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 PI-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-Blotchel (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.

PараметуATION 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; Kernicke 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 PI-Rhoades (PI-Rh) alleles can spontaneously change to weaker expression states (B' and Pl') that are strongly paramutagenic; 100% of the allele transmitted from B-I/B' and PI-Rh/Pl' individuals are B' or Pl', respectively (Coe 1966; Hollick et al. 1995). In contrast, the paramutable 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-stiplad (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 PI-Rh and R-r alleles. Paramuntant 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 PI-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-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 qualitatively 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 Klarsfeld 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 hybrid (Meyer et al. 1993) and Nicotiana tabacum (Park et al. 1996). In sharp contrast, one of the strongest repressors 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 PI-Rh allele. Despite extensive DNA sequence identity, we find that the epigenetically regulated PI-Blotchel (PI-BH) allele (Coccolone 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 PI-Rhoades (herein designated PI-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 PI’ allele originally isolated as a spontaneous derivative of the PI-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 PI-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 PI-Rh/Pl’ and PI’/Pl-Rh plants to B1/B1; Pl’/Pl-Rh; R-r/B-r testers. Material for sunlight exposure experiments and restriction endonuclease comparisons was obtained from PI-Rh/Pl’-Rh and PI’/ Pl-Rh plants derived by crosses of PI-Rh/Pl-Rh and Pl’/Pl-Rh plants to a common PI-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 B1 allele and a functional allele of the r locus required for anthocyanin pigmentation (R or 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-m1 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 Xhol, pl-specific 3’ clone have been previously described (Coccolone and Cone 1993). The 5’ pl-specific clone (pJH1) is a 850-bp HindIII 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 EcoRI linkers (pPlcDNA) provided by Karen Cone. The 5’ most 505-bp EcoRI-BglI fragment from pPlcDNA was first treated with T4 DNA polymerase to generate blunt ends and then cloned into the Smal site of pBluescriptII SK (+) (Stratagene, La Jolla, CA) to generate pJH 7. The BglI site used in the pPlcDNA subcloning lies between the third exon Ncol and Ncol 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 phylloicum 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 1-2 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 32P-UTP-labeled RNA transcripts of both pl (pH 7 linearized with Xbal) and maize actin (pMACl linearized with EcoRI). Bulk RNA (5 μg/sample) was hybridized with 106 cpm of each radiolabeled transcript overnight at 37°C and then digested for 20 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 32P-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 (20, 15, 12, 8.3); additional details of these analyses are available upon request. Differences in anthocyanins and pl RNA transcription rates were measured for each individual nuclei preparation. In the third experiment, three ears of each genotype were pooled (16, 12, 9); subsequent experiments, three ears of each genotype were pooled (16, 12, 7, 3.5, 1.8, 1.2); 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 P1 samples by the various enzymes with the 5' and 3' hybridization probes Acd 5' (3.9); Acd 3' (1.2, 0.6); Acd 5' (20, 15, 12, 8.3); Acd 3' (1.1); BglII 5' (7.9, 2.4); BglII 3' (8, 7, 2.6); BstNI 5' (6.0, 8.8); BstNI 3' (1.8, 1.2); Dde 5' (>12); Dde 3' (6.6, 4.2, 2.1); Eagl 5' (>12); Eagl 3' (1.1); EcoRII 5' (16, 12); EcoRII 3' (16, 12, 7, 3.5, 1.8, 1.2); HindIII 5' (0.85); HindIII 3' (0.85); HindIII 2' (12); HindII 5' (0.35, 0.3); HindII 3' (2.8); Hpal 5' (>23); Hpal 3' (10, 7.5, 3.2, 1.8, 1.6); MspI 5' (17, 9.9, 5.9, 5.3); MspI 3' (10, 7.5, 3.2, 1.8, 1.6); NciI 5' (>23); NciI 3' (23, 11); NciI 5' (5.3); NciI 3' (9.7); NsiI 5' (2.4, 1.7); NsiI 3' (3.3, 1.1); Pstl 5' (~35); Pstl 3' (>40, ~25, 12); PvuI 5' (>23); PvuI 3' (~23); PvuI 3' (14, 11.5, 8.2, 5.1, 5.2, 2.6, 2.5); SalI 5' (>15); SalI 3' (>23); Sau96I 5' (2.1); SbaI 96I 3' (4.8, 1.8, 0.9); XhoI 5' (17, 14, 12, 7.6, 6.2, 6.2, 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; Holllick 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 P1 (Holllick et al. 1995). In mature leaf sheath, husks, and culm, there appears to be a homogeneous reduction of pigment in all epidermal
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
and flanking sequences found in all *pl* alleles characterized to date (Cone et al. 1993a) 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 *PＩ’/Pl-Rh* samples (Figure 3B) show *pl* RNA is 18.6-fold higher in *Pl-Rh*/Pl-Rh vs. *PＩ’/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.

*pl* RNA expression patterns are different between *Pl-Rh* and *PＩ’* 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 *PＩ’/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 *PＩ’/Pl-Rh* husks varied 12-fold. Correspondingly, the differences between *pl* RNA levels in *Pl-Rh*/Pl-Rh vs. *PＩ’/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 *PＩ’/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 *PＩ’/Pl-Rh* ears, we wondered if *pl* RNA expression was light dependent. Most *pl* alleles

**Figure 2.—*Pl-Rh* vs. *PＩ’* 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; *PＩ’/Pl-Rh* (*PＩ’*) 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; *PＩ’/Pl-Rh* (*PＩ’*) 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; *PＩ’/Pl-Rh* (*PＩ’*) 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 *PＩ’/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, PＩ’ 9); *pl*, (Pl-Rh 17, PＩ’ 15); *a1*, (Pl-Rh 9, PＩ’ 9); *c2*, (Pl-Rh 9, PＩ’ 9); *bz1*, (Pl-Rh 12, PＩ’ 12).
PI’ alleles required light signals by comparing the pigmentation occurring when PI’ explant ears are exposed or masked to light. In the first set of experiments, outer husk leaves from PI’/PI-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 PI’/PI-Rh ears responded by producing anthocyanin pigments while the masked halves remained unpigmented. We next used Northern blot hybridization to measure light-dependent pl RNA accumulation in PI-Rh/PI-Rh and PI’/PI-Rh explant ears. In the absence of light (fully masked ears), pl RNA from the fourth husk leaf is much greater in PI-Rh/PI-Rh than PI’/PI-Rh plants (Figure 4B). However, pl RNA levels increased in PI’/PI-Rh husks following 36 hr of light exposure while pl RNA levels remained relatively unchanged in PI-Rh/PI-Rh husks following the same treatment (Figure 4B). Thus paramutation fundamentally alters the regulation of the PI-Rh allele such that RNA accumulation in the PI’ state is responsive to light signals. It should be noted that even though the PI’ allele is light responsive in husk tissues, it does not display another pigment patterns typical of sun-red pI 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 PI-Rh and the light-dependent PI’ allele. We used 21 enzymes for this analysis and 17/21 enzymes were methylation sensitive. Two pl-specific genomic frag-
ments were used in the analyses; one is nearly 6 kb upstream of the predicted PI-Rh transcription start site and the other is at the 3' end of the pl coding sequence. Much of the sequences 5' of the PI-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 PI-Rh/PI-Rh plant and a single PI'/PI-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 MspI/HpaII and EcoRI/BstNI 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 PI-Rh/PI-Rh and PI'/PI-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 PI-Rh/PI-Rh and PI'/PI-Rh samples at the 3' HpaII sites previously shown to be differentially methylated between the strongly expressed PI-Rh allele and the weakly expressed PI-Bh allele (Cocciolone and Cone 1993).

The PI-Bh allele is not paramagenic: The PI-Bh and PI-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 PI-Rh and PI-Bh, PI-Bh is only weakly expressed (Cocciolone and Cone 1993). PI-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 PI-Bh and PI', we tested whether or not the PI-Bh allele could participate in pl paramutation. We asked whether or not PI-Bh was paramutagenic by crossing PI-Bh homozygous plants by homozygous PI-Rh stocks and examining the anther phenotypes seen in F1 progeny. A single bag of pollen from our standard PI-Rh stock was used to pollinate both a single ear from a PI-Bh stock and a single ear from our standard pl-sunred (pl-sr) stock. None of the progeny from these pollinations displayed a strong PI' phenotype. The ACS distributions from the two families (Table 1) are nearly identical, indicating that the few weaker-pigmented individuals seen in the PI-Bh/PI-Rh family are due to spontaneous instability of the PI-Rh allele.

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

Figure 5.—PI-Rh and PI' alleles are structurally similar. Genomic restriction map shared between PI-Rh and PI' 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.
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 PI’ 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 PI’ anther phenotypes; i.e., both Pl-Bh/ Pl-Rh and PI'/ 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 PI'/ Pl-Bh and PI'/ Pl-Rh 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 PI’ phenotypes; however, 14 families had both PI’ and PI-Rh phenotypes present in roughly a 1:1 ratio (Figure 6C). These data indicate that Pl-Bh behaves like a neutral allele following exposure to PI’ alleles.

**Table 1**

Pl-Blotched is not paramutagenic

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<th>Genotype</th>
<th>Anther color score</th>
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<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Pl-Bh/ Pl-Rh</td>
<td>1</td>
</tr>
<tr>
<td>pl-sr/ Pl-Rh</td>
<td>1</td>
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<td>7</td>
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</table>

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.

**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 PI’ 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 PI’ phenotype and half had a Pl-Rh phenotype.

Pl paramutation occurs independently of paramutation interactions at h and r: We recognized that paramutagenic PI’ 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 PI-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/ PI’ types were outcrossed as males to recessive r testers to see if R-r activity was reduced following exposure to PI’. By visual inspection, aleurone pigmentation by R-r was similar following exposure to either Pl-Rh/ PI’ (6 testcross ears) or Pl-Rh/ pl-sr (11 testcross ears). Thus the paramutable R-r allele appears insensitive to the paramutagenic action of PI’. We also tested the reciprocal situation where the paramutable PI-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 other 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...
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/Pl-R-r and six (R-mb/Pl-R-r) total ears. No plants had anther color scores <5.

### 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 P1′ 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 P1′, are regulated by light, one hypothesis is that differences in anther pigmentation patterns reflect differential abilities of the P1′ 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 (k9) and the rate of degradation (k0) where \[ \text{[RNA]} = k_9 / k_0 \] (Kenney and Lee 1982). Prior to attaining steady state, however, measured RNA levels are a function of time and degradation rate; \[ \text{[RNA]} = k_9 / k_0 (1 - e^{-kt}) \] (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

### TABLE 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Anther color score</th>
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<tr>
<td>R-st/Pl-R-r</td>
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</tr>
<tr>
<td>R-mb/Pl-R-r</td>
<td>6</td>
</tr>
<tr>
<td>pl paramutation is locus specific</td>
<td></td>
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<td>pl paramutation</td>
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<td>6</td>
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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 DNA, 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 auto-regulatory 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.
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 PI-Rh allele (Cone et al. 1993b). There are 226 bp of matching sequence information available between PI-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 PI-Bh sequences are identical to PI-Rh in this 5’ region (Hoekenga 1998), our finding that PI-Bh does not behave like PI-Rh in paramutation does not support this idea at the pl locus. If doppia-like sequences are sufficient for paramutation interactions between PI-Rh and Pl’, then PI-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 PI-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 PI-Rh is masked by light-insensitive transcriptional control. However, if PI-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 lhcb 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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