recombinant phage on an *E. coli* strain that is defective for several restriction enzymes that recognize and cleave methylated sequences (Mrr$^-$, McrA$^-$, McrB$^-$). This suggested that sequences near or within the *Pl-Bh* gene might be methylated.

To compare the methylation levels between *Pl* and *Pl-Bh*, genomic DNA from each genotype was digested with *Msp*I and *Hpa*II, an isochizomer pair that differ in their sensitivity to methylation. Both enzymes recognize the sequence CCGG, but *Hpa*II cleaves this sequence only when the internal cytosine is unmethylated, whereas *Msp*I cleaves this sequence regardless of the methylation state of the internal cytosine. DNA isolated from *Pl* and *Pl-Bh* seedlings was digested with either *Msp*I or *Hpa*II, fractionated on agarose gels, blotted to nitrocellulose, and hybridized with a *Pl*-specific probe derived from the 3′ end of the gene. As shown in Figure 8A digestion of *Pl* and *Pl-Bh* with *Msp*I yielded the same fragments, indicating that the positions of the *Msp*I sites are the same in both alleles. From the *Msp*I digestion pattern and the sequence of both genes, we constructed a map of all the existing *Msp*I sites in the 3′-flanking region of the gene (Figure 8B). To determine which of these *Msp*I sites is methylated, samples were digested with *Hpa*II, and the pattern and intensity of the bands was compared between the two digests. Reduction in the intensity of a band in the *Hpa*II-digested samples, relative to *Msp*I-digested samples reflects methylation of specific CCGG sequence(s) that give rise to that DNA fragment. Digestion of wild-type *Pl* DNA with *Hpa*II revealed a reduction in the intensity of bands corresponding to fragments of 1800, 670 and 426 bp and the absence of the 1500-bp fragment. This indicates that at least some of the sites giving rise to these bands are methylated in wild-type DNA. Digestion of *Pl-Bh* DNA with *Hpa*II revealed a drastic reduction in the intensity of all four of these bands, indicating that the sites that give rise to these bands are almost completely methylated in *Pl-Bh* DNA. Based on these results, we conclude that in both alleles a subset of CCGG sites is unmethylated on the internal cytosines (indicated by arrows in Figure 8B). Another subset of sites (indicated by asterisks) is methylated in both alleles, but hypermethylated in *Pl-Bh*.

The level of Pl mRNA is reduced in Pl-Bh plants: In

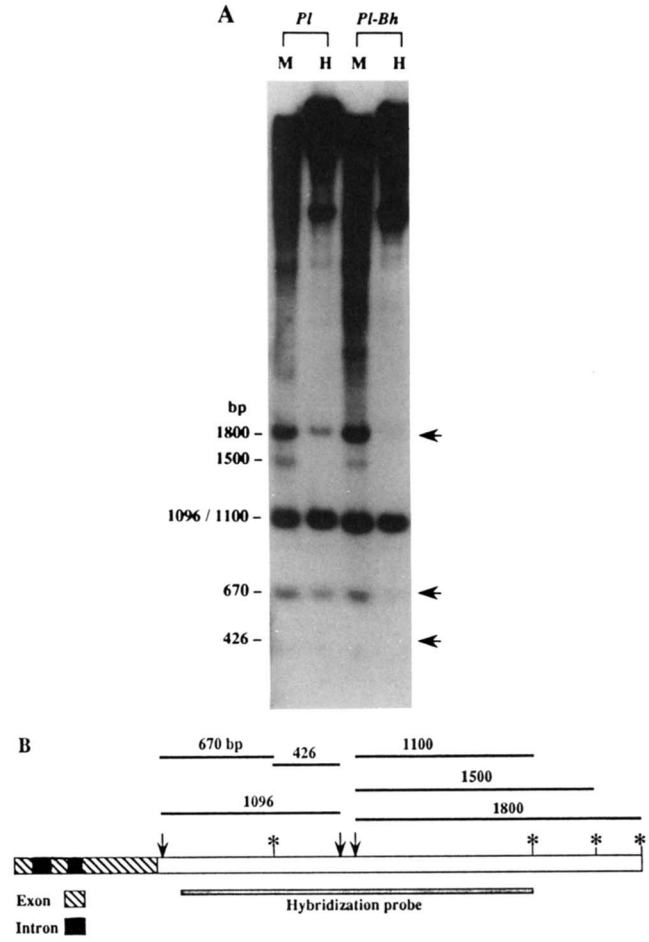

FIGURE 8.—Analysis of cytosine methylation of genomic DNA from *Pl* and *Pl-Bh* alleles. (A) Blot of DNA from seedlings of plants homozygous for either *Pl* or *Pl-Bh*. Identical results were obtained for DNA isolated from husk tissue. DNA samples were digested with the restriction endonucleases *Msp*I (M) and *Hpa*II (H), fractionated on agarose gels and blotted to nitrocellulose. Blots were hybridized with the 1.1-kb *Xho*I fragment derived from the 3′ untranslated region of the gene (Figure 2). Arrows indicate bands whose intensities in *Hpa*II digests differ between *Pl* and *Pl-Bh*. (B) Diagram showing the location and methylation pattern of *Msp*I/*Hpa*II sites in the 3′ flanking region of *Pl-Bh*. Restriction fragments and their sizes in bp are shown above the schematic representation of the *Pl-Bh* gene. Arrows indicate sites that are not methylated. Asterisks (*) indicate sites that are methylated.

most eukaryotic systems, DNA methylation is correlated with a decrease in gene expression (HERGERSBERGER 1991). To determine whether the hypermethylation of *Pl-Bh* is correlated with down-regulation of its expression, we measured the levels of *Pl* RNA in husks of plants carrying either *Pl-Bh* or *Pl*. Total RNA was isolated from uniformly pigmented husks of wild-type plants, and from heavily blotched sectors and sparsely blotched sectors of husk from *Pl-Bh* plants. To assay and compare the levels of *Pl*-specific RNA within the different tissues, we used a PCR-based method. The RNA was converted to first-strand cDNA by reverse transcription and then used as a template for PCR amplification. *Pl*-specific sequences

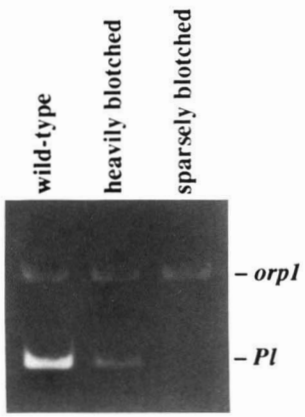

FIGURE 9.—Quantitative analysis of *Pl* mRNA levels in husk tissue. Total RNA was isolated from purple husks from plants carrying the wild-type *Pl* allele, and from heavily blotched and sparsely blotched husks from plants carrying *Pl-Bh*. RNA was converted to first strand cDNA for amplification by PCR. Two primer sets were used in each reaction, one specific for *Pl* and the other specific for *orp1*, a constitutively expressed gene. The sizes of the amplified products are: *Pl*, 170 bp; *orp1*, 207 bp.

were amplified using an upstream primer located at the 3′ end of the first exon and a downstream primer located at the 3′ end of the second exon. A second primer set specific for the maize *orange pericarp-1* (*orp1*) gene, encoding tryptophan synthase β, was included in the same amplification reaction to serve as a reference standard for normalizing the amount of input RNA in each reaction. Figure 9 shows that the level of *orp1* product was roughly equal between samples; however, the amount of *Pl*-specific product varied. The relative levels of *Pl* mRNA were estimated from the photographic negative for Figure 9 by scanning with a densitometer and normalizing to the levels of *orp1* mRNA. Based on these results, wild-type *Pl* husks contain about three times as much *Pl* mRNA as the heavily blotched sectors of *Pl-Bh* husks, and about 50 times as much *Pl* mRNA as the sparsely blotched sectors of *Pl-Bh* husks. These results indicate that the degree of pigmentation is correlated with *Pl* mRNA levels, and suggests that pigmentation can serve as a visual indicator of *Pl* expression in *Pl-Bh* plants. Furthermore, the reduced level of *Pl* mRNA in blotchy husks relative to the level in wild-type husks, supports the idea that hypermethylation of the *Pl-Bh* is correlated with decreased *Pl* gene expression from this allele.

DISCUSSION

A number of lines of evidence lead to the conclusion that *Bh* is an allele of the anthocyanin regulatory gene *Pl*. Allelism tests conducted by crossing *Bh* stocks to *pl* tester lines revealed that *Bh* leads to an unusual phenotype in the vegetative and floral organs of the plant. Plants carrying *Bh* display a blotchy pattern of pigmentation unlike the uniform purple pigmentation associated with wild-type *Pl* alleles and unlike the uniform sun-red pigmentation associated with recessive *pl* alleles. The blotchy phenotype appears to be dominant to *pl*, because blotching is evident in *Bh/pl* heterozygotes. The blotchy phenotype is masked by the wild-type phenotype in *Bh/Pl* heterozygotes, indicating that blotching in plant tissues is not due to the presence of a dominant *trans*-acting factor capable of modifying expression of the wild-type allele to yield a blotchy phenotype. Thus, by genetic criteria, *Bh* is allelic with *Pl*.

The results of molecular experiments also argue that *Bh* is an allele of *Pl*. In restriction fragment length polymorphism analysis of more than 200 individual plants from two different F_2 populations segregating for the *Bh* gene, the blotchy plant phenotype always co-segregated with the *Pl* allele contributed by the blotched parents. In addition, hybridization of genomic DNA from plants carrying *Bh*, with probes derived from the *Pl* gene and its kernel-specific homolog *C1*, revealed no third homologous sequence that might represent a functionally similar, but distinct, *Bh* gene. Examination of RNA expression in plants carrying the *Bh* gene showed that a *Pl*-specific mRNA was present in blotchy aleurones (an organ not pigmented by other *Pl* alleles) and in blotchy husks. Moreover, the level of *Pl* mRNA in blotched husks was proportional to the degree of pigmentation, *e.g.*, heavily blotched regions from blotchy husks contained more *Pl* mRNA than did sparsely blotched regions. This observation is certainly consistent with the interpretation that anthocyanin synthesis in blotched plants is controlled by a *Pl* gene. Taken together, the genetic and molecular results support the conclusion that *Bh* and *Pl* are allelic. We have designated the blotchy allele *Pl-Bh*.

The early descriptions of *Bh* focused on the blotchy pigmentation in the kernel, whereas blotchy expression in the vegetative and floral organs was not reported. The failure to note expression in plant organs was most likely due to the fact that some *Pl-Bh* stocks did not carry the appropriate alleles of the *R/B* gene family for activating anthocyanin synthesis in organs other than the kernel. In some cases however, blotchy expression in the plant organs appears to have been misclassified as wild-type *Pl* expression. We have observed that in some genetic backgrounds *Pl-Bh* leads to very heavily pigmented plants that could easily be classified as wild type at first glance.

The overall characteristics of the blotched phenotype are virtually the same in all blotchy organs. The distribution of pigmented sectors within any organ is random and varies from plant to plant. A distinguishing characteristic of the blotched phenotype is that the degree of pigmentation varies on a cell-by-cell basis, yielding cells that range from darkly pigmented

to unpigmented. Because the degree of pigmentation within a blotched plant correlates with *Pl* mRNA levels, it is likely that the cell-to-cell variation in pigmentation is due to cell-to-cell variation in *Pl-Bh* gene transcription. There is precedence for the dosage of regulatory genes affecting the levels of anthocyanin production. For example plants carrying two doses of *B* are more darkly pigmented than plants carrying a single dose (COE 1966). A similar effect of dosage has also been documented for the *C1* locus in the kernel (COE 1962). Another characteristic common to the blotched phenotype is that the pigmented cells tend to be clustered into sectors, as if early in development a decision is made in some cell lineages to express *Pl-Bh*. This "activated state" is inherited clonally to produce sectors of pigmented cells. In some cells, *Pl-Bh* expression is activated late in development, giving rise to the small pigmented sectors and scattered pigmented cells seen in sparsely blotched areas of the plant body and aleurone.

Although the general features of the blotched phenotype are similar in the aleurone and the plant body, there is no correlation between the degree of blotching within organs of the same plant. For example, plants displaying heavy blotching in vegetative and floral organs can produce kernels with very few pigmented cells; conversely, sparsely blotched plants can produce kernels with many pigmented sectors. This observation suggests that there are distinct organ-specific modifiers of *Pl-Bh* expression. In fact, MANGELSDORF (1958, 1959, 1960) mapped two genes, on chromosomes *4L* and *9L*, that are involved in modifying blotchy expression in the aleurone. The presence of modifiers is especially evident when scoring blotching in the aleurone; outcrossing of *Pl-Bh* plants often results in severe suppression of blotching. Suppression may be so extreme that blotching is reduced to a single sector with only a few pigmented cells. The degree of blotching in the aleurone can also be influenced by environmental conditions. Kernels taken from the same ear and grown under different conditions, *i.e.*, field *vs.* greenhouse, can produce self-pollinated ears with very different levels of kernel blotching. We have observed that warmer growth conditions suppress blotching, possibly because the kernels dry down before anthocyanin synthesis reaches its peak.

The blotched phenotype in maize shares many characteristics with position effect variegation (PEV) in Drosophila. The classical example of PEV is variegation associated with rearrangements of the *X* chromosome, in which an inversion places the euchromatic *white* gene (necessary for production of red eye color pigment) adjacent to the heterochromatic region near the centromere (reviewed in HENIKOFF 1990; PARO 1990). Variegated expression is seen in the eye as numerous spots or clones of colored cells on a mutant white background. The presence of clones indicates that some developmental decision is made early in formation of the eye and that the determined state is inherited through subsequent cell divisions. The determined state appears to be somewhat random in that exactly which cells are mutant or wild type differs from eye to eye, suggesting that the mechanism controlling PEV has a stochastic component. Changes of the DNA environment, rather than changes in the euchromatic locus, are responsible for the variegated phenotype, since translocation of variegating loci away from heterochromatin regions restores the non-variegating phenotype. The currently accepted model for PEV proposes that early in development, heterochromatin forms and spreads along the chromosome to a variable extent in different cells; spreading of heterochromatin past the euchromatin break point causes the repression of neighboring euchromatic genes. Once the variable repression is established, its inheritance is both stable and clonal, and it gives rise to a variegated phenotype. Initiation of heterochromatin assembly is postulated to occur at a specific initiation point and spread in a polarized fashion. The termination of heterochromatin formation is thought to be controlled more or less randomly by mass action, such that the extent of heterochromatin assembly is dictated by the localized concentration of free heterochromatin proteins. In support of this idea, suppressor mutations for PEV have been shown to affect genes that encode heterochromatin-associated proteins (PARO 1990; GATTI and PIMPINELLI 1992).

A similar type of initiation and spreading mechanism is thought to be responsible for inactivation of genes on mammalian *X* chromosomes (reviewed in LYON 1991, 1992; RIGGS and PEIFER 1992). *X* chromosome inactivation results in dosage compensation of *X*-linked genes by the transcriptional inactivation of all but one of the *X* chromosomes in a diploid somatic cell. Inactivation is thought to initiate at the *X*-inactivation center, which has been mapped in some species, and then spread along the chromosome in both directions. The mechanism directing this wave of gene inactivation is not understood. In eutherian mammals, the end result is that specific sites within the promoter regions of the inactivated genes are hypermethylated relative to the same sites on the active *X* chromosome. A mechanism for the initiation and spreading of DNA methylation has been proposed by SZYF (1991). In this model, specific DNA sequences serve as centers of methylation. Protein factors bind to these sequences and increase the affinity of nearby sites for DNA methyltransferases. The methylated sites may then in turn be bound by factors that exhibit increased affinity for methylated DNA, such as the methylated DNA-binding proteins described recently

(BOYES and BIRD 1991, 1992). This binding limits the accessibility of the gene to *trans*-acting factors that are essential for expression and also enhances the susceptibility of adjoining sites to DNA methyltransferase activity. The end result is a cooperative wave of *de novo* methylation which spreads along the DNA and serves to inhibit the expression of one or more genes.

The cell-to-cell variability of *Pl-Bh* expression is reminiscent of the behavior of an inactive *Activator* (*Ac*) transposable element at the *waxy* (*wx*) locus in maize (MCCLINTOCK 1978). In the *wx-m7* allele, the transposable element *Ac* is inserted in the promoter of the gene. The ability of the *Ac* element to transpose is controlled by DNA methylation of the element (CHOMET, WESSLER and DELLAPORTA 1987). When *Ac* is active (unmethylated), it can excise from *wx* and produce somatic sectors having the wild-type level of *wx* expression. When the *Ac* is inactive (methylated), it leads to a variegated pattern of expression in which cells express *wx* to different degrees. The inactive state of *Ac* and thus the variegated pattern of *wx* expression are heritable from generation to generation. The mechanism controlling the variable expression is unclear, but it appears to be related to the positional effect of *Ac* insertion in promoter elements crucial for gene expression, since *Ac* insertions in other positions in *wx* (for example, the *wx-m5* and *wx-m9* alleles; MCCLINTOCK 1963; WEIL *et al.* 1992) do not lead to this pattern of variable expression.

The mechanisms controlling PEV, *X* inactivation, and transposable element activity are particularly interesting in light of our discovery that *Pl* alleles are differentially methylated. We have shown that although the *Pl-Bh* gene is virtually identical in nucleotide sequence to its wild-type counterpart, its pattern of methylation is distinct. *Pl-Bh* contains a number of sites that are hypermethylated relative to the same sites in the wild-type allele. The methylation status of *Pl* is inversely correlated with the level of *Pl* mRNA expression, in that *Pl* mRNA levels in blotchy husks are significantly lower than *Pl* mRNA levels from wild-type purple husks. This correlation is consistent with the hypothesis that DNA methylation serves to down-regulate *Pl* expression in the *Pl-Bh* allele.

By analogy to PEV, *X* inactivation, and the control of transpoable element activity, we suggest a model to explain both the variegation and the altered organ-specificity associated with *Pl-Bh* expression. We propose that expression of *Pl-Bh* is silenced by DNA methylation. We envision that DNA methylation results from initiation of a modification signal at a *cis*-acting sequence (the center of modification, CM) located some distance away from the gene. The modifying signal is propagated toward *Pl-Bh* in a stochastic manner, so that the DNA of some cells becomes methylated and the DNA of other cells is unmethylated. Once established, the methylation pattern is clonally inherited to produce pigmented sectors. We predict that the methylation pattern involves multiple sites, such that methylation of only a few of these sites or incomplete methylation of crucial sites would allow low level expression of *Pl-Bh*. If we consider the role of the *Pl* gene product as a probable transcription factor essential for activating at least six structural genes in the anthocyanin pathway, then low level expression from *Pl-Bh* might lead to the production of suboptimal numbers of PL protein molecules for full activation of the anthocyanin target genes. This "threshold effect" of *Pl* expression in turn could account for the cell-to-cell variability in anthocyanin synthesis characteristic of cells within pigmented sectors. DNA methylation could also explain the activation of *Pl-Bh* expression in the kernel, an organ not pigmented by most other *Pl* alleles. Because methylation has been shown to prevent the association of some DNA-binding proteins with their target sequences (INAMDAR, EHRLICH and EHRLICH 1991), it is possible that methylation of *Pl-Bh* interferes with the binding of some repressor that normally prevents expression of *Pl* in the kernel. Alternatively, methylation could activate a cryptic kernel-specific enhancer, thus resulting in *Pl-Bh* expression in the aleurone.

According to this model, *Pl-Bh* can be considered as a wild-type *Pl* gene whose expression is modified by the tightly linked *cis*-acting CM. Potential evidence for the existence of such a controlling sequence can be gained from reconsidering the results of earlier genetic mapping experiments. RHOADES (1948) mapped *Bh* as a distinct gene located 1 cM distal to *Pl*). Since our data argue that *Bh* is actually an allele of *Pl*, the mapping results could be reinterpreted to suggest that the *cis*-linked "gene" RHOADES called *Bh* is actually the CM. His mapping experiment was performed by crossing a tester line homozygous for recessive *bh* and *pl* alleles (colorless kernel and plant) with a line carrying *Bh Pl* (blotchy kernel and pigmented plant). Based on our studies of plants descended from RHOADES' *Bh Pl* stock, we believe that the *Bh Pl* plants in this cross did not carry a dominant *Pl* allele; rather the plant pigmentation (probably blotchy) was caused by *Pl-Bh*. In view of this idea, we cannot be sure what the genotype of RHOADES' recombinant classes (non-blotchy kernel, purple plant and blotchy kernel, non-blotchy plant) actually were. Apparently no progeny tests were performed to verify the genotype of these recombinants. Therefore, the recombinants could have represented misclassifications. An alternative hypothesis is that the recombinants did exhibit a phenotypic combination different from the parental lines, perhaps as a result of recombination between the proposed CM and the *Pl-Bh* gene. By our model, unlinking the CM from *Pl-Bh*

would produce a wild-type phenotype (non-blotched kernel, purple plant) identical to that of one of RHOADES' recombinant classes. Clearly, the resolution of this paradox will depend on the results of further experiments using both molecular and genetic methods to detect recombination between *Pl-Bh* and any *cis*-linked regulatory sequences that control its expression.

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