Allelic Interactions Heritably Alter the Activity of a Metastable Maize *pl* Allele

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ABSTRACT

The maize *pl* locus encodes a transcriptional activator of anthocyanin biosynthetic genes. The *Pl-Rhoades* (*Pl-Rh*) allele confers robust purple anthocyanin pigment in several tissues. Spontaneous derivatives of *Pl-Rh*, termed *Pl'-mahogany* (*Pl'-mah*), arise that confer reduced pigment and are meiotically heritable. These derivatives influence other *Pl-Rh* alleles such that only *Pl'-mah* alleles are transmitted from a *Pl-Rh/Pl'-mah* heterozygote. Genetic crosses establish that chromosomal segregation distortion does not explain this exclusive transmission and suggest that *Pl-Rh* invariably changes to *Pl'-mah* when exposed to *Pl'-mah*. Such behavior is a hallmark of paramutation. Cosegregation experiments demonstrate that this paramutagenic activity is genetically linked to the *pl* locus. By visually quantifying *pl* action through successive crosses, we find that phenotypic expression is inversely related to paramutagenic strength. In addition, the paramutagenic state is metastable; reversion to a nonparamutagenic *Pl-Rh* state can occur. The behavior of *Pl-Rh* is unique, yet it shares characteristics with paramutation at two other maize loci, *b* and *r*. Previous analysis of *b* and *r* paramutation revealed extensive differences and led to suggestions of distinct molecular mechanisms. Consideration of the common features of all three systems reinvigorates the interpretation that the mechanistic processes of these three allelic interactions are similar.

PARAMUTATION, which is defined as an interaction between alleles that leads to a heritable change in one of the alleles at high frequency, has been characterized in a limited number of systems (BRINK 1973). The *b* and *r* genes of maize represent two examples of paramutation for which the gene is cloned and the function of the gene product is known. The predicted basic-helix-loop-helix proteins produced by these genes are very similar (LUDWIG *et al.* 1989; RADICELLA *et al.* 1991), and genetic and transient transformation experiments indicate that the gene products are functionally interchangeable transcriptional activators of the anthocyanin biosynthetic pathway (STYLES *et al.* 1973; GOFF *et al.* 1990; LUDWIG *et al.* 1990). Transcription of anthocyanin biosynthetic genes depends on a functional basic-helix-loop-helix protein (encoded by the *b* or *r* genes) and a functional myb-related protein (encoded by the *c1* or *pl* genes) (reviewed in DOONER *et al.* 1991; GOFF *et al.* 1992). The equivalent genes are typically expressed in different tissues because they contain distinct regulatory sequences that influence their developmental and tissue-specific expression (LUDWIG and WESSLER 1990; RADICELLA *et al.* 1992; CONE *et al.* 1993). Thus, despite distinct tissue-specific regulatory sequences, paramutation occurs at both *b* and *r*.

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For most cases of paramutation, only particular alleles of a given locus show such behavior (BRINK 1973). In both *r* and *b* paramutation the presence of one allele (paramutagenic) leads to a heritable change in the other allele (paramutable) such that the pigmentation activity of the affected allele is heritably reduced. At *b*, the reduced activity discerned by visible pigment levels accurately reflects changes in *de novo* transcription (PATTERSON *et al.* 1993). Despite the common heritable change in gene activity, there are associated allelic behaviors and molecular differences that may, or may not, be directly relevant to the paramutation process (reviewed in COE 1966; BRINK 1973; PATTERSON and CHANDLER 1995a,b). Such differences have led to the suggestion that the process of paramutation is mechanistically distinct for these two loci. Alternatively, *b* and *r* paramutation may occur via similar mechanisms, yet differ due to nuances related to gene structure, chromosome context, genomic organization, or spatial and temporal occurrence of the process. The study of other examples of paramutation should aid in the development of unifying principles that define the basic process.

In this paper, we describe the identification of a novel anthocyanin phenotype called mahogany and demonstrate that this phenotype is linked to *pl* (*purple plant*), a gene unrelated to *b* and *r*. Genetic characterization of this *pl* allele suggests that it is a paramutant derivative of the *Pl-Rhoades* (*Pl-Rh*) allele. Results of our genetic analyses show that this behavior at *pl* shares characteristics with both *b* and *r* paramutation but it is not exclusively like either one. Spontaneous paramutant de-

rivatives of *Pl-Rh* arise and some of these are characteristically metastable. We show that paramutation-like allelic interactions heritably affect the activity of this *pl* allele. Comparisons of the allelic behaviors seen at *b*, *r* and *pl* are discussed within the context of a mechanistically related paramutation process.

MATERIALS AND METHODS

Maize stocks: The mahogany phenotype was originally observed in a W23 genetic background homozygous for the *B-I*, *R-r* and *Pl-Rh* alleles (see RESULTS for phenotypic description). Mahogany plants were identified from a bag of seed containing the progeny from two ears. Thirteen seeds from the two ears were planted, and two had the mahogany phenotype.The mahogany plants were previously reported to have the genotype *B-mahogany R-r Pl-Rh* (COE 1979), but mapping experiments described in RESULTS indicate these plants were *B-I R-r Pl'-mahogany* (*Pl'-mah*). The stocks used to examine *Pl-Rh* stability (Figure 3A) have *R-r, Pl-Rh,* and *b-W23* in the W23 genetic background. The recessive *pl* allele used in these experiments was derived from a W23/K55 stock, and we hereafter refer to this allele as *pl-W23*. *pl-W23* confers a moderate to weak sunlight-dependent pigmentation in tissues where it is expressed in combination with either a functional *b* or *r* product. *pl-W23* expression in the anthers is readily distinguishable from *Pl'-mah* in that the former causes uniform pale pigmentation whereas the latter causes mottled pigmentation. The *salmon silks* (*sm*) stock (stock number 611A) was obtained from the Maize Genetics Stock Center (Urbana, IL).

The *R-r* "allele" used in these experiments consists of two linked *r* genes, called the seed (*R*) and plant (*-r*) components (STADLER and NUFFER 1953; DOONER and KERMICLE 1971). These components share extensive sequence identity to each other and other *r* alleles (LUDWIG *et al.* 1989; PERROT and CONE 1989; ROBBINS *et al.* 1991; CONSONNI *et al.* 1993) and to *b* (CHANDLER *et al.* 1989; RADICELLA *et al.* 1991). Aleurone pigmentation is due to the expression of the seed component whereas pigment formation in anthers and seedling sheath is due to the expression of the plant component.

Confidence intervals in mapping: The maximum map distance, where there is a 5% probability of not exchanging two genetic markers in 42 possible events, is calculated by the following binomial, $p^0(1-p)^{42} < 0.05$. Map distance, *p*, is represented in centimorgans.

Anther scoring: Based on the existing variation seen amongst spontaneous paramutant derivatives, a 1-7 anther color classification was defined: 1, no color or, at most, a few spots of red pigment; 2, slightly mottled distribution of red pigment on the top of the lobes (opposite the side of filament attachment), but fully yellow on the bottom; 3, medium mottled distribution of pigment on the top, but fully yellow on the bottom; 4, strongly mottled tops and mostly yellow bottoms; 5, almost fully pigmented tops but mottled bottoms; 6, solid, or nearly solid, red throughout except in-between the locule chambers; and 7, all visible tissue solid red. Examples of these anther color classes are shown in Figure 3B. Plants used for scoring were numbered and anthers were visually inspected from three individual florets sampled from multiple tassel branches of each individual plant. Anthers were teased out of florets where extrusion of the anthers was beginning to occur and then assigned an anther color score (ACS) based on the visible anthocyanin phenotypes listed above. Anthers sampled from different tassel locations mostly had the same ACS throughout an individual tassel; rare exceptions had anther color scores that only differed by one gradation. An overall average ACS was given in cases where there was variation.

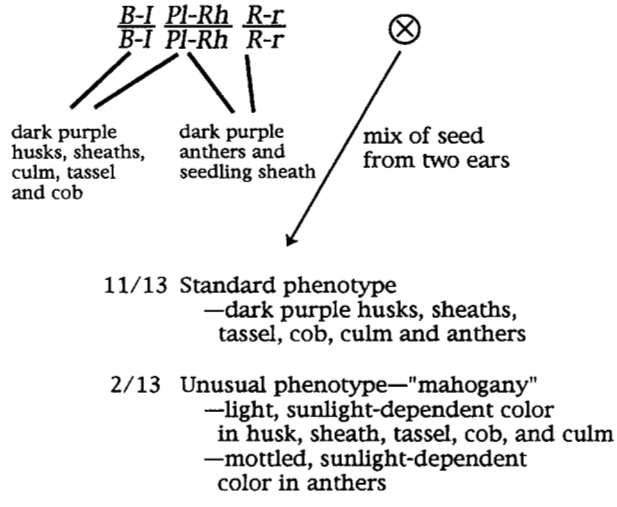


FIGURE 1.—Identification of plants with the mahogany phenotype.

For data presented in Figure 3A, all the plants were scored separately by two individuals and the independently assessed ACS for each plant were then compared. The rare discrepancies never varied by more than one ACS interval. Tassels that were resampled at a later date were normally scored with the same ACS indicating that both early and late maturing spikelets reflected the same pigmentation activity.

DNA blots: Methods for DNA isolation, blotting, and hybridization were as described (COCCIOLONE and CONE 1993). Blots were hybridized with a 200-bp *Xho*I-*Dra*I fragment isolated from the 3′ end of a *Pl-Rh* cDNA clone that was radiolabelled by random priming (CONE *et al.* 1993). This probe does not cross hybridize with the duplicate *c1* gene (CONE *et al.* 1993).

RESULTS

Exceptional anthocyanin phenotypes arise spontaneously: An unusual variant was isolated from plants with the genotype of *B-I/B-I, R-r/R-r, Pl-Rh/Pl-Rh, C1/C1*. The expected phenotype for this genotype is intense pigment in sheath, culm, tassel glumes and branches, cob, pericarp and husk due to the expression of *B-I* and *Pl-Rh*; intense pigment in the anthers and seedling sheaths and roots due to the expression of *R-r* and *Pl-Rh*; and intense pigment in the aleurone layer of the seed due to the expression of *R-r* and *C1*. This intense pigment phenotype will be henceforth referred to as the "standard" phenotype. As shown in Figure 1, two of 13 progeny from self-pollination of plants with the standard phenotype had an altered phenotype, termed "mahogany". These variants had pigment in all of the same tissues as their standard sibs, but differed in two ways. First, the variants had less pigment in each tissue in which pigment is influenced by *pl* (see Figure 2 for comparison of anther, mature plant, and seedling sheath color). Second, the formation of pigment was sunlight dependent, that is, pigment was substantially less in tissues not directly exposed to sunlight. In Figure 2C, the green tissue on the sheath from the mahogany plant (between the arrows) is tissue that was covered

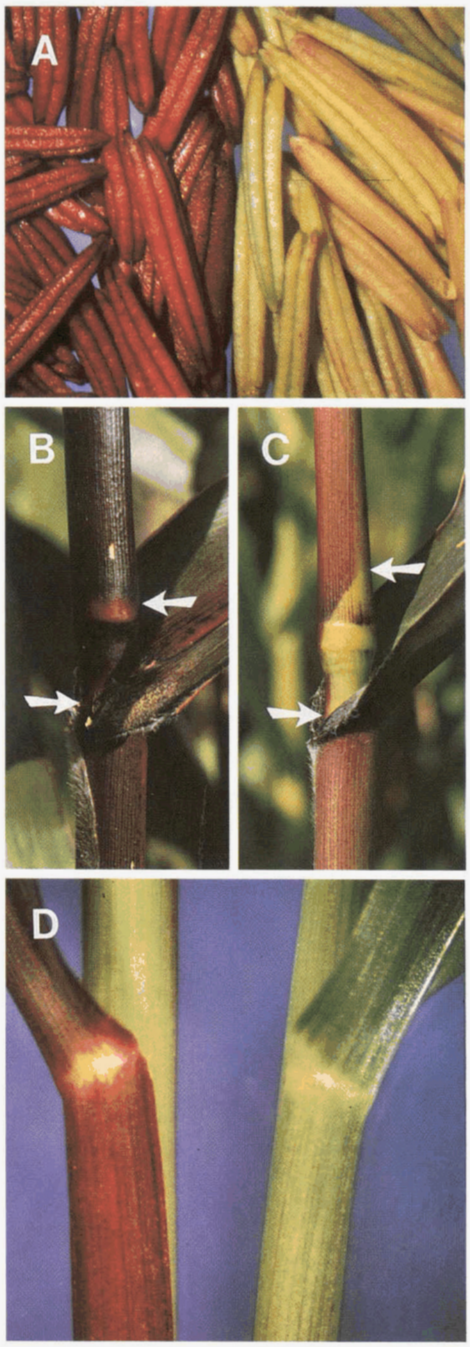


FIGURE 2.—Examples of the standard and mahogany phenotypes. (A) Anthers from standard and mahogany individuals, respectively. (B and C) Comparison of stalks from mature standard (B) and mahogany (C) plants. Arrows delimit portions of the culm and sheath that were covered by basipetal sheath and blade tissue. (D) Comparison of first sheaths from standard (left) and mahogany (right) seedlings.

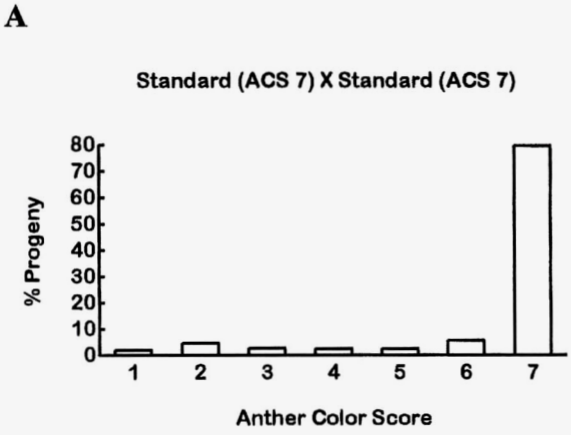
by the immediately basipetal sheath. This tissue has no detectable pigment yet develops color after exposure to sunlight. The sunlight-dependent pigment is mahogany colored. In contrast, the sheath from the standard plant is uniformly dark even where similarly covered (indicated by arrows in Figure 2B). The standard and mahogany plants had equivalent pigmentation in the aleurone, where *C1,* but not *Pl-Rh,* is expressed.

We found anther scoring to be the easiest and most quantitative assessment of pigmentation activity. Tissues requiring *B-I* for pigmentation are not reliably scored due to spontaneous paramutation at *b.* Because the plant component of *R-r* (MATERIALS AND METHODS) does not undergo notable spontaneous paramutation, anther tissues that require *R-r* function could be used as reliable indicators of *pl* activity. The remaining analyses refer predominantly to anther pigmentation.

To assess the frequency with which the mahogany phenotype arises, anther phenotypes from 34 additional families of the same genetic background as the original isolate were examined. Of the 385 progeny derived from self-pollinations of standard plants, 79 (21%) had less anther pigmentation than that of their standard parents. The amount of anther pigment varied considerably between individuals but was very uniform within any particular plant. To quantify this continuum of anther pigmentation, we developed a seven class scoring scale (MATERIALS AND METHODS). Each individual plant was assigned an ACS to reflect the amount of pigment seen in the anthers; a score of 1 indicates little to no pigment whereas a score of 7 indicates standard-like coloration (examples of each ACS are presented in Figure 3B). The ACS of the spontaneous derivatives span the entire 1–6 range without any notable bias for a particular ACS (Figure 3A).

The mahogany phenotype shows exclusive transmission: Heterozygotes derived from mahogany and standard parental plants have the mahogany phenotype; no plants with the standard phenotypes have yet been found ($>10^3$ plants). The fact that the mahogany phenotype is observed in the F_1 seedling sheaths suggests that the dominant action of mahogany occurs early in ontogeny. In addition to this dominant reduction in pigment in all F_1 tissues, all segregant progeny have the mahogany phenotype. To illustrate this point, F_1 heterozygotes (ACS 1-3) were backcrossed by standard plants (ACS 7) and anthers of the resultant progeny were then scored. Of 678 progeny from 14 individual crosses, 99% had ACS of 1-3 and the remaining 1% had ACS of 4. No plants had ACS indicative of the standard phenotype (ACS 7). On a larger scale (3184 progeny from 130 families), no standard phenotypes have been recovered from either self-crosses of mahogany plants (79 families) or backcrosses of mahogany plants by standard plants (51 families). These genetic tests with standard stocks indicate that the mahogany phenotype is exceedingly stable, completely dominant, and exclusively transmitted through meiosis.

The mahogany phenotype is linked to the *pl* locus: Because anthocyanin pigmentation was affected, the mahogany plants might have been altered in any of the biosynthetic or regulatory genes required for anthocyanin synthesis. However, the most likely candidate was the *Pl-Rh* allele, because the mahogany plants differ from the standard plants in all tissues in which pigmen-



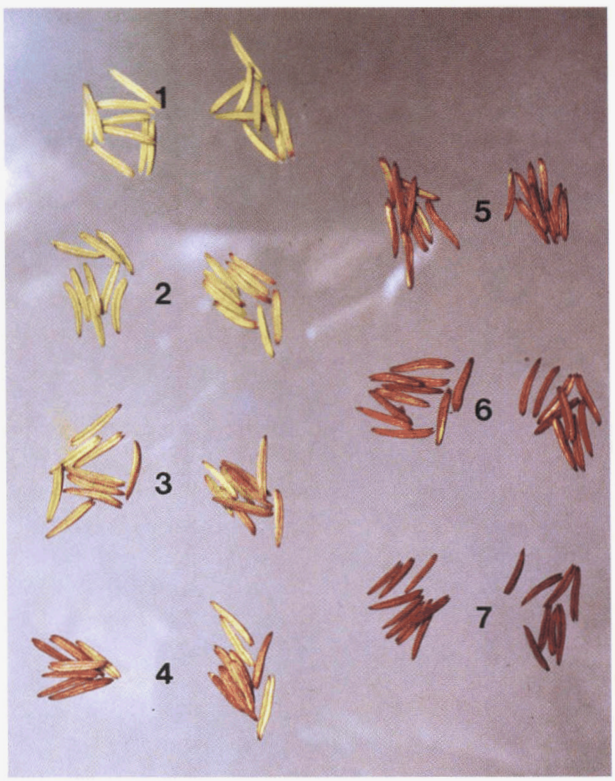


FIGURE 3.—Distribution and anther phenotypes of *Pl-Rh* spontaneous derivatives. (A) From crosses amongst standard phenotypes (*Pl-Rh/Pl-Rh*) the distribution of progeny with the indicated anther color scores is presented as percentages of the total population of 385 progeny from 34 families. (B) Anthers classified on the basis of visual anthocyanin pigments (see MATERIALS AND METHODS for details) were assigned specific anther color scores (ACS). Examples of ACS ranging from 1–7 are presented.

tation is controlled by *Pl-Rh*, and only in those tissues. A lesion in a biosynthetic gene or one of the regulatory genes, *c1* or *r*, was unlikely because these genes are required for the production of seed color, which is unaffected in mahogany plants.

Genetic linkage was tested by asking whether the mahogany phenotype cosegregated with a specific *pl* allele.

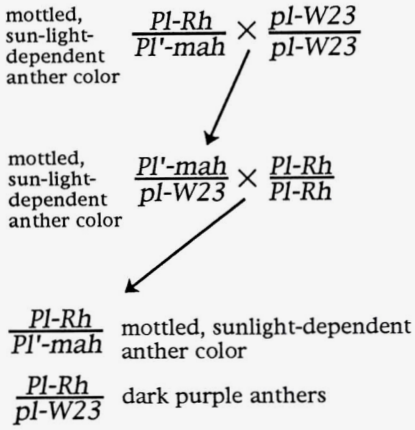


FIGURE 4.—Expected genotypes and phenotypes if mahogany phenotype is linked to the *pl* locus. One of the original mahogany plants was crossed to a sib with the standard phenotype. This cross would be expected to produce the genotype shown on the top left, *Pl-Rh/Pl'-mah*. This plant was crossed by a *pl-W23/pl-W23* stock, and the resulting progeny crossed by *Pl-Rh/Pl-Rh* plants.

The crosses performed to test for linkage are shown in Figure 4 and are briefly outlined below. Only the *pl* genotypes of the plants are shown; all plants had *r* alleles that allowed for scoring of the mahogany anther phenotype. Assuming the mahogany phenotype was due to a derivative allele of *Pl-Rh*, we designated it *Pl'-mahogany* (*Pl'-mah*). A plant of putative genotype *Pl'-mah/Pl-Rh* was crossed by a plant homozygous for *pl-W23*. *pl-W23* is an allele of *pl* that is recessive to *Pl-Rh* for anther color and is not paramutagenic. Preliminary segregation experiments also indicated that *pl-W23* was not paramutable. The anther phenotype produced by *pl-W23* (uniform pale pink color) can be distinguished from that of the mahogany phenotype by scoring anthers for mottling, which is produced by *Pl'-mah* but not *pl-W23*. The *Pl'-mah/pl-W23* F_1 plant was then crossed by a *Pl-Rh/Pl-Rh* standard plant. If the mahogany phenotype is linked to *pl*, and the hypothesis about genotypes is correct, two classes of progeny should be present: *Pl'-mah/Pl-Rh* (mahogany phenotype) and *pl-W23/Pl-Rh* (standard phenotype). Anther colors of the plants were scored and four categories were distinguished, ranging from lightly mottled to completely dark anthers (Table 1). Genotypes were established by restriction fragment

TABLE 1

Linkage analysis of the mahogany phenotype

No. of plants	Anther phenotype[a]	RFLP genotype
12	Lightly mottled	*Pl'-mah/Pl-Rh*
3	Medium mottled	*Pl'-mah/Pl-Rh*
3	Dark mottled	*pl-W23/Pl-Rh*
24	Solid	*pl-W23/Pl-Rh*

[a] Phenotypes were assessed before implimenting the 1–7 scoring scheme.

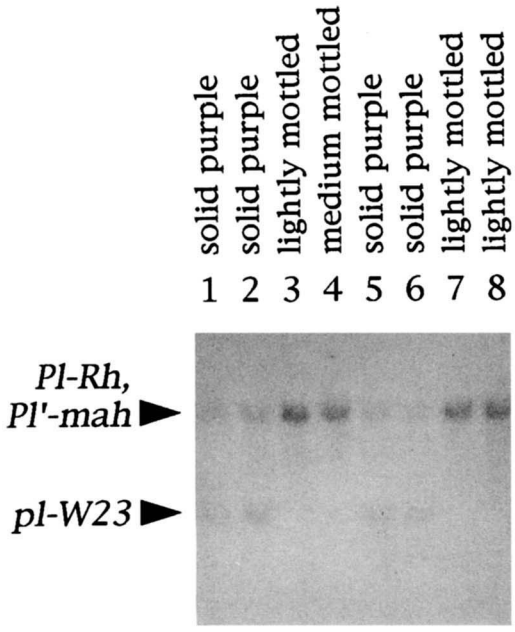


FIGURE 5.—RFLP analysis of *pl* genotype. Genomic DNA from the plants in the final generation shown in Figure 4 was digested with *Hin*dIII, electrophoresed, blotted, and the resulting blot probed with a 0.2-kb *Xho*I/*Dra*I probe generated from *Pl-Rh* (MATERIALS AND METHODS). Previous digests (not shown) demonstrated that both *Pl-Rh* and the *pl* allele found in the mahogany plants generate a ~12-kb fragment, while *pl-W23* generates a ~6.6-kb fragment. The anther phenotypes of the plants from which the DNA was prepared are shown above each lane. Presented data are a representative sample of 42 plants that were examined.

length polymorphism (RFLP) analysis (Figure 5). Previous RFLP studies had shown that *Pl-Rh* has the same restriction pattern as the *pl* allele found in mahogany plants, but is distinguishable from recessive *pl* alleles such as *pl-W23* (CONE *et al.* 1993; K. CONE, unpublished data). Plants in the two phenotypically darkest categories were *Pl-Rh/pl-W23*, and plants in the two lightest categories were *Pl'-mah/Pl-Rh* (Table 1, Figure 5). Thus, *pl* genotypes segregated with the anther phenotypes predicted by the hypotheses that the mahogany phenotype was linked to the *pl* locus and that *pl-W23* is neutral with respect to any effects on *Pl-Rh*. The fact that all plants with the *Pl'-mah/Pl-Rh* genotype produced only the lighter mottled mahogany anthers demonstrates that the dominant character of the mahogany phenotype is linked to the *Pl'-mah* allele. Given that the number of individuals examined was small (42) this genetic activity could formally be as much as seven centimorgans from *pl* (see MATERIALS AND METHODS for calculation) at the 5% limit. The three *Pl-Rh/pl-W23* plants that were scored as having a dark mottled phenotype (Table 1) may represent spontaneous derivatives of *Pl-Rh*. Similar cosegregation experiments demonstrated that the mahogany phenotype is not linked to either *b* or *r* (G. I. PATTERSON and V. L. CHANDLER, unpublished data).

Exclusive transmission of the mahogany phenotype is not due to segregation distortion: Exclusive transmission of the *Pl'-mah* chromosome from a *Pl'-mah/Pl-Rh* plant could account for the fact that all progeny receive the *Pl'-mah* allele. To address this possibility, we used a genetically linked morphological marker to follow the individual chromosomes. The *salmon silks* (*sm*) locus is located 10 cM distal to the *pl* locus on 6L. In the presence of dominant *p* alleles, *P-rr* or *P-wr*, recessive mutations at *sm* result in accumulation of an orange-pink salmon color in the silks (COE *et al.* 1988). Our *Pl'-mah* stocks contain neither the *sm* allele nor the appropriate alleles at *p* necessary for salmon-colored silks. A *sm, P-rr* stock with the standard dark anther phenotype was crossed to our mahogany stock (*Pl-Rh/Pl'-mah*; ACS 1) and the resultant F_1 plants were self-pollinated to generate segregating populations. All 16 F_1 plants had a mahogany phenotype consistent with the idea that the *sm* stock contains an allele at *pl* sensitive to paramutation. A total of 316 F_2 plants (six families derived from the self-pollinated ears of six mahogany F_1 plants) were scored for both anther and silk color. All F_2 plants had the mahogany phenotype and 61 of these had salmon colored silks. Assuming normal segregation, the expected frequency for salmon silk color would be the product of the frequencies for obtaining a dominant *p* allele (0.75) and two recessive *sm* alleles (0.25), 59 out of 316 plants. The close agreement between the expected and observed frequencies of *sm* individuals ($\chi^2 = 0.00016$) demonstrates that the chromosome containing the *sm* marker is segregating normally. This indicates that the chromosome containing *Pl-Rh* upon entry into the F_1 cross exits carrying *Pl'-mah*. In the absence of segregation distortion, we suggest that presence of the *Pl'-mah* allele somehow results in the alteration of the *Pl-Rh* allele so that it is now indistinguishable from *Pl'-mah*. These observations are consistent with the hypothesis that *Pl-Rh* is a paramutable allele and that *Pl'-mah* is a corresponding paramutant derivative that is paramutagenic.

***Pl-Rh* derivatives are metastable:** As previously indicated, derivatives with reduced pigmentation can spontaneously arise from the standard phenotype (Figure 3A). To better define the properties of such allelic variants, examples representing each anther color score in Figure 3A were subsequently crossed. Some plants were self-pollinated to test the heritability of the particular anther color potential, and other plants were crossed by *Pl-Rh* sibs (ACS 7) to test whether or not the spontaneous derivatives could influence the activity of a naive (not previously exposed to such derivatives) *Pl-Rh* allele. Anther color scores of the resultant progeny are shown in Figure 6, A and B. The anther color classes can be subdivided based on the stability of the pigment phenotype and their efficiency to induce a change in *Pl-Rh*. Upon self-pollinations, spontaneous derivatives with ACS of 1-4 exclusively transmit a similar or lower ACS

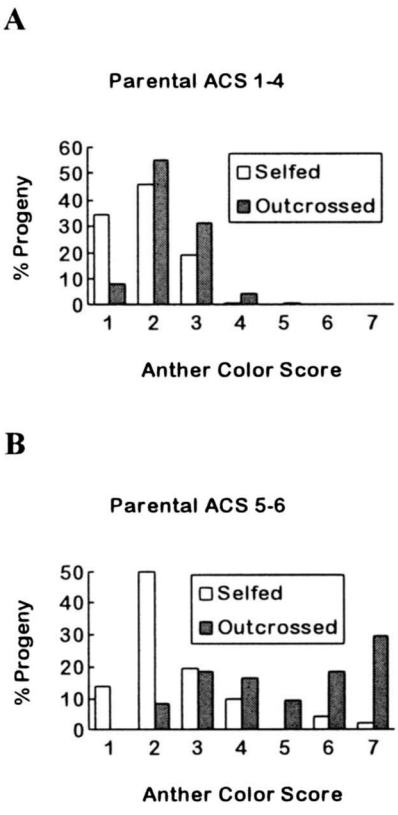


FIGURE 6.—Stability and paramutagenicity of *Pl-Rh* and its spontaneous derivatives. The source of spontaneous *Pl-Rh* derivatives is described in RESULTS and is graphically represented in Figure 3A. (A) The distribution of anther color scores from progeny derived by either self-pollination or through outcrosses by *Pl-Rh/Pl-Rh* are compared for parent plants with anther color scores between 1 and 4. A total of 124 plants from 18 self-pollinated families and 274 plants from 31 outcross families were scored for this experiment. (B) The same comparison as made in (A) is presented for parental plants with anther color scores of 5 or 6. A total of 92 plants from 10 self-pollinated families and 98 plants from 11 outcross families were scored for this experiment.

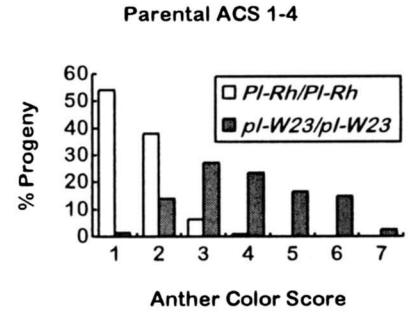


FIGURE 7.—Metastability of *Pl'-mah*. Related *Pl-Rh/Pl'-mah* F_1 plants were crossed by the indicated testers and the anthers of the resultant progeny were scored. The histogram illustrates the frequency of progeny with the indicated anther color scores. A total of 678 plants from 14 individual *Pl'-mah/Pl-Rh* × *Pl-Rh/Pl-Rh* crosses are compared with 356 plants from seven individual *Pl'-mah/Pl-Rh* × *pl-W23/pl-W23* crosses.

to their offspring demonstrating that reduced pigment levels are highly heritable. Although the average ACS of progeny derived from backcrosses by *Pl-Rh* is slightly greater than from self crosses, no offspring with a standard phenotype (ACS of 7) are recovered (Figure 6A). Thus, alleles that express low anther color scores are strongly paramutagenic in subsequent generations. Based on the spontaneous acquisition of a highly penetrant paramutagenic state, these exceptional derivatives of *Pl-Rh* are indistinguishable from the originally identified *Pl'-mah* allele. In contrast to the fully penetrant phenotype associated with an ACS of 1-4, the ACS 5-6 classes were more variable. Plants with an ACS of 5-6 frequently transmitted a higher ACS to their progeny of self-pollinations (Figure 6B), suggesting that the pigmentation activity of these derivatives is not irreversibly reduced. Upon backcrossing by *Pl-Rh*, ACS 5-6 individuals were still able to incite paramutation, as evidenced by the fact that a large portion of the progeny had an anther color score of 1-4 (Figure 6B). However, ACS 5-6 individuals caused paramutation less efficiently than the ACS 1-4 individuals, as evidenced by the recovery of progeny that had an ACS of 7 (Figure 6B). Four ACS 7 plants in Figure 6B were subsequently self-pollinated and these transmitted the fully pigmented phenotype to their offspring (data not shown). The same plants (ACS 7, Figure 6B), when outcrossed to *Pl'-mah/Pl'-mah* plants, exclusively produced progeny with the mahogany phenotype, demonstrating that these revertant alleles remain fully sensitive to *Pl'-mah*. Thus slightly paramutagenic *Pl-Rh* derivatives (ACS 5-6) are able to revert to *Pl-Rh* (ACS 7).

The spontaneous instability together with the correlation between anther pigmentation and paramutagenicity indicates that the *Pl-Rh* allele can exist in multiple genetic "states". The fully expressed state (ACS 7) is relatively stable (Figure 3A); stocks exist where no spontaneous derivatives (ACS 1-6) have been observed (0/307 progeny of 18 individual crosses) (J. B. HOLLICK and V. L. CHANDLER, unpublished observations). The weakest expression state characterized by a 1-4 ACS is very stable and highly paramutagenic. The intermediate state (ACS 5-6) appears to be metastable as both ACS 7 and ACS 1-4 progeny can be transmitted from those plants.

Genetic metastability of *Pl'-mah* is enhanced in stocks containing an insensitive *pl* allele: As previously mentioned, the *pl-W23* allele is insensitive to causing or responding to paramutation. However, exposure of *Pl'-mah* alleles to a stock containing *pl-W23* increases the pigmentation potential and decreases the paramutagenic abilities of the *Pl'-mah* alleles. Unlike outcrosses by *Pl-Rh* individuals, progeny with the standard dark phenotype (ACS 7) can be recovered when mahogany plants (ACS 1-4) are outcrossed by *pl-W23/pl-W23* individuals (Figure 7). These standard dark progeny behave like naive *Pl-Rh* plants in subsequent testcrosses; they usually transmit the standard phenotype through two generations of crosses to *Pl-Rh/Pl-Rh* plants, and they

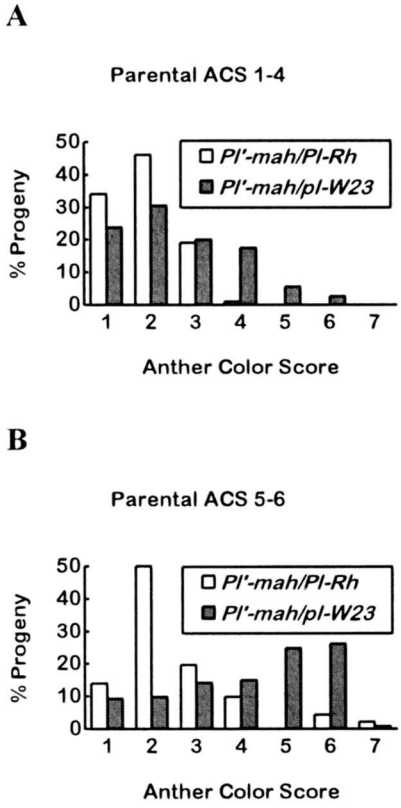


FIGURE 8.—Stability and paramutagenicity of *Pl'-mah* in stocks containing a paramutation-neutral *pl* allele. (A) Progeny derived from self-pollinations of either *Pl'-mah/Pl-Rh* or *Pl'-mah/pl-W23* parents with anther color scores of 1–4 are plotted together for comparison. *Pl'-mah/pl-W23* parents are derived from material represented in Figure 7. Data for the *Pl'-mah/Pl-Rh* parents are reproduced from Figure 6A. Data for the *Pl'-mah/pl-W23* parents are compiled from 160 progeny of 28 crosses. (B) A similar comparison as made in (A) is presented for parents with anther color scores of 5 or 6. Data for the *Pl'-mah/Pl-Rh* parents are reproduced from Figure 6B. Data for the *Pl'-mah/pl-W23* parents are compiled from 141 progeny of 24 crosses. Progeny with pink, nonmottled anthers characteristic of the *pl-W23/pl-W23* genotype are not included.

exclusively transmit the mahogany phenotype after crosses to *Pl'-mah* plants. In addition, progeny with anther color scores greater than those of their parents (ACS 1-4) occur at a high frequency relative to crosses with *Pl-Rh* (57.5% *vs.* 1% progeny with an ACS greater than 1-4; Figure 7). The same effect is displayed among the progeny from self-pollinations of these *Pl'-mah/pl-W23* F_1 plants. Comparisons of anther color score distributions for the progeny of *Pl'-mah/Pl-Rh vs. Pl'-mah/pl-W23* parent plants (Figure 8, A and B) indicate that exposure to this *pl-W23* stock increases the pigmenting potential of *Pl'-mah*. Note that progeny from self-pollination of a *Pl'-mah/pl-W23* plant are segregating three *pl* genotypes; *Pl'-mah/Pl'-mah, Pl'-mah/pl-W23,* and *pl-W23/pl-W23*. The first two genotypes were phenotypically indistinguishable and therefore pooled together whereas the third genotype was distinctive (see MATERIALS AND METHODS) and excluded from the data presented in Figure 8. Additional self crosses of this material allowed the first two genotypes to be distinguished; no detectable bias for particular anther color scores could be attributable to particular genotypes (J. B. HOLLICK and V. L. CHANDLER, unpublished observation). Thus in the presence of *Pl-Rh*, the ACS 1-4 state of *Pl'-mah* is stable yet the same *Pl'-mah* allele appears to be predominantly metastable in the presence of *pl-W23* as both higher and lower anther color scores can be transmitted.

DISCUSSION

In this report, we demonstrate that a novel anthocyanin phenotype shows linkage to the *pl* locus and designate this new allele *Pl'-mahogany*. This allele has the unusual property that only *Pl'-mah* is transmitted from a *Pl'-mah/Pl-Rh* heterozygote. This preferential inheritance is not due to segregation distortion because a closely linked genetic marker distal to *Pl-Rh* is transmitted normally. Thus, *Pl-Rh* changes into *Pl'-mah* at a frequency of 100% in the *Pl-Rh/Pl'-mah* heterozygote. Such behavior fits the description of paramutation. *Pl-Rh* is an inherently unstable paramutable allele that can spontaneously change to paramutagenic derivatives. The paramutagenic strength of these derivatives is inversely correlated with the phenotypic expression of anther pigment. Derivatives with weaker anther pigmentation (ACS 1-4) are highly efficient at reducing the heritable activity of associated *Pl-Rh* alleles. In contrast, derivatives with only slightly reduced anther pigment relative to *Pl-Rh* (ACS 5-6) are less efficient at causing paramutation of *Pl-Rh*. The paramutagenic derivatives of *Pl-Rh* are also metastable in that their pigmentation and associated paramutagenic activities can heritably change in either direction. This metastable property is most notable when allelic activity is intermediate (ACS 5-6) and full reversion of the paramutagenic state can occur. Exceptions to the relative stability of the lightly mottled anther phenotype (ACS 1-4) are observed in crosses to a related stock carrying a paramutation-neutral *pl* allele, *pl-W23*.

Specific behavioral aspects of *pl* paramutation are similar to those seen at either the *b* and *r* loci but not both. In fact, the *pl* system exhibits exactly those features that have been used to distinguish *b* and *r* paramutation. These similarities and differences are compared below.

Inherent instability of paramutable alleles: B-Intense (B-I), Pl-Rh, and standard *R-r* (*R-r*) are the paramutable alleles of the *b, pl,* and *r* loci, respectively. All these alleles exhibit genetic instability but to different extents. *B-I* normally confers intense pigmentation in mature plant tissues, but *B-I* can spontaneously change to a drastically weaker colored paramutagenic derivative, *B'* (COE 1966). The frequency with which this change occurs

varies between $\sim 10^{-1}$ to 10^{-2} (COE 1966; PATTERSON *et al.* 1995; K. KUBO, G. I. PATTERSON and V. L. CHANDLER, unpublished observations). No intermediate phenotypes are seen. *Pl-Rh* also spontaneously changes to weaker derivatives yet intermediate states are detectable. The frequency of all spontaneous events is roughly similar to that seen with *B-I*. *R-r* confers mottled pigmentation to the aleurone layer of the seed when transmitted through the male lineage. The amount of mottling can be precisely quantified by visually scoring individual kernels from testcross ears and then averaging the scores to assess the activity of the transmitted allele (BRINK 1956). *R-r* activity is metastable; pigmentation activity can spontaneously increase, or decrease, in successive generations depending on the complementary allele with which it is maintained (STYLES and BRINK 1966). Although the range of *R-r* activity as assessed on a per kernel basis can be quite large, the averaged activity per ear changes only slightly (BROWN and BRINK 1960). However, drastic reductions in *R-r* activity occur rapidly through heterozygosity with other, distinctively paramutagenic, *r* alleles. Such affected *R-r* alleles are designated *R-r'*. *R-stippled* (*R-st*) is one such paramutagenic *r* allele that can cause this change. Although *R-r* activity can spontaneously fluctuate, acquisition of the *R-r'* state requires exposure to a paramutagenic *r* allele. Thus, while paramutable alleles of *b*, *pl* and *r* are inherently unstable, only *B-I* and *Pl-Rh* spontaneously change to very weak derivatives that are themselves strongly paramutagenic.

The innate ability to heritably change gene activity appears to be a prerequisite feature of paramutable alleles. The magnitude with which spontaneous changes occur is notably different between the various alleles. This difference might be related to an allelic, or chromosome, structure that dictates how easily the change can take place. Both *B'* and *B-I* are simple, with only one coding region each that contains no detectable molecular differences (PATTERSON *et al.* 1993). *Pl-Rh* and *Pl'-mah* are similarly simplex although they both contain a 3' duplication of ~1 kb of 3' untranslated and 3' flanking sequences (CONE *et al.* 1993; J. B. HOLLICK and V. L. CHANDLER, unpublished results). In contrast, *R-st* and *R-r* are complex at both genetic and molecular levels (DOONER and KERMICLE 1971; EGGELSTON *et al.* 1995). Molecularly simple *B-I* and *Pl-Rh* alleles may undergo rapid changes in activity states while analogous changes at the more complex *R-r* allele may be relatively inhibited. Alternatively, different chromosomal locations or associated elements may be more prone to such alterations. Applying such models, *B-I* would be most efficiently changed to *B'*; intermediate states may never be detected for this reason. In contrast, a full range of spontaneous *Pl-Rh* derivatives are detectable due, perhaps, to a slower progressive change. The seemingly random distribution of derivatives with various anther color scores (Figure 3A) might reflect the timing during somatic development that the process is initiated. As the male and female reproductive structures are separated by both developmental time and space (JOHRI and COE 1983), this idea is potentially testable through comparison of alleles that are transmitted through reciprocal crosses to a common *Pl-Rh* tester. In comparison to *B-I* and *Pl-Rh*, changes at *R-r* would occur more slowly or less efficiently. This idea is consistent with the observation that relatively large scale spontaneous changes in *R-r* pigmenting potential are only achieved after multiple generations (STYLES and BRINK 1966).

Stability of paramutant derivatives: Independent of their origin (spontaneous versus exposure to an established paramutagenic allele) paramutant alleles can be extremely stable. *B'* does not spontaneously revert (COE 1966; PATTERSON *et al.* 1993; K. KUBO, G. I. PATTERSON and V. L. CHANDLER, unpublished observations). *Pl'-mah* (ACS 1-4) is very stable when maintained with *Pl-Rh* alleles (Figures 6A and 7). Although *R-r'* spontaneously reverts (BRINK 1956), *R-r''* (two generations of exposure to paramutagenic alleles) does not (MIKULA 1961). Thus the change from *B-I* to a stable *B'* appears to occur in a single discrete interval whereas the change of *R-r* to a stable paramutant derivative is progressive and involves metastable intermediates. The states of *Pl'-mah* ACS 1-4 and *R-r''* may be analogous to the exceedingly stable *B'*. Thus weak gene activity appears to be favored. Once such a stable state is established, it may not be readily reversible. In contrast, intermediate allelic states like *Pl'-mah* (ACS 5-6) and *R-r'* are metastable; they can fully revert to, or change towards, higher gene activity. Potentially related to the ease with which such changes occur, *Pl'-mah* (ACS 5-6) can revert to *Pl-Rh* in the span of a single generation while similar reversions of *R-r'* require multiple generations (BRINK 1964). These differences may reflect inherent properties of the various alleles that influence the stability of particular allelic states. The above comparisons of these allelic states suggests a continuum of stability; *B'* > *Pl'-mah* (ACS 1-4) ~*R-r''* > *Pl'-mah* (ACS 5-6) > *R-r'* > *B-I* ~*Pl-Rh* > *R-r*.

An additional indicator of metastability is the influence of neutral alleles on the stability of a given allelic state. At *r*, it has been established that the pigmenting activity of both *R-r* and *R-r'* is influenced by exposure to paramutation-neutral alleles like *r-g*. The pigmenting potential of both *R-r* and *R-r'* is heritably increased when either heterozygous with *r-g* or hemizygous via a deletion (STYLES and BRINK 1966, 1969). These findings demonstrate that *r-g* is inert with respect to effects on *R-r* stability and expression. In the absence of *R-r*, the innate propensity for *R-r'* change is toward higher states of expression. Results of crosses with stocks containing the *pl-W23* allele show a similar effect; the pigmentation activity of *Pl'-mah* alleles increases substantially when *pl-W23* is present in comparison to crosses where *pl-W23* is absent (Figures 7 and 8). Although related, the *pl-*

W23 and *Pl-Rh* stocks (K55/W23 *vs.* W23) are not isogenic so we cannot attribute this effect to the *pl-W23* allele itself. The potential parallel with *r* paramutation is nonetheless intriguing. Without ascribing the observed effect on *pl-W23* or potential nuclear background effects, however, such effects on reversion or metastability are not seen with *B′*. *B′* is stable in both the K55 and W23 backgrounds, even when heterozygous with *b-W23* or other *b* alleles (COE 1966). Based on the effect of hemizygosity on *R-r′* activity mentioned above, metastability may be more related to the absence of contact with a paramutable partner than to association with specific paramutation-neutral alleles. The reason metastability is not seen with *B′* may relate to the extreme stability of this allelic state. If the presence of paramutable alleles contributes to the stability of the paramutant state, then one might expect spontaneous paramutation of *B-I* and *Pl-Rh* to occur much more frequently in *B-I/B-I* and *Pl-Rh/Pl-Rh* stocks than in *B-I/b-W23* or *Pl-Rh/pl-W23* stocks. COE (1966) reported observations at *b* supporting this idea, and we are currently pursuing similar studies on both *b* and *pl* to further test this prediction.

F_1 phenotypic suppression: When *B′* is combined with *B-I*, the F_1 heterozygote displays a phenotype that is similar to that of a *B′/B′* homozygote (COE 1966; PATTERSON and CHANDLER 1995b). Transcription of *b* is correspondingly reduced (PATTERSON *et al.* 1993). The phenotypic expression of *Pl-Rh* is also notably suppressed in *Pl′-mah/Pl-Rh* heterozygotes. In contrast to the reduced pigmentation of *B′/B-I* and *Pl′-mah/Pl-Rh* plants, the F_1 heterozygote of *R-r′/R-r* has fully colored aleurone tissue in the seed; reduced activity is not detected. Even in *R-st/R-r* heterozygotes, *R-r* action is not notably altered in aleurone tissue (MCWHIRTER and BRINK 1963) yet following sporophytic development *R-r* is sexually transmitted in a less active state. Failure to detect suppressed aleurone expression may be due to the distinctive process of triploid endosperm development or may indicate that the suppression process requires a requisite number of cell cycles to become manifest. There are certain paramutable *r* alleles that do exhibit F_1 phenotypic suppression. One particular paramutable *R-g* allele confers coloration to both aleurone and seedling tissue. Tissues of *R-st/R-g* seedlings are less pigmented than those of *r-g/R-g* (BRINK *et al.* 1970) but the amount of detectable suppression is slight in comparison with the *b* and *pl* examples. In contrast to *R-st*, the *R-r′* allele alone is too weak to have a detectable effect on naive *R-g* in the initial *R-r′/R-g* heterozygote.

Exactly when during ontogeny exposure to a strongly paramutagenic partner leads to a heritable change has been the subject of debate (SASTRY *et al.* 1965; COE 1968; PATTERSON and CHANDLER 1995b). Phenotypic suppression seen in the initial F_1 heterozygotes between paramutable and paramutagenic alleles would seem to indicate that the allelic changes take place relatively early. However, visually detectable suppression of *B-I* in *B-I/B′* plants may not necessarily reflect the heritable alteration of *B-I* as irradiation-induced loss of the *B′* chromosome during embryonic development gives rise to large phenotypically *B-I* sectors (COE 1966). Alternatively, the heritable alteration could take place relatively late in development as long as it occurs prior to the differentiation of cells producing pigment (PATTERSON and CHANDLER 1995b). It thus remains unresolved whether allelic suppression in the soma and meiotically heritable suppression are separable events. This question is relevant not only to paramutation but also to cases of transgene *trans*-inactivation and co-suppression (MATZKE and MATZKE 1993) where allelic interactions lead to reduced gene expression yet do not always incite stable heritable changes.

The mahogany phenotype is particularly well suited to address the timing of heritable alteration. Because the F_1 phenotype is visible in the seedling and mature plant, irradiation-induced loss of the *Pl′-mah* allele at various times during embryogenesis and seedling germination should allow visual comparison of meristematic-derived sectors (early in ontogeny, large sectors) to those that arise immediately before terminal differentiation of the epidermal cells (late in ontogeny, small sectors). The size of the smallest sector that retains a *Pl-Rh* phenotype would demarcate when in development the somatically heritable change to *Pl′-mah* is seen. Additionally, there is a clonal lineage relationship between the pigmented endothecium cells of the anther and its microsporangium (DAWE and FREELING 1990). Thus the size of the smallest endothecium sector that still transmits *Pl-Rh* alleles from the associated anthers should indicate the approximate time during ontogeny when the meiotically heritable change occurs.

Paramutagenic strength: As stated above, a definitive feature of paramutation is the heritable alteration of a paramutable allele in the presence of a paramutagenic allele. *B′* is exceedingly paramutagenic; *B-I* is almost never transmitted from a *B′/B-I* heterozygote (COE 1966; PATTERSON *et al.* 1993, 1995; K. KUBO and V. L. CHANDLER, unpublished observations). Presence of *B′* causes a heritable alteration of *B-I* so that it is virtually indistinguishable from *B′*. Concomitant with the reduced pigmentation in *B′*, there is a 10–20-fold decrease in *de novo b* transcription compared with *B-I* (PATTERSON *et al.* 1993). In contrast to this discrete change in *B-I*, changes in *R-r* expression can span a continuous range. Paramutagenic strength of *r* alleles is defined by the magnitude of the phenotypic change imparted to *R-r* (MCWHIRTER and BRINK 1962). By such tests, *R-st* is extremely paramutagenic; the *R-r′* recovered from *R-st/R-r* confers much less aleurone pigmentation than the parental *R-r*. Unlike the *B′/B-I* system, *R-st* is not a spontaneous derivative of *R-r*; *R-st* is distinctive from *R-r* and has no known nonparamutagenic progenitors (BRINK 1973). Results of our genetic tests suggest that

the paramutagenic strength of spontaneous *Pl'-mah* alleles is inversely related to their pigmentation potential (Figure 6). This finding implies the involvement of a quantitative repressor in the process related to heritable suppression. A similar conclusion is drawn from the paramutagenicity of *R-st* and its multiple *R-self colored* (*R-sc*; fully colored aleurone) derivatives. Primarily as a result of unequal crossing over, fully colored derivatives of *R-st* occur at a high frequency (ASHMAN 1960). A test of 83 such derivatives revealed that the paramutagenic activity could be decreased, eliminated, or even increased such that a continuum of activity could be represented (MCWHIRTER and BRINK 1962). This actually reflects the fact that the paramutagenic activity of *R-st* is physically distributed over a large region of DNA that includes multiple duplications of the *r* gene coding and intervening sequences (KERMICLE *et al.* 1995). Although the discrete behavior of *B-I* and *B'* appears at odds with the proposed involvement of quantitative repressors, *B-I* and *B'* may utilize the same type of repressive elements yet be so sensitive to their action that intermediate states are not seen.

Subsequent paramutation: "New" *B'* alleles (previously *B-I*) derived from a *B-I/B'* heterozygote are equally efficient as "old" *B'* alleles in directing paramutation of *B-I* to *B'* in subsequent generations (COE 1966; PATTERSON and CHANDLER 1995b). Similarly, all progeny of a *Pl'-mah/Pl-Rh* × *Pl-Rh/Pl-Rh* mating have a clear mahogany phenotype (ACS 1-4; Figure 7). Overlap between the *Pl'-mah* and *Pl-Rh* phenotypes is not seen. The "new" *Pl'-mah* (*Pl-Rh* in the previous generation) efficiently alters a naive *Pl-Rh* allele, which is similar to "new" *B'* altering *B-I*. In contrast with this strong paramutagenic activity, *R-r'* alleles derived from a *R-st/R-r* heterozygote are only weakly paramutagenic. The average aleurone color score phenotype of "new" *R-r'/R-g* is different from that of *R-r/R-g*, but the distribution of these color score phenotypes overlap substantially (BROWN and BRINK 1960). Thus despite a large reduction in gene action, *R-r'* alleles fail to acquire a strong paramutagenic function. Detectable paramutagenic activity at *R-r'* is subsequently lost in successive generations due in part to its propensity for reversion (BRINK 1956). The changes in *R-r* induced by a single exposure with *R-st* appear to be transitory. The *R-r'* state is thus inherently unstable both in terms of pigmentation potential and paramutagenic action.

Discussions of *b* and *r* paramutation have often emphasized the large differences between the phenomenology at the two genes and led to the suggestion that paramutation may be mechanistically different at the two genes (COE 1966; BRINK 1973; PATTERSON and CHANDLER 1995a). The behavior of *Pl-Rh* shares similarities with precisely those features that, to this point, have discriminated *b* and *r* paramutation; namely spontaneous acquisition of a detectably paramutagenic state, strong phenotypic suppression in the F_1 heterozygote, and allelic metastability. This finding illustrates that those previously distinguishing features need not reflect disparate paramutation-like mechanisms. The continuum of allelic stabilities discerned through comparisons of *pl* with both *b* and *r* indicates that the basic process of paramutation may be mechanistically similar at all three maize loci. We suspect that the differences between these alleles arise from intrinsic properties relating to their utilization of common molecular components. One testable prediction of such a model is that at least some *trans*-acting mutations which disrupt or impair paramutation at one locus should affect the process at the other loci as well. Paramutation at *pl* is an attractive genetic target for isolating such mutations. In maintenance with *Pl-Rh*, the *Pl'-mah* (ACS 1-4) allele is exceedingly stable and paramutagenic. The F_1 suppression is detectable in germinated seedlings (Figure 2D), allowing large numbers of mutational events to be screened. In addition to finding *trans*-acting suppressors of *pl* paramutation, such mutation strategies should help define the relevant *cis*-linked regions that specifically mediate the process between the *Pl-Rh* and *Pl'-mah* alleles.

It is interesting that paramutation in maize has only been described for the anthocyanin regulatory genes. Before the study described here, *b* and *r* were the only maize genes well documented to undergo paramutation. Given that these genes encoded functionally equivalent proteins, it was not surprising that they shared this feature as well. The observation that a structurally unrelated anthocyanin gene, *pl*, can also undergo paramutation suggests that paramutation may be more prevalent than previously thought. There are hints that another gene, *p*, which regulates the related phlobophene pigment pathway may also undergo paramutation (DAS and MESSING 1994; J. MESSING, personal communication). We suspect that the pigment pathways in maize are particularly suited to identifying phenomena such as paramutation. The pigments are not essential and variants produce phenotypes that are easily identified by visual inspection. We suspect paramutation has been observed at the regulatory genes because the pathways are very sensitive to regulatory gene expression, enabling even subtle changes in the levels of a regulatory protein to be visually detected.

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