Paramutation Alters Regulatory Control of the Maize *pl* Locus

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ABSTRACT

The maize *purple plant* (*pl*) locus encodes a transcription factor required for anthocyanin pigment synthesis in vegetative and floral tissues. The strongly expressed *Pl-Rhoades* (*Pl-Rh*) allele is unstable, spontaneously changing to weaker expression states (*Pl′*) at low frequencies and exclusively changing to *Pl′* in *Pl′/Pl-Rh* heterozygotes. The weakly expressed *Pl′* state is mitotically and meiotically stable, yet reversible. This type of allele-dependent, heritable alteration of gene control is called paramutation. Expression studies herein demonstrate that visible differences in anthocyanin pigment levels mirror *pl* RNA abundance and that *pl* paramutation is associated with reduced transcription of the *pl* gene. This transcriptional alteration is accompanied by acquisition of light-dependent regulation. Restriction endonuclease mapping indicates that these changes in *pl* gene regulation are not associated with detectable DNA alterations or with extensive changes in cytosine methylation patterns. Genetic tests show that *Pl-Blotched* (*Pl-Bh*), a structurally similar *pl* allele encoding an identical *pl* RNA and PL protein, does not participate in *pl* paramutation. This result suggests that if *cis*-acting sequences are required for *pl* paramutation they are distinct from the protein coding and immediately adjacent regions. A model is discussed in which *pl* paramutation results in heritable changes of chromatin structure that fundamentally alter regulatory interactions occurring during plant development.

PARAMUTATION describes a process whereby expression of one allele is heritably altered by another (Hollick *et al.* 1997). The best-characterized examples of paramutation interactions occur in *Zea mays* between alleles of three genes encoding transcriptional regulators of the anthocyanin pigment biosynthetic pathway (Brink 1956; Coe 1966; Hollick *et al.* 1995). The *r* (*red*) and *b* (*booster*) loci encode functionally duplicate basic helix-loop-helix (b-HLH) proteins (Ludwig *et al.* 1989; Goff *et al.* 1990; Radicella *et al.* 1991) and the *pl* locus encodes a myb-like protein (Cone *et al.* 1993a). In plant tissues, purple anthocyanin pigmentation requires combined action of PL and one or the other b-HLH factors encoded by either *r* or *b* (reviewed in Dooner *et al.* 1991; Goff *et al.* 1992).

Alleles susceptible to changes in gene expression by allelic interactions are termed "paramutable" while alleles that promote this instability are termed "paramutagenic." Alleles that are neither paramutable nor paramutagenic are termed "neutral." Most paramutagenic *r* alleles are structurally complex and clearly different from paramutable *r* alleles (Eggleston *et al.* 1995; Kermicle *et al.* 1995). In contrast, paramutagenic alleles of the *b* and *pl* loci consist of single coding regions and they arise spontaneously from corresponding paramutable alleles (Coe 1966; Patterson *et al.* 1993; Hollick *et al.* 1995).

In addition to structural differences between paramutable and paramutagenic alleles of the *r*, *b*, and *pl* loci, specific details of spontaneous paramutation, paramutagenic strength, and stability of the paramutant state have led to considerable debate about whether or not these examples are mechanistically related (Coe 1966; Brink 1973; Hollick *et al.* 1995). For instance, the paramutable *B-Intense* (*B-I*) and *Pl-Rhoades* (*Pl-Rh*) alleles can spontaneously change to weaker expression states (*B′* and *Pl′*) that are strongly paramutagenic; 100% of the alleles transmitted from *B-I/B′* and *Pl-Rh/Pl′* individuals are *B′* or *Pl′*, respectively (Coe 1966; Hollick *et al.* 1995). In contrast, the paramutable *R-r:standard* (*R-r*) allele is relatively stable; its expression is strongly reduced only following exposure to strongly paramutagenic, structurally distinct *r* alleles such as *R-stippled* (*R-st*) or *R-marbled* (*R-mb*; Brink 1956; Brink and Weyers 1957). Paramutant *R-r′* is only weakly paramutagenic; it is weakly effective in causing reduced expression of another paramutable *R-r* allele in a *R-r/R-r′* heterozygote (Brown and Brink 1960).

In contrast to the differences listed above between *pl* and *r* paramutation, the two examples share the ability of paramutant *Pl′* and *R-r′* to revert to fully active, non-

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paramutagenic *Pl-Rh* and *R-r* alleles. Paramutant *B′* alleles are extremely stable and have never been observed to change back to a fully active, nonparamutagenic state (Coe 1966; Patterson *et al.* 1995; G. Patterson, K. Kubo, J. Dorweiler, J. Hollick and V. Chandler, unpublished observations). Both *Pl′* and *R-r′* can show increased pigment and change back to *Pl-Rh* and *R-r* in subsequent generations if they are carried heterozygous with neutral *pl* or *r* alleles, respectively (Styles and Brink 1966; Hollick and Chandler 1998). A further similarity between *pl* and *r* is that the amount of pigment expressed is inversely related to paramutagenic strength; weaker pigmenting states are more paramutagenic (Styles 1967; Hollick *et al.* 1995). Both *Pl′* and *R-r′* alleles can exist in quantitatively distinct expression states, and in the *Pl′* case these expression states appear to be fixed early in development and maintained throughout somatic development (Chandler *et al.* 1996).

Given the phenomenological differences and similarities between these various examples of maize paramutation it remains an open question as to whether they share a common molecular mechanism. Somehow, interacting alleles recognize each other and effect some type of regulatory change. Following this initial establishment event, the altered allele is maintained in a repressed expression state by a mitotically and meiotically heritable mechanism. Cytosine methylation is arguably the best-characterized example of a heritable epigenetic change. However, meiotic inheritance of epigenetic information in organisms lacking cytosine methylation (*Schizosaccharomyces pombe* and *Drosophila melanogaster*) is clearly due to a distinct mechanism, hypothesized to be chromatin based (Grewal and Klar 1996; Cavalli and Paro 1998). Increased cytosine methylation is correlated with decreased expression of paramutant *r* alleles (Walker 1998) and also paramutation-like transcriptional silencing of certain transgenes in *Petunia hybrida* (Meyer *et al.* 1993) and *Nicotiana tobacum* (Park *et al.* 1996). In sharp contrast, one of the strongest repressions of transcriptional activity, that occurring in paramutant *B′*, is not associated with changes in cytosine methylation within the 5′ protein coding region nor within 9 kb of the 5′ flanking region (Patterson *et al.* 1993).

In this article, we examine the structure, cytosine methylation profile, and transcription of *pl* alleles undergoing paramutation. These experiments reveal additional molecular parallels between *b* and *pl* paramutation and indicate that *pl* paramutation results in light-responsive regulation of the normally light-independent *Pl-Rh* allele. Despite extensive DNA sequence identity, we find that the epigenetically regulated *Pl-Blotched* (*Pl-Bh*) allele (Cocciolone and Cone 1993; Hoekenga 1998) does not participate in *pl* paramutation consistent with the interpretation that critical *cis*-acting sequences are not within the coding and immediate flanking regions.

MATERIALS AND METHODS

Genetic stocks: The *Pl-Rhoades* (herein designated *Pl-Rh*) and *Pl′* (previously described as *Pl′-mahogany*; *Pl′-mah*) alleles were maintained in a W23 inbred background and in four other genetic backgrounds of mixed parentage. The *Pl′* allele originally isolated as a spontaneous derivative of the *Pl-Rh* allele (Hollick *et al.* 1995) was used for the initial set of *in vitro* transcription assays, sunlight exposure experiments, DNA comparisons, and *Pl-Bh* paramutation tests. Additional *Pl′* alleles obtained from spontaneous paramutation of *Pl-Rh* alleles were used for all subsequent experiments. Anthocyanin and RNA extractions from anthers were carried out with a single family of W23 material displaying a high frequency of spontaneous paramutation. Material for *in vitro* transcription experiments was produced by backcrossing related *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* plants to *B-I*/*B-I*; *Pl-Rh*/*Pl-Rh*; *R-r*/*R-r* testers. Material for sunlight exposure experiments and restriction endonuclease comparisons was obtained from *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* plants derived by crosses of *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* plants to a common *Pl-Rh*/*Pl-Rh* stock. The *pl-sunred* (*pl-sr*) stock used for the *Pl-Bh* paramutagenicity tests has been previously described (Hollick *et al.* 1995). The *Pl-Bh* stocks were provided by the Maize Genetics Cooperation Stock Center, accession no. 607A (Urbana, IL) and by Karen Cone (University of Missouri, Columbia). Material for the *r* paramutation tests was in either K55 or K55/W23 hybrid backgrounds. Unless otherwise stated, all stocks were also homozygous for the *B-I* allele and a functional allele of the *r* locus required for anther pigmentation (*R-r* or *r-r*).

Anthocyanin measurements: For each measurement, a total of 12 freshly extruded anthers (3 from each of four primary florets) for each anther color score (ACS) were ground in 1-ml aliquots of 70% ethanol, 1% HCl solution in 1.5-ml microfuge tubes using disposable plastic pestles (Kontes). Absorbance at 530 nm was measured for extracts from ACS 1–4 and dilutions (1:10) of extracts from ACS 5–7. The number of plants sampled for each ACS is in parentheses: ACS1 (2); 2 (3); 3 (3); 4 (2); 5 (3); 6 (2); 7 (2).

DNA materials: DNA was isolated from immature cobs and purified by CsCl-density centrifugation (Rivin *et al.* 1982). Most of the maize clones (Patterson *et al.* 1993) and the 1.1-kb *Xho*I, *pl*-specific 3′ clone have been previously described (Cocciolone and Cone 1993). The 5′ *pl*-specific clone (pJH1) is a 850-bp *Hin*dIII subclone from a *Pl-Rh* genomic clone (Cone *et al.* 1993a). The *pl*-specific clone (pJH7) used for making radiolabeled RNA transcripts is a subclone of 5′ sequences from a *Pl-Rh* cDNA clone with *Eco*RI linkers (pPlcDNA) provided by Karen Cone. The 5′-most 505-bp *Eco*RI-*Bgl*I fragment from pPlcDNA was first treated with T4 DNA polymerase to generate blunt ends and then cloned into the *Sma*I site of pBluescriptII SK+ (Stratagene, La Jolla, CA) to generate pJH7. The *Bgl*I site used in the pPlcDNA subcloning lies between the third exon *Nae*I and *Nci*I sites listed in Figure 5. Two different *pl* clones gave similar relative hybridization values for the *in vitro* transcription assays; pPlcDNA was used in the first two experiments and pJH7 was used for the last three experiments.

RNA measurements: Anther RNA was prepared using Trizol (GIBCO-BRL, Gaithersburg, MD) reagent according to manufacturer protocols except that initial tissue homogenization was carried out in Trizol reagent in microfuge tubes using disposable plastic pestles and a small amount of washed sand (Sigma, St. Louis). A total of 12 anthers were used for each

individual RNA sample. RNA was prepared from husk tissues for RNase-protection experiments using Trizol reagent according to manufacturer protocols.

Husk tissues were prepared from mature ears at silk emergence as follows: (1) ears were transported to the laboratory, (2) the prophyllum and outer two husk leaves were removed, (3) the bottom of the ear from the base of the cob to the tip of the shank was cut off, and (4) the cob and silks were removed. Following this ear preparation, all the husk leaves or individual husk leaves were immersed in liquid nitrogen and ground to a fine powder with a mortar and pestle. RNA samples were obtained with Trizol extractions from 1-g quantities of homogenized husk leaf powder. *pl* RNA was quantified using RNase-protection analysis (Gilman 1993) as follows. *In vitro* transcription reactions with T3 RNA polymerase were used to generate ^{32}P-UTP-labeled RNA transcripts of both *pl* (pJH7 linearized with *Xba*I) and maize *actin* (pMAc1 linearized with *Eco*RI). Bulk RNA (5 μg/sample) was hybridized with 10^5 cpm of each radiolabeled transcript overnight at 42° and then digested for 30 min with 60 units RNase T1 (GIBCO-BRL). Digestion products were separated by electrophoresis using 12% polyacrylamide gels. Protected RNA fragments were quantified using a Phosphoimager (Research Dynamics). *pl* RNA levels were normalized to the most prevalent *actin* RNA protected fragment.

Northern blot hybridizations with total RNA were performed as described (Cocciolone and Cone 1993) using the indicated ^{32}P-labeled DNA clones as hybridization probes. Hybridization results were quantified using an AMBIS (San Diego) radioactive detection system.

***In vitro* transcription assays:** Nuclei isolations and transcription assays were performed as previously described (Cone *et al.* 1993a). Husk samples were prepared from mature ears as described in the RNA measurements section. Five separate experiments were conducted. In the first two experiments, single ears were used for each nuclei preparation. In subsequent experiments, three ears of each genotype were pooled for each individual nuclei preparation. In the third experiment, *pl*, *bz1*, and *ubiquitin* (*uq*) transcription rates were measured. In the last two experiments, *pl* and *uq* transcription rates were measured.

To obtain nuclei and RNA from the same samples, husk tissue was homogenized in a blender, an aliquot of the liquid from the blended material was removed, and RNA was isolated by a guanidinium thiocyanate method (Chirgwin *et al.* 1979). Nuclei were fractionated using a discontinuous Percoll/Hexylene Glycol step gradient (Cone *et al.* 1993a). Approximately 1×10^7 nuclei were used per ^{32}P-UTP labeling reactions. The amount of hybridization was measured by quantification of radioactivity using either the AMBIS radioactive detection system for the first experiment or Phosphoimager (Research Dynamics) for all subsequent experiments. Hybridization to *uq* was used to control for differences in efficiency of each transcription reaction, and hybridization to plasmid vectors ptz19 or pBS (SK+) was used as a control for background hybridization. Quantification was based on the intensity of hybridization of the labeled RNA to the DNA clone of interest, subtracting the value for hybridization to the plasmid controls, and dividing by the value for the *uq* clone subtracted for the plasmid control.

Light treatments: The first three outer husk leaves were removed from mature *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* ears to expose the fourth husk leaf. Explant ears were enclosed in plastic wrap and placed in a shallow baking dish such that the shank was immersed in water during the treatment. Light exposure was 48 hr on an 18-hr light, 6-hr dark regimen at 166 microeinsteins per square meter per second (Biospherical Instruments Inc., San Diego, model QSL-100). RNA was isolated from the exposed or masked husk leaves using a guanidinium thiocyanate procedure (Chirgwin *et al.* 1979). Northern blot analysis was carried out as previously described (Patterson *et al.* 1993) using pPlcDNA as a probe.

DNA gel blot analysis: DNA gel blot analysis and hybridization were performed as described (Patterson *et al.* 1993) except that hybridization signals were assessed by visual inspection. Single enzyme digests of *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* genomic DNA were compared with gel blot analysis using the 3′ 1.1-kb and 5′ pJH1 *pl*-specific fragments sequentially as probes. Digests of a 15-kb *Pl-Rh* genomic clone (Cone *et al.* 1993a) were also included for each enzyme for reference and mapping purposes. The following enzymes were tested: ***Acc*I**, *Ava*I, *Bgl*II, ***Bst*NI**, *Dde*I, *Eag*I, *Eco*RII, ***Hin*dIII**, *Hin*fI, *Hpa*II, *Msp*I, *Nae*I, *Nci*I, *Nco*I, ***Nsi*I**, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sau*96I, *Xho*I. Enzymes listed in boldface type are not cytosine methylation sensitive. Both *Bgl*II and *Dde*I are context specific in their methylation sensitivity. We used *Pl-Rh* sequence information (Cone *et al.* 1993b), restriction map information obtained during subcloning experiments, and gel blot hybridization results for the 15-kb *Pl-Rh* reference clone to map 36 unique sites tested by our analyses. The estimated number of unmapped sites tested is based on the results of the gel blot hybridization for the 15-kb *Pl-Rh* reference clone. We assumed that $n + 1$ restriction sites were tested for n number of hybridizing fragments identified from the *Pl-Rh* reference clone. Based on fragment sizes, some of the unmapped restriction sites were potentially identified by both the 5′ and 3′ probes; in these cases we conservatively assumed that only one shared site was tested rather than two independent sites. The following list summarizes restriction fragment sizes in kilobases identified in both *Pl-Rh* and *Pl′* samples by the various enzymes with the 5′ and 3′ hybridization probes: *Acc*I 5′ (3.9); *Acc*I 3′ (1.2, 0.6); *Ava*I 5′ (20, 15, 12, 8.3); *Ava*I 3′ (1.1); *Bgl*II 5′ (7.9, 2.4); *Bgl*II 3′ (8, 7, 2.6); *Bst*NI 5′ (6, 0.8); *Bst*NI 3′ (1.8, 1.2); *Dde*I 5′ (>12); *Dde*I 3′ (6.6, 4, 2.1); *Eag*I 5′ (>12); *Eag*I 3′ (1.1); *Eco*RII 5′ (16, 12); *Eco*RII 3′ (16, 12, 7, 3.5, 1.8, 1.2); *Hin*dIII 5′ (0.85); *Hin*dIII 3′ (12); *Hin*fI 5′ (0.35, 0.3); *Hin*fI 3′ (2.8); *Hpa*II 5′ (>23); *Hpa*II 3′ (10, 7.5, 3.2, 1.8, 1.6); *Msp*I 5′ (17, 9.9, 5.9, 5.3); *Msp*I 3′ (10, 7.5, 3.2, 1.8, 1.6); *Nae*I 5′ (18); *Nae*I 3′ (8.5); *Nci*I 5′ (>23); *Nci*I 3′ (>23, 11); *Nco*I 5′ (5.3); *Nco*I 3′ (9.7); *Nsi*I 5′ (2.4, 1.7); *Nsi*I 3′ (3.3, 1.1); *Pst*I 5′ (∼35); *Pst*I 3′ (>40, ∼25, 1.2); *Pvu*I 5′ (>23); *Pvu*I 3′ (>23); *Pvu*II 3′ (14, 11.5, 8.2, 5.1, 5, 2.6, 2.5); *Sal*I 5′ (>15); *Sal*I 3′ (>23); *Sau*96I 5′ (2.1); *Sau*96I 3′ (4.8, 1.8, 0.9); *Xho*I 5′ (17, 14, 12, 7.6, 6.2, 6, 2.2, 1.8). Additional details of these analyses are available upon request.

RESULTS

Differences in anther pigment mirror quantitative differences in anthocyanins and *pl* RNA: Based on pigment phenotypes, the *Pl-Rh* allele is strongly and uniformly expressed in seedling leaf sheaths, aboveground prop roots, leaf sheath and auricle, husks, culm, glumes, and anthers of the aerial sporophyte (Coe *et al.* 1988; Hollick *et al.* 1995). Previous experiments show that *pl* RNA is produced from the *Pl-Rh* allele in mature husks and in both shoots and roots of 7- to 8-day-old germinated seedlings (Cocciolone and Cone 1993; Cone *et al.* 1993b). All plant tissues show reduced pigmentation when *Pl-Rh* changes to *Pl′* (Hollick *et al.* 1995). In mature leaf sheath, husks, and culm, there appears to be a homogeneous reduction of pigment in all epidermal

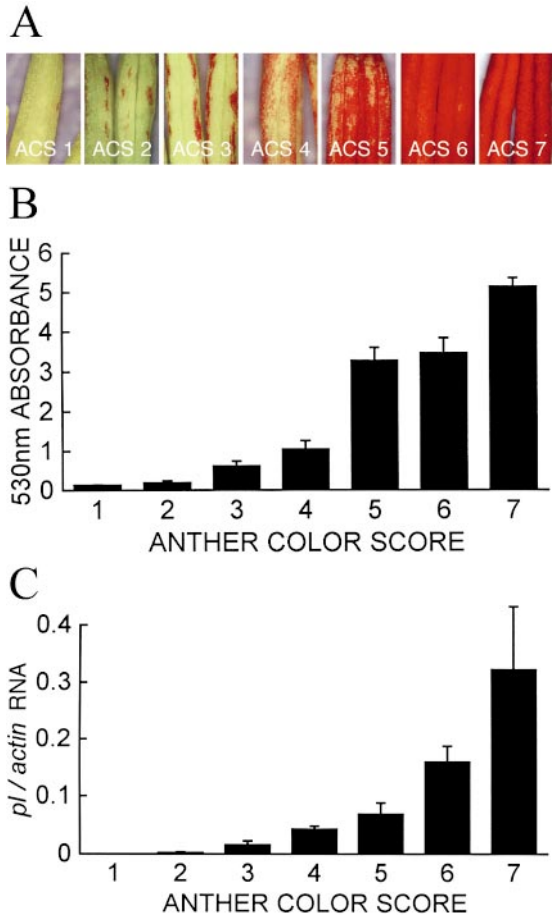

Figure 1.—Quantitative states of *Pl-Rh* and *Pl′* expression in mature anthers. (A) Close-up views of individual anthers representing each anther color score (ACS). (B) Anthocyanin quantification, ± standard error, measured by absorbance at 530 nm for ACS 1–7 extracts. (C) RNase-protection quantification, ± standard error, of *pl* RNA relative to *actin* RNA levels for ACS 1–7 anthers. The numbers of anther samples measured for each ACS are in parentheses: ACS 1, (3); ACS 2, (3); ACS 3, (3); ACS 4, (2); ACS 5, (3); ACS 6, (2); ACS 7, (2).

cells. Anther pigmentation, however, is reduced in a variegated manner. A 1–7 graded ACS has been used to quantify pigment levels conferred by *Pl-Rh* or weakly expressed derivatives (Figure 1A). In plants with an ACS 1, all the anthers of the tassel (several thousand anthers per tassel) are weakly pigmented and this pigment is restricted to a few longitudinally arranged cells of the epidermis. The ACS 2 and ACS 3 classes have progressively more pigmented cells still confined to the epidermis but concentrated in specific regions of the anther lobes. The ACS 4 class has more pigmented cells, including a few of the transversely oriented subepidermal cells. The ACS 5 and ACS 6 classes have progressively more pigmented subepidermal cells. All epidermal and subepidermal cells of the anther produce pigment in the ACS 7 class.

The amount of anthocyanin pigments extracted and measured from anthers directly correlates with these phenotypic classes (Figure 1B) and the number of pigmented anther cells. To test whether the pigment differences reflect differences in *pl* RNA abundance, *pl* RNA levels were quantified using RNase protection analysis with total anther RNA (Figure 1C). Because *pl* paramutation does not appear to affect the expression of the *actin* gene, *actin* RNA levels were used to normalize *pl* RNA levels. Results show that *pl* RNA is undetectable in ACS 1 anther samples yet increases proportionally as the amount of pigment increases. *Pl′* plants with an ACS of ≤3 were used in all subsequent experiments.

***pl* paramutation affects transcriptional activity:** Although exterior husk leaves of *Pl′/Pl-Rh* ears are only slightly less pigmented than those of *Pl-Rh/Pl-Rh* ears (Figure 2A), interior husk leaves of *Pl′/Pl-Rh* ears are virtually devoid of pigment. In contrast, all interior husk leaves of *Pl-Rh/Pl-Rh* ears can be strongly pigmented. In whole husk samples (all husk leaves except the two most exterior; see materials and methods), RNase-protection experiments showed that *pl* RNA levels are ~100-fold higher in *Pl-Rh/Pl-Rh vs. Pl′/Pl-Rh* husks (Figure 2B). There was no indication of novel-sized *pl* RNA transcripts in *Pl′/Pl-Rh* RNA samples by either RNase-protection (Figure 2B) or Northern blot hybridizations (Patterson 1993). Differences in RNA abundance could be due to changes in transcription rate, changes in RNA stability, or both. To address this issue, we used *in vitro* transcription assays with isolated husk nuclei to measure relative *pl* transcription rates in *Pl-Rh/Pl-Rh* and *Pl′/Pl-Rh* material. We also measured relative transcription rates for *b* (the other transcriptional activator required for husk pigmentation), *a1*, *c2*, and *bz1* (genes encoding enzymes required for anthocyanin biosynthesis that are transcriptionally regulated by B and PL proteins), and *light-harvesting chlorophyll a/b-protein* (*lhcp*; a phytochrome-regulated gene not involved with anthocyanin biosynthesis). In every experiment, hybridization values were normalized to maize *ubiquitin* levels. Results of these experiments (Figure 2, C and D) show that *pl* paramutation correlates with a 3-fold reduction of *pl* transcription while transcription of the *b* allele (*B-I*) remains constant. Transcription rates of the *a1*, *c2*, and *bz1* genes were decreased while the transcription rate of *lhcp* increased 2-fold. Thus *pl* paramutation results in decreased transcriptional activity of the *pl* gene averaged among all interior husk leaves.

Comparisons of transcription rates with RNA levels: To directly compare transcription rates with RNA levels, total RNA was isolated from aliquots of the initial husk homogenates used to isolate the nuclei used in the first *in vitro* transcription experiment. The *pl*, *a1*, *c2*, and *actin* RNA levels were measured using Northern blot hybridizations (Figure 3A). The predominant *pl* transcript is 1.2 kb and an additional low-abundance 2-kb RNA species is also seen in *Pl-Rh/Pl-Rh* RNA samples and in *Pl′/Pl-Rh* RNA samples on longer exposures (not shown). The abundance of both RNA species changes

in concert upon *pl* paramutation. The primary *pl* coding region should produce a 1-kb transcript (Cone *et al.* 1993a). There is a 1.1-kb direct duplication of 3′ coding and flanking sequences found in all *pl* alleles characterized to date (Cone *et al.* 1993b) that could account for the longer *pl* transcripts through improper transcriptional termination and/or alternate splicing. Northern blot hybridizations were quantified and normalized to *actin* RNA levels. Comparisons between *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* samples (Figure 3B) show *pl* RNA is 18.6-fold higher in *Pl-Rh*/*Pl-Rh vs. Pl′*/*Pl-Rh* husks, *a1* RNA is 17.3-fold higher, *c2* RNA is 14.6-fold higher, and *bz1* RNA is 13-fold higher. Although trends are consistent, in no cases do absolute differences in transcription rates match the differences in RNA levels: *pl*, 3 *vs.* 18.6; *a1*, 11 *vs.* 17.3; *c2*, 7 *vs.* 14.6; and *bz1*, 4 *vs.* 13.

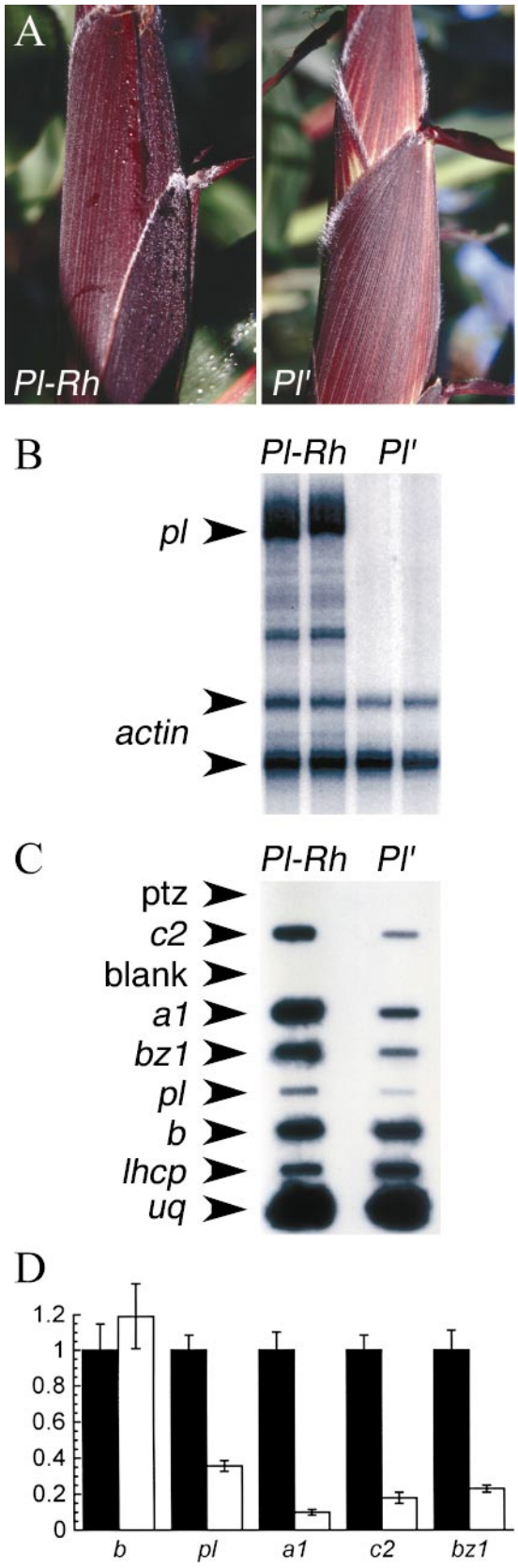

Figure 2.—*Pl-Rh vs. Pl′* expression in mature husk tissues. (A) Comparison of darkly colored *B-I*/*B-I*; *Pl-Rh*/*Pl-Rh* (*Pl-Rh*) ears and mahogany-colored *B-I*/*B-I*; *Pl′*/*Pl-Rh* (*Pl′*) ears. (B) RNase-protection comparison of *pl* and *actin* RNA levels from husks of *B-I*/*B-I*; *Pl-Rh*/*Pl-Rh* (*Pl-Rh*) and *B-I*/*B-I*; *Pl′*/*Pl-Rh* (*Pl′*) ears. (C) *In vitro* transcription data comparing relative transcription rates of *c2*, *a1*, *bz1*, *pl*, *b*, *lhcp*, and *ubiquitin* (*uq*) genes in *B-I*/*B-I*; *Pl-Rh*/*Pl-Rh* (*Pl-Rh*) and *B-I*/*B-I*; *Pl′*/*Pl-Rh* (*Pl′*) husk tissues. ptz (ptz19) is a plasmid control. (D) Results of five separate *in vitro* transcription experiments. Relative transcription rates for the various genes are compared between *Pl-Rh*/*Pl-Rh* (solid bars) and *Pl′*/*Pl-Rh* (open bars) samples. For each gene, expression values, ± standard error, are normalized to *ubiquitin* levels and then represented as a fraction of expression seen in *Pl-Rh*/*Pl-Rh* samples (set to unit value). The number of individual nuclei samples tested for each gene is as follows: *b*, (*Pl-Rh* 9, *Pl′* 9); *pl*, (*Pl-Rh* 17, *Pl′* 15); *a1*, (*Pl-Rh* 9, *Pl′* 9); *c2*, (*Pl-Rh* 9, *Pl′* 9); *bz1*, (*Pl-Rh* 12, *Pl′* 12).

_pl_ RNA expression patterns are different between _Pl-Rh_ and _Pl′_ ears: Both nuclei for *in vitro* transcription assays and RNA for quantification were purified from homogenates of ~8–12 husk leaves of a given ear. Because each husk leaf is distinct in both developmental age and in its proximity to the exterior of the ear we wondered if *pl* RNA expression patterns were similar in both *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* ears. To address this issue, we fractionated husk samples by individual leaves and measured *pl* RNA levels by quantifying results of an RNase-protection analysis (Figure 3C). When normalized to *actin* RNA levels, *pl* RNA levels in the oldest and most exterior *Pl-Rh*/*Pl-Rh* husk leaf tested were only 2.4-fold higher than the level found in the most interior leaf tested. In contrast, *pl* RNA levels between the same leaves of *Pl′*/*Pl-Rh* husks varied 12-fold. Correspondingly, the differences between *pl* RNA levels in *Pl-Rh*/*Pl-Rh vs. Pl′*/*Pl-Rh* husk leaves are smallest in exterior leaves (22-fold) and greatest in interior leaves (105-fold). Thus *pl* RNA is highly expressed in all *Pl-Rh*/*Pl-Rh* husk leaves while *pl* RNA expression is confined to more exterior *Pl′*/*Pl-Rh* husk leaves.

_pl_ paramutation affects the response to light signals: Because *pl* RNA was preferentially found in the most exterior husk leaves of *Pl′*/*Pl-Rh* ears, we wondered if *pl* RNA expression was light dependent. Most *pl* alleles

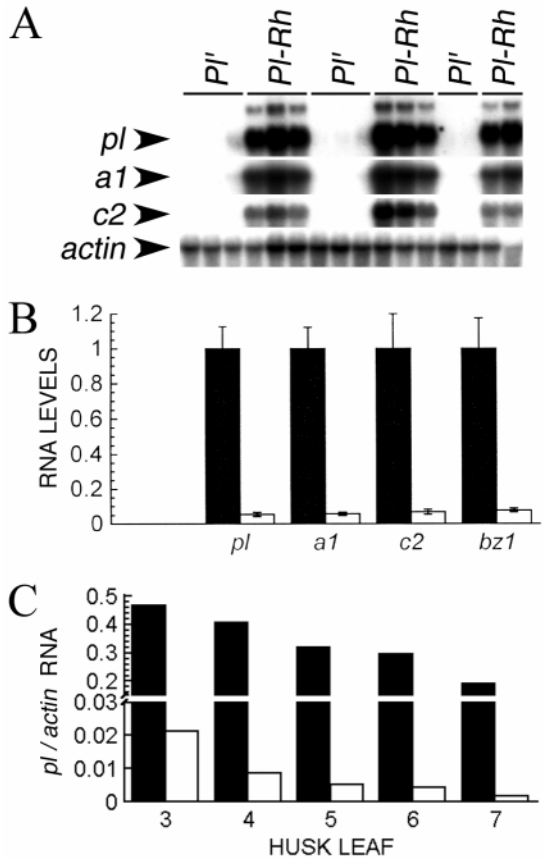

Figure 3.—Comparisons of *Pl-Rh* and *Pl′* expression in mature husk tissues. (A) Northern blot analysis of *pl*, *a1*, *c2*, and *actin* RNA levels from *Pl-Rh*/*Pl-Rh* (*Pl-Rh*) and *Pl′*/*Pl-Rh* (*Pl′*) husk extracts used for the first *in vitro* transcription experiment. (B) Quantification of RNA from the eight husk homogenates used for the first *in vitro* transcription experiment. RNA levels are compared between *Pl-Rh*/*Pl-Rh* (solid bars) and *Pl′*/*Pl-Rh* (open bars) samples. For each gene, expression values, ± standard error, are normalized to *actin* levels and then represented as a fraction of the expression seen in *Pl-Rh*/*Pl-Rh* samples (set to unit value). (C) Heterogeneity in *pl* RNA levels seen in husk samples. RNase-protection assays were used to quantify the amount of *pl* RNA found in individual husk leaves of *Pl-Rh*/*Pl-Rh* (solid bars) and *Pl′*/*Pl-Rh* (open bars) ears. Values of *pl* RNA are given relative to *actin* RNA for husk leaves 3–7 where the third leaf is the oldest and most exterior leaf. *y*-axis measurements representing two different scales are divided at 0.03 to emphasize differences among the *Pl′*/*Pl-Rh* leaf samples.

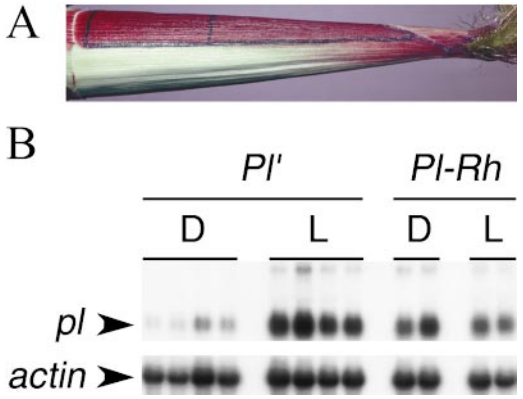

Figure 4.—The *Pl′* allele is light responsive. (A) Example of a *B-I*/*B-I*; *Pl′*/*Pl-Rh* ear where half of the fourth husk leaf has been exposed for two cycles of 18 hr light (L)/6 hr dark (D); the unpigmented section of the ear was covered by tin foil during the exposure. (B) Northern blot analysis of *pl* and *actin* RNA levels from the fourth husk leaf of *Pl-Rh*/*Pl-Rh* (*Pl-Rh*) and *Pl′*/*Pl-Rh* (*Pl′*) explant ears receiving two cycles of 18L/6D either fully shielded (D) or fully exposed (L).

are termed "sun red" as their expression is dependent on, or augmented by, light. Consistent with this regulation, canonical light-responsive elements are found in the immediate 5′ region of all sun-red *pl* alleles examined to date (Cone *et al.* 1993b). The *Pl-Rh* allele is structurally distinct from the sun red class and does not have this light-responsive element in the same promoter-proximal position. In addition, *pl* RNA measurements in dark-grown seedlings show that the *Pl-Rh* allele does not require light signals for *pl* RNA accumulation (Cone *et al.* 1993b). We tested whether expression of *Pl′* alleles required light signals by comparing the pigmentation occurring when *Pl′* explant ears are exposed or masked to light. In the first set of experiments, outer husk leaves from *Pl′*/*Pl-Rh* ears were removed to reveal the fourth husk leaf and then half of the ear was covered with a strip of tin foil held in place by enclosing the ear in transparent plastic wrap. After 48 hr (two 18-hr periods of light), the foil was removed and the ear was photographed (Figure 4A). Exposed regions of *Pl′*/*Pl-Rh* ears responded by producing anthocyanin pigments while the masked halves remained unpigmented. We next used Northern blot hybridizations to measure light-dependent *pl* RNA accumulation in *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* explant ears. In the absence of light (fully masked ears), *pl* RNA from the fourth husk leaf is much greater in *Pl-Rh*/*Pl-Rh* than *Pl′*/*Pl-Rh* plants (Figure 4B). However, *pl* RNA levels increased in *Pl′*/*Pl-Rh* husks following 36 hr of light exposure while *pl* RNA levels remained relatively unchanged in *Pl-Rh*/*Pl-Rh* husks following the same treatment (Figure 4B). Thus paramutation fundamentally alters the regulation of the *Pl-Rh* allele such that RNA accumulation in the *Pl′* state is responsive to light signals. It should be noted that even though the *Pl′* allele is light responsive in husk tissues, it does not display anther pigment patterns typical of sun-red *pl* alleles (Coe *et al.* 1988; Hollick and Chandler 1998).

***pl* paramutation is not associated with global changes in DNA sequence or cytosine methylation:** We carried out comparative genomic Southern blot hybridizations to determine if DNA sequence/organization or DNA methylation was different between the strongly expressed *Pl-Rh* and the light-dependent *Pl′* allele. We used 21 enzymes for this analysis and 17/21 enzymes were methylation sensitive. Two *pl*-specific genomic frag-

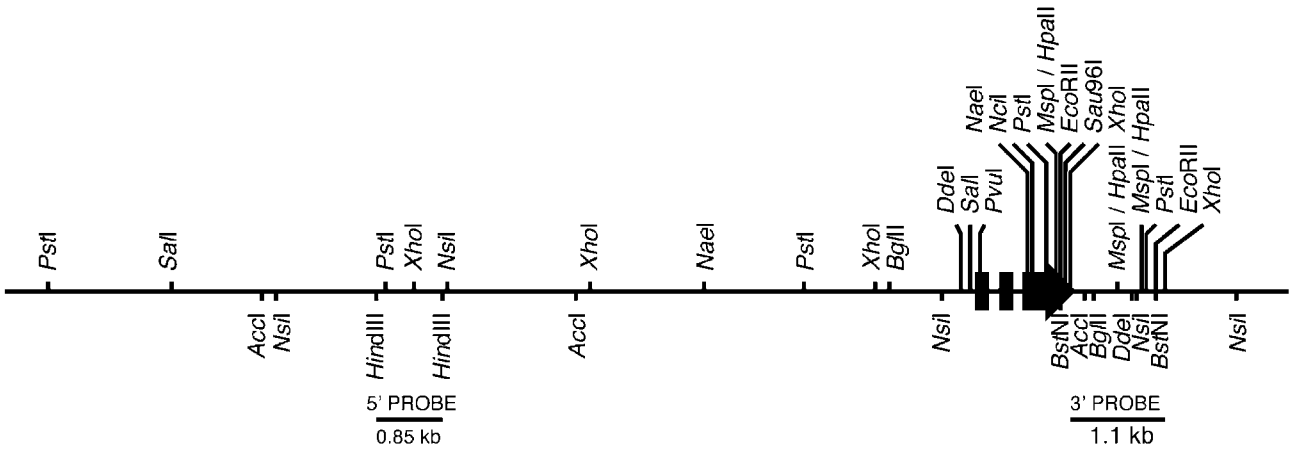

Figure 5.—*Pl-Rh* and *Pl′* alleles are structurally similar. Genomic restriction map shared between *Pl-Rh* and *Pl′* alleles is illustrated. The three *pl* coding region exons and direction of transcription are indicated with solid boxes and the arrowhead. Positions of the 850-bp 5′ and 1.1-kb 3′ probes used in hybridization reactions are indicated below the map. The 36 sites listed represent mapped restriction endonuclease sites examined in our analysis. At least 58 additional sites tested by our analysis are not indicated since they could not be unambiguously mapped. Methylation-sensitive enzyme recognition sites are listed above the map and methylation-insensitive enzyme sites are listed below the map. See materials and methods for additional details.

ments were used in the analyses; one is nearly 6 kb upstream of the predicted *Pl-Rh* transcription start site and the other is at the 3′ end of the *pl* coding sequence. Much of the sequences 5′ of the *Pl-Rh* protein coding region are highly repetitive within the maize genome (Cone *et al.* 1993b) but the 5′ fragment used in these hybridization analyses represents a unique sequence.

DNA samples isolated from immature cobs of a single *Pl-Rh*/*Pl-Rh* plant and a single *Pl′*/*Pl-Rh* plant were compared using single-enzyme digests with both 5′ and 3′ hybridization probes. In every experiment, the pattern and hybridization intensity of digested DNA fragments was identical between the two samples. Comparative digests using the isoschizomer pairs *Msp*I/*Hpa*II and *Eco*RII/*Bst*NI indicated complete cytosine methylation of those sites tested by the 5′ hybridization probe yet relatively little methylation of those sites tested near the 3′ end of the *pl* coding region. Although moderate to extensive cytosine methylation was detected throughout the genomic region examined in our analyses, there were no differences between *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* samples. We conservatively estimate that our analysis tested 94 unique restriction sites and that 73 of these were tested by methylation-sensitive enzymes (see materials and methods). These restriction sites collectively encompass ~15 kb of the *pl* genomic region, 12 kb 5′ and 2 kb 3′ of the *pl* coding sequence. Restriction sites that could be unambiguously positioned on the physical map are indicated in Figure 5. We also found no methylation differences between *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* samples at the 3′ *Hpa*II sites previously shown to be differentially methylated between the strongly expressed *Pl-Rh* allele and the weakly expressed *Pl-Bh* allele (Cocciolone and Cone 1993).

The *Pl-Bh* allele is not paramutagenic: The *Pl-Bh* and *Pl-Rh* alleles are very similar at the DNA level. The two alleles are predicted to produce an identical PL protein and there are only 10 dispersed nucleotide differences confined to the 5′ and 3′ flanking regions over 5.5 kb of compared sequence (Hoekenga 1998). Despite the high sequence identity between *Pl-Rh* and *Pl-Bh*, *Pl-Bh* is only weakly expressed (Cocciolone and Cone 1993). *Pl-Bh* displays variegated patterns of expression in the aleurone layer of the seed, in most tissues of the seedling, mature plant, and in the anthers. Given the structural and phenotypic similarities between *Pl-Bh* and *Pl′*, we tested whether or not the *Pl-Bh* allele could participate in *pl* paramutation. We asked whether or not *Pl-Bh* was paramutagenic by crossing *Pl-Bh* homozygous plants by homozygous *Pl-Rh* stocks and examining the anther phenotypes seen in F_1 progeny. A single bag of pollen from our standard *Pl-Rh* stock was used to pollinate both a single ear from a *Pl-Bh* stock and a single ear from our standard *pl-sunred* (*pl-sr*) stock. None of the progeny from these pollinations displayed a strong *Pl′* phenotype. The ACS distributions from the two families (Table 1) are nearly identical, indicating that the few weaker-pigmented individuals seen in the *Pl-Bh*/*Pl-Rh* family are due to spontaneous instability of the *Pl-Rh* allele.

The *Pl-Bh* allele behaves like a neutral allele following exposure to paramutagenic *Pl′*: We asked whether *Pl-Bh*, like *Pl-Rh*, could become paramutagenic following exposure to *Pl′* using a series of genetic crosses. Thirty-one sibling plants that were either *Pl-Bh*/*pl-sr* or *Pl-Bh*/*Pl′* were crossed by a uniform *Pl-Rh*/*Pl-Rh* stock to test whether or not *Pl-Bh* became paramutagenic (Figure 6A). Although it was not known which plants were *Pl-Bh*/*pl-sr* and which were *Pl-Bh*/*Pl′* to begin with, we could identify these retrospectively based on the results.

TABLE 1

***Pl-Blotched* is not paramutagenic**

	Anther color score		
Genotype	5	6	7
Pl-Bh/*Pl-Rh*	1	5	12
pl-sr/*Pl-Rh*	0	3	15

Single ears from *Pl-Bh*/*Pl-Bh* and *pl-sr*/*pl-sr* plants were crossed with pollen from a single *Pl-Rh*/*Pl-Rh* plant and the progeny were grown to maturity. Progeny with the given genotypes were assigned anther color scores based on visible anther phenotypes. Data in the table refer to the number of progeny with a given anther color score. No progeny plants had an anther color score <5.

Because *pl-sr* and *Pl-Bh* are not paramutagenic by themselves (Hollick *et al.* 1995; Table 1, this study), all the progeny from crosses between *Pl-Bh*/*pl-sr* and *Pl-Rh*/*Pl-Rh* plants should primarily display *Pl-Rh* anther phenotypes; *i.e.*, both *Pl-Bh*/*Pl-Rh* and *pl-sr*/*Pl-Rh* progeny should have an ACS 7 phenotype. Because half of the 31 sibling plants used in the analysis should be *Pl-Bh*/*pl-sr*, we observed as expected that half (17/31) of all the families resulting from crosses to *Pl-Rh*/*Pl-Rh* plants had individuals with primarily *Pl-Rh* (ACS 7) phenotypes (Figure 6B). The expectation for the remaining families depends on whether or not *Pl-Bh* becomes paramutagenic. If *Pl-Bh* becomes as strongly paramutagenic as *Pl′* in the *Pl′*/*Pl-Bh* heterozygote, then all the progeny from crosses between *Pl′*/*Pl-Bh* and *Pl-Rh*/*Pl-Rh* plants should exclusively display *Pl′* anther phenotypes; *i.e.*, both *Pl-Bh*/*Pl-Rh* and *Pl′*/*Pl-Rh* progeny should have an ACS 1–4 phenotype. However, if *Pl-Bh* does not acquire any paramutagenic activity, then half of the progeny from crosses between *Pl′*/*Pl-Bh* and *Pl-Rh*/*Pl-Rh* plants should have ACS 1–4 phenotypes (*Pl′*/*Pl-Rh*) and half should have ACS 7 phenotypes (*Pl-Bh*/*Pl-Rh*). None of the 31 families examined had exclusively *Pl′* phenotypes; however, 14 families had both *Pl′* and *Pl-Rh* phenotypes present in roughly a 1:1 ratio (Figure 6C). These data indicate that *Pl-Bh* behaves like a neutral *pl* allele following exposure to *Pl′* alleles.

***pl* paramutation occurs independently of paramutation interactions at *b* and *r*:** We recognized that paramutagenic *Pl′* alleles could be maintained together with paramutable *B-I* alleles without causing *B-I* to change to *B′* (Figure 2, A and D) and that paramutable *Pl-Rh* alleles could be maintained together with paramutagenic *B′* alleles (>10^3 plants have been examined). We asked whether the same locus independence also applied for paramutation at the *pl* and *r* loci.

Plants from a family homozygous for the paramutable *R-r* allele and segregating *Pl-Rh*/*pl-sr* and *Pl-Rh*/*Pl′* types were outcrossed as males to recessive *r* testers to see if *R-r* activity was reduced following exposure to *Pl′*. By visual inspection, aleurone pigmentation by *R-r* was

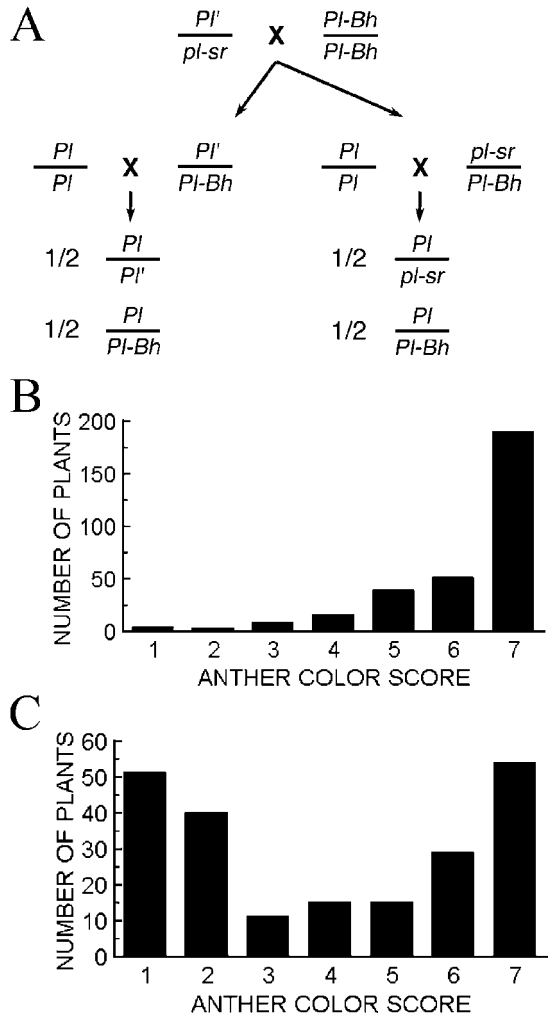

Figure 6.—*Pl-Bh* behaves like a neutral allele. (A) Pedigree illustrating genetic tests used to determine if *Pl-Bh* becomes paramutagenic following exposure to the highly paramutagenic *Pl′* allele. (B) Histogram representing the distribution of anther color scores seen in 17 families from the pedigree where the majority of the individuals had ACS 7 anthers. (C) Histogram representing the distribution of anther color scores seen in 14 families from the pedigree where approximately half of all the individuals had a clear *Pl′* phenotype and half had a *Pl-Rh* phenotype.

similar following exposure to either *Pl-Rh*/*Pl′* (6 testcross ears) or *Pl-Rh*/*pl-sr* (11 testcross ears). Thus the paramutable *R-r* allele appears insensitive to the paramutagenic action of *Pl′*. We also tested the reciprocal situation where the paramutable *Pl-Rh* allele was exposed to the paramutagenic *r* alleles, *R-st* and *R-mb*. Stocks homozygous for *pl-sr*, and either *R-st* or *R-mb*, were crossed by our standard *Pl-Rh*/*Pl-Rh*, *R-r*/*R-r* stock and then anther color scores were recorded for the resulting progeny. There was no indication of induced *pl* paramutation in the five *pl-sr*/*Pl-Rh*, *R-st*/*R-r* families or in the six *pl-sr*/*Pl-Rh*, *R-mb*/*R-r* families scored (Table 2). Thus we conclude that *r* paramutation occurs independently of *pl* paramutation and vice versa. This result is consistent

TABLE 2

***pl* paramutation is locus specific**

	Anther color score		
Genotype	5	6	7
R-st/*R-r*	1	0	37
R-mb/*R-r*	0	1	52

Individual *pl-sr*/*Pl-Rh* plants with the indicated *r* genotype were assigned anther color scores based on visible anther pigmentation. Data in the table represents the number of individual plants with a given anther color score grown from five (*R-st*/*R-r*) and six (*R-mb*/*R-r*) total ears. No plants had anther color scores <5.

with previous studies showing the allele dependence of paramutation interactions (Brink *et al.* 1960; Coe 1966; Hollick *et al.* 1995) and the independence of paramutation interactions between the *b* and *r* loci (Coe *et al.* 1988).

DISCUSSION

Our results suggest that paramutation occurring at the maize *pl* locus creates a novel regulation in addition to reducing gene expression. Paramutation changes the highly expressed, light-insensitive *Pl-Rh* allele to a weakly expressed, light-dependent *Pl′* derivative in husk tissues, implying a basic shift in how regulatory elements in the promoter region are utilized. This alteration in gene regulation correlates with changes in transcription rates when measured in whole husk samples. Analogous to paramutation occurring at the maize *b* locus, these regulatory changes are not associated with detectable alterations in DNA structure or cytosine methylation patterns over the 15-kb region examined.

Our expression analyses demonstrate that quantitative levels of anther pigment, as measured by visual scoring or extractable anthocyanin levels, directly reflect *pl* RNA levels. Thus, quantitative changes in *pl* transcript levels are simply and accurately assayed by visual inspection. Paramutation of the *Pl-Rh* allele results in variegated patterns of expression in anther tissues that are reminiscent of examples of position effect variegation and transdominant suppression described in Drosophila (reviewed in Weiler and Wakimoto 1995). Mechanistic parallels between these examples and paramutation have already been discussed (Patterson *et al.* 1993; Henikoff and Comai 1998). The particular patterns of pigmented cells are reproducible, suggesting that certain regions of the anthers have a stronger potential for *pl* expression. The signals responsible for this patterning are unknown, although preferential pigmentation occurs in regions of the anthers that lie closest to exterior light during floret development. As most *pl* alleles, including *Pl′*, are regulated by light, one hypothesis is that differences in anther pigmentation patterns reflect differential abilities of the *Pl′* alleles in individual anther cells to respond to light signals.

Our *in vitro* transcription assays with isolated husk nuclei show that *pl* paramutation causes alterations in *pl* transcription. There were no effects on *b* transcription but there were corresponding reductions in the transcription of the *a1*, *c2*, and *bz1* structural genes that are transcriptionally regulated by the B and PL proteins. Our results are analogous to previous *in vitro* transcription experiments showing that *b* paramutation reduces *b* transcription and reduces transcription of the structural genes but does not affect the transcription of *pl* (Patterson *et al.* 1993). In both studies transcription of the structural genes (*a1*, *c2*, and *bz1*) encoding biosynthetic enzymes parallels transcription of the *b* and *pl* genes encoding transcriptional regulatory proteins.

Based on our RNA measurements, the differences in *pl* transcription rates (3-fold) are much less than the differences in *pl* RNA levels (18.4-fold). We also observed lower transcription rates relative to RNA levels of the genes transcriptionally regulated by the B and PL proteins (*a1*, *c2*, and *bz1*). The lower transcription rates of *pl*, *a1*, *c2*, and *bz1* relative to their measured RNA levels imply that transcriptional rate changes are accompanied by either differences in RNA stability, temporal differences in transcriptional control, or a combination of both. Under steady-state conditions, measured RNA levels are a function of both the rate of synthesis (k_S) and the rate of degradation (k_D) where [RNA] = k_S/k_D (Kenney and Lee 1982). Prior to attaining steady state, however, measured RNA levels are a function of time and degradation rate; $[RNA]_t = k_S/k_D\ (1 - e^{-k_D t})$ (Price *et al.* 1962). If our RNA measurements reflect steady-state conditions, then in addition to our observed 3-fold decrease in the rate of *pl* RNA synthesis, the rate of *pl* RNA degradation must be increased roughly 6-fold in *Pl′*/*Pl-Rh* tissues. If our RNA measurements reflect RNA levels prior to steady-state conditions and there are no differences in the rates of *pl* RNA degradation, then *pl* RNA synthesis in *Pl′*/*Pl-Rh* husks must begin at a point in development later than in *Pl-Rh*/*Pl-Rh* husks. The latter scenario is consistent with our finding that *pl* RNA levels begin to be detected in *Pl′*/*Pl-Rh* husk leaf number 7 (Figure 3C) and that anthocyanin production begins much earlier in *Pl-Rh*/*Pl-Rh* husk development. The observation that *pl* RNA levels continue to increase relative to *actin* RNA levels during both *Pl′*/*Pl-Rh* and *Pl-Rh*/*Pl-Rh* husk leaf maturation is further indication that our *pl* RNA measurements do not reflect steady-state levels. Our data therefore suggest that the difference between *pl* transcription rates and measured RNA levels may be accounted for by temporal differences in transcriptional control of *pl* during ear shoot development.

Results comparing changes in transcription and changes in RNA levels upon *pl* paramutation differ from

the results obtained with *b* paramutation. Previous comparisons for *b* paramutation showed a 10-fold difference in *b* transcription rates and a 5-fold difference in *b* RNA levels (Patterson *et al.* 1993). The differences in the transcription rates of the *a1*, *c2*, and *bz1* genes closely matched the differences seen in *a1*, *c2*, and *bz1* RNA levels after *b* paramutation (Patterson *et al.* 1993). We suspect that *b* RNA levels were at, or near, steady-state levels in the tissues used for the previous study, whereas the *pl* RNA levels were not near steady state in the current study (as discussed above). However, our data do not rule out the alternate interpretation that *pl* paramutation affects both transcriptional and post-transcriptional regulatory mechanisms, whereas *b* paramutation exclusively affects transcriptional regulation.

Paramutation occurring at the *pl* locus does not share key features commonly used to define examples of post-transcriptional gene silencing (PTGS; Depicker and Van Montagu 1997). *In vitro* transcription assays with isolated nuclei illustrate that PTGS is associated with either no changes or increases in transcription rates. *pl* paramutation results in a threefold reduction in transcription rate. Documented examples of induced PTGS are not meiotically heritable. The *Pl′* allele is meiotically stable. PTGS is often correlated with increased cytosine methylation in corresponding coding regions. *pl* paramutation occurs without any apparent alterations in cytosine methylation patterns. While our results do not eliminate the possibility that post-transcriptional mechanisms are involved with *pl* paramutation, our finding that the *Pl-Bh* allele does not participate in paramutation demonstrates that the *pl* RNA itself is insufficient to catalyze or initiate paramutation. Taken together, the current evidence does not favor the hypothesis that *pl* paramutation is due to a post-transcriptional regulatory mechanism.

Similar to *b* paramutation, differences in *pl* transcription were not accompanied by changes in DNA sequence or cytosine methylation patterns within or flanking the transcribed region. Given the reversibility of *Pl′* and its ability to stably exist in discrete quantitative expression states, we doubt that *pl* paramutation is the result of direct DNA sequence alterations. However, the formal possibility of DNA changes cannot be adequately addressed until the functional *cis*-acting sequences are defined. It is possible that important regulatory sequences are positioned 5′ or 3′ of the region examined. While we found no evidence that *pl* paramutation affects global DNA methylation patterns in the proximate regions of the affected gene, our results do not discount the involvement of subtle changes in cytosine methylation. Paramutation of the *R-r* allele is associated with differences in cytosine methylation that are confined to a 3.4-kb region centered on a small promoter region of two divergently oriented *r* gene coding regions. Although no promoter sequences were tested, all eight methylation-sensitive restriction sites tested in the 5′ coding regions of these divergently oriented *r* genes showed increased methylation upon *r* paramutation (Walker 1998). If *pl* paramutation was associated with the same magnitude of methylation changes seen at the *r* locus we would have detected them.

We also did not detect any differences between the *Pl-Rh*/*Pl-Rh* and *Pl′*/*Pl-Rh* samples with the same enzyme and probe that recognizes methylation differences between the strongly expressed *Pl-Rh* allele and the weakly expressed *Pl-Bh* allele (Cocciolone and Cone 1993). Thus, despite 99.8% sequence identity over a 5.5-kb region, the *Pl-Bh* allele does not appear to be silenced by a similar mechanism as *Pl′*. This is consistent with results of our genetic tests indicating that the *Pl-Bh* allele does not participate in paramutation. While our tests do not exclude the possibility that *Pl-Bh* can attain mild paramutagenic activity, it is clear that the two alleles have distinct epigenetic behaviors (Cocciolone and Cone 1993; this study).

The observation that *Pl-Bh* behaves like a neutral allele suggests that neither the PL protein, the *pl* RNA, nor the proximate 5′ and 3′ flanking regions are sufficient for paramutation interactions. Because PL is a transcriptional regulatory protein, it seems possible that an autoregulatory loop, whereby the transcription factor regulates its own gene expression through interactions at the promoter, could be established and maintained with high fidelity. This is especially attractive for PL, as it binds DNA directly (Sainz *et al.* 1997). If this autoregulatory model is true, then our results imply that *Pl-Bh* lacks the necessary *cis*-acting elements to participate in paramutation. Another model supposes that paramutation leads to a prion state (abnormal, yet stable, protein conformation) of the PL protein itself. The inheritance and inductive properties of prion proteins so far described (Tuite and Lindquist 1996) closely models the behaviors seen in *pl* paramutation. Our finding that the *Pl-Bh* allele, which encodes an identical PL protein to *Pl-Rh*, does not become strongly paramutagenic following exposure to *Pl′* is not consistent with a PL protein prion model.

Attention has recently been focused on the involvement of RNA in mediating homology-dependent silencing interactions. In most cases, these silencing mechanisms occur at the post-transcriptional level. However, a paramutation-like transcriptional silencing behavior in *Phytophthora infestans* heterokaryons is RNA mediated (van West *et al.* 1999). Our current studies do not directly address this issue for *pl* paramutation. However, despite 100% identity in the transcribed region, the *Pl-Bh* allele does not appear to participate in paramutation interactions with *Pl′* and *Pl-Rh*. If *pl* paramutation utilizes specific RNA sequences, we would expect the *Pl-Bh* allele to participate in such allelic interactions.

It has been suggested that transposable elements directly, or indirectly, mediate paramutation interactions (Martienssen 1996; Matzke *et al.* 1996; Hollick *et al.*

1997). Remnants of a CACTA-like element called *doppia* appear to be involved in the acquisition, and/or maintenance, of paramutagenic activity in the maize *R-r* allele. A deletion derivative of *R-r* that removes these *doppia* sequences and a small portion of the flanking *r* gene coding regions shows reduced ability to become paramutagenic following prolonged exposure to paramutagenic *R-st* (Kermicle 1996). Intriguingly, CACTA-like element sequences with 77% identity to *doppia* sequences at *R-r* reside −129 bp 5′ of the predicted transcription start site of the *Pl-Rh* allele (Cone *et al.* 1993b). There are 226 bp of matching sequence information available between *Pl-Rh* and the *doppia* sequences found in *R-r* (Cone *et al.* 1993b; Walker *et al.* 1995). These sequences share 11 (12-bp) repeats with only five total nucleotide differences and there are 49/94 identities over the remainder of the compared sequence. It is tempting to speculate that these common sequences at *r* and *pl* are responsible for either the establishment and/or maintenance of paramutation. Because *Pl-Bh* sequences are identical to *Pl-Rh* in this 5′ region (Hoekenga 1998), our finding that *Pl-Bh* does not behave like *Pl-Rh* in paramutation does not support this idea at the *pl* locus. If *doppia*-like sequences are sufficient for paramutation interactions between *Pl-Rh* and *Pl′*, then *Pl-Bh* alleles must utilize these *doppia*-like sequences in distinct ways. We also found no obvious paramutation interactions between paramutagenic and paramutable alleles found at the *r* and *pl* loci, suggesting that locus-specific sequences, not simply common *doppia* sequences, are required to mediate the establishment and/or maintenance of paramutation at the individual loci.

Because *pl* paramutation affects transcriptional activity, either recruitment, promoter clearance, or elongation of polII RNA polymerase is affected. The observation that *Pl′* is now light dependent implies that *pl* paramutation affects interactions with light-regulated factors. Fully expressed *Pl-Rh* alleles presumably contain light-responsive *cis*-linked elements that are unable to affect, or remain unutilized for, transcriptional control. It is also formally possible that light-responsive regulation of *Pl-Rh* is masked by light-insensitive transcriptional control. However, if *Pl-Rh* were able to respond to light signals to the same extent as *Pl′*, we would expect to observe an obvious increase in *pl* RNA following light treatment (Figure 4B). We know that *pl* paramutation does not retard the plant's general perception of light. In fact, presumably due to the reduction in light-absorbing anthocyanin pigments, the phytochrome-regulated *lhcp* gene is actually transcribed at a twofold higher rate. Thus one possibility is that *pl* paramutation leads to heritable alterations of the *pl* gene regulatory environment that supports interactions with light-regulated, and possibly other developmentally regulated, signals.

Because the alteration of *pl* gene regulation occurs without any obvious changes in DNA structure and cytosine methylation within or nearby the coding region, we favor a model whereby *pl* paramutation involves remodeling of proximate chromatin into a stable configuration that is both heritable and leads to altered transcriptional regulation. Because *Pl′* alleles can regain high levels of activity when they are heterozygous with neutral *pl* alleles or when they are hemizygous (Hollick and Chandler 1998), allelic interactions likely stabilize this chromatin structure. The alterations in chromatin structure may include, but are not limited to, states of histone modifications, novel complexes of chromatin-associated proteins, general chromosome condensation, and specific transcription factors themselves (Chandler *et al.* 1996).

We are currently using directed genetic screens to identify components required to stably maintain the *Pl′* paramutant state. Our working model predicts that these components will encode proteins involved in chromatin remodeling, modification, and structure. Further, if paramutation at the *r*, *b*, and *pl* loci are mechanistically related, then we expect certain mutations to affect paramutation interactions at all three loci.

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