DOI: 10.1534/genetics.105.045260

Rmr6 Maintains Meiotic Inheritance of Paramutant States in *Zea mays*

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Manuscript received May 6, 2005
Accepted for publication July 8, 2005

ABSTRACT

Paramutation generates heritable changes affecting regulation of specific alleles found at several *Zea mays* (maize) loci that encode transcriptional regulators of anthocyanin biosynthetic genes. Although the direction and extent of paramutation is influenced by poorly understood allelic interactions occurring in diploid sporophytes, two *required to maintain repression* loci (*rmr1* and *rmr2*), as well as *mediator of paramutation1* (*mop1*), affect this process at the *purple plant1* (*pl1*) locus. Here we show that the *rmr6* locus is required for faithful transmission of weakly expressed paramutant states previously established at both *pl1* and *red1* (*r1*) loci. Transcriptional repression occurring at both *pl1* and *booster1* (*b1*) loci as a result of paramutation also requires *Rmr6* action. Reversions to highly expressed, nonparamutant states at both *r1* and *pl1* occur in plants homozygous for *rmr6* mutations. Pedigree analysis of reverted *pl1* alleles reveals variable latent susceptibilities to spontaneous paramutation in future generations, suggesting a quantitative nature of *Rmr6*-based alterations. Genetic tests demonstrate that *Rmr6* encodes a common component required for establishing paramutations at diverse maize loci. Our analyses at *pl1* and *r1* suggest that this establishment requires *Rmr6*-dependent somatic maintenance of meiotically heritable epigenetic marks.

MEIOTICALLY heritable alterations in gene regulation conditioned by specific allelic interactions are known as paramutations (Brink 1958). At least four distinct examples of paramutations occur in *Zea mays* (maize) (Hollick *et al.* 1997; Chandler *et al.* 2000; Chandler and Stam 2004). Specific alleles exhibit this unusual inheritance behavior at the *red1* (*r1*), *booster1* (*b1*), *pericarp1* (*p1*), and *purple plant1* (*pl1*) loci (Brink 1956; Coe 1966; Das and Messing 1994; Hollick *et al.* 1995), all of which encode transcriptional regulatory proteins of flavonoid biosynthetic pathways. In general, these unique alleles can exist in a dynamic range of regulatory states manifest as heritable differences in visual patterns and levels of colorful red or purple pigments. Both the heritable switching and the stability of these states are influenced by the homologous allele in diploid sporophytes (Coe 1966; Styles and Brink 1966; Das and Messing 1994; Hollick and Chandler 1998). Such allelic interactions affecting inheritance of regulatory information has important implications for breeding efforts and represents a potential novel resource of heritable variation in plants (Kermicle and Alleman 1990; Hollick and Chandler 1998).

The *pl1* locus encodes a R2R3 MYB domain protein (Cone *et al.* 1993) that, in combination with basic helix-loop-helix domain proteins encoded by either *b1* or *r1* loci, promotes transcription of genes encoding enzymes required for anthocyanin biosynthesis (Goff *et al.* 1992). The *Pl1-Rhoades* allele confers the highest pigment levels of any previously described *pl1* allele. The high-expression reference state selected in culture (denoted *Pl-Rh*) is, however, unstable and can spontaneously change to weaker expression states referred to as *Pl′* (Figure 1A; Hollick *et al.* 1995). A continuum of *Pl1-Rhoades* expression states can be achieved and is quantified using a visual 1–7 graded anther color score (ACS; Figure 1B; Hollick *et al.* 1995). Different *pl1* RNA levels are directly correlated with these anther phenotypes and are considered to reflect differences in transcription rates as measured from isolated husk nuclei (Hollick *et al.* 2000).

Pigment levels produced from various *Pl1-Rhoades* expression states are inversely correlated with levels of an experimentally defined activity—termed paramutagenicity—that facilitates heritable changes of the homologous *Pl1-Rhoades* expression state in *trans* (Hollick *et al.* 1995). *Pl′* states representing ACS 1–4 classes are highly paramutagenic to the *Pl-Rh* (ACS 7) reference state: when *Pl1-Rhoades* alleles of both *Pl′* and *Pl-Rh* states are combined in a diploid sporophyte, only *Pl1-Rhoades* alleles of ACS 1–4 *Pl′* states are sexually transmitted (Figure 1C; Hollick *et al.* 1995). This is the general behavior of paramutation; certain allelic interactions lead to directed, and heritable, changes in gene action. A similar example at the *sulfurea* locus of *Lycopersicum esculentum* affecting chlorophyll levels (Hagemann 1969) indicates that paramutation is not confined to maize or to regulators of flavonoid biosynthesis. Parallels

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noted among diverse examples of non-Mendelian inheritance patterns suggest that paramutation may be a general feature of many eukaryotic genomes (Chandler and Stam 2004).

Several *trans*-acting components of the paramutation process have been identified by mutational analysis. Recessive mutations in *mediator of paramutation1* (*mop1*; Dorweiler *et al.* 2000) and *required to maintain repression1* and *-2* (*rmr1*, *rmr2*; Hollick and Chandler 2001) lead to elevated *pl1* RNA levels and corresponding increases in pigmentation from *Pl1-Rhoades* alleles of *Pl′* state, suggesting involvement of *Mop1*, *Rmr1*, and *Rmr2* functions in somatic maintenance of transcriptional repression. Indeed, *Mop1* aids somatic maintenance of transcriptional repression of paramutant *b1* alleles (*B1-Intense* in the *B′* state) and is required to establish meiotically heritable paramutant states at both *b1* and *r1* loci (Dorweiler *et al.* 2000). One distinction of *b1* paramutation is the remarkable stability of *B′* states; regardless of somatic phenotype, *B′* states are faithfully transmitted, even from plants homozygous for *mop1* mutations (reviewed in Chandler *et al.* 2002). In contrast, *Pl′* and paramutant *R-r:standard* (*R-r′*) are reversible (Styles and Brink 1966; Hollick and Chandler 1998). *Pl1-Rhoades* alleles of *Pl′* state can heritably revert to fully active *Pl-Rh* in hemizygous condition (Hollick and Chandler 1998; J. Hollick, unpublished results) in heterozygous combination with structurally distinct *pl1* alleles (Figure 1C; Hollick and Chandler 1998) or after passage through *mop1* (Dorweiler *et al.* 2000), *rmr1*, or *rmr2* homozygous mutant plants (Hollick and Chandler 2001). Mutant analyses of *pl1* paramutation can therefore distinguish between functions required to maintain somatic repression and functions affecting meiotically transmissible information.

Although paramutation is often described as an example of homology-dependent gene silencing (Martienssen 1996; Matzke *et al.* 1996, 2002; Chandler and Stam 2004), it is important to recognize that paramutation at *pl1* and *r1* alters specific regulatory properties. The *Pl1-Rhoades* allele transitions from light-independent (*Pl-Rh*) to light-dependent (*Pl′*) regulatory modes, but transcription is still detectable from *Pl′* states (Hollick *et al.* 2000). Similarly, the seed-specific component of *R-r* haplotypes is conditioned by paternal imprinting to produce a variable and irregular mottled pigment pattern to the kernel aleurone, and paramutation to the *R-r′* state intensifies this effect (Kermicle 1970). In contrast, uniform seed pigmentation characterizes both *R-r* and *R-r′* states following female transmission. The ability of both *R-r:standard* and *Pl1-Rhoades* to adopt a range of quantitatively discrete regulatory states combined with the highly reversible nature of these states strongly suggests that genetic mechanisms operate on *R-r:standard* and *Pl1-Rhoades* to define meiotically heritable epigenetic states. Herein we present a mutational analysis detailing functions of a novel maize locus, *required to maintain repression6* (*rmr6*), encoding a key component of this genetic mechanism.

MATERIALS AND METHODS

Maize nomenclature: Following established guidelines (http://www.agron.missouri.edu/maize_nomenclature.html), chromosomes, loci, alleles, and allelic states are all designated by italic type. Phenotypes conferred by specific allelic state genotypes are designated by non-italic type. Chromosome translocation breakpoints are designated "*T*," while the same regions in structurally normal chromosomes are designated "+." When known, specific alleles are designated with hyphenated extensions (*i.e.*, *Pl1-Rhoades*). Dominant alleles either are abbreviated as "+" or begin with uppercase letters while recessive alleles are all lowercase. The prime symbol, "′," refers to a paramutant allelic state. Sporophyte genotypes are written with pistillate (female)-derived chromosomes, alleles, and allelic states preceding those derived from staminate (male) parents. In previous publications, *Pl-Rh* and *Pl′-mahogany* (*Pl′-mah* or *Pl′*) denoted distinct *pl1* alleles. Given the apparent lack of nucleotide polymorphisms, the continuum of quantitative expression states, and the high incidence of reversibility, the two extreme forms are discussed as representing alternative regulatory states of a single allele. Here, *Pl1-Rh* denotes the single *pl1* allele that is able to exist in either *Pl-Rh* or *Pl′* states of regulatory control.

Genetic stocks: All stocks contain dominant and functional alleles required for anthocyanin pigment production in anther tissues unless otherwise noted. Specific *b1*, *pl1*, and *r1* regulatory loci genotypes are noted since these directly affect pigment patterns used in stock syntheses and analyses. The *Pl1-Rhoades* and *B1-Intense* alleles used here derive from a full-color W23 line developed by Ed Coe, Jr. (USDA-ARS, University of Missouri, Columbia, MO) and maintained in several lines obtained from the Maize Genetics Cooperation Stock Center (USDA-ARS, University of Illinois, Urbana, IL) and Vicki Chandler (University of Arizona, Tucson, AZ). Elite inbred lines A619 and A632 were provided by the North Central Plant Introduction Station (USDA-ARS, Ames, IA). *Pl1-Rhoades* alleles of *Pl-Rh* state were introgressed into A619 and A632 inbred lines through recurrent backcrosses to the inbred lines as pistillate parents. Lines homozygous for *Pl1-Rhoades* alleles of *Pl-Rh* state, *Pl-Rh/Pl-Rh* (A619) and *Pl-Rh/Pl-Rh* (A632), were established with the following color factor genotypes: *b1-A619*, *Pl1-Rhoades* (*Pl-Rh*), *R-r*, (>98% A619); *B1-A632*, *Pl1-Rhoades* (*Pl-Rh*), *R1-A632* (>93% A632). Spontaneous paramutation of *Pl1-Rhoades* from *Pl-Rh* to *Pl′* states occurs in the A632-converted line at modest frequency and provided isogenic *Pl′/Pl′* individuals used in experiments represented in Table 2. Similar isogenic siblings were crossed to Coe's full-color W23 line [*B1-Intense* (*B-I*); *Pl1-Rhoades* (*Pl-Rh*); *R-r*] to generate *Pl-Rh/Pl-Rh* and *Pl′/Pl′* (W23/A632) hybrids used in one experiment described in Table 2. The *Pl-Rh/Pl-Rh* (W23/CO159) stock is a recombinant inbred line derived using the CO159 inbred and has the following color factor genotype: *b1-CO159*, *Pl1-Rhoades* (*Pl-Rh*), *R-r*. The source of the mutant alleles ems97406 and ems98225 is identical to that described in Hollick and Chandler (2001). The *Pl′/Pl′ T6-9* translocation stock was derived by isolating recombination events between the *Pl1-Rhoades* allele and the *6L* breakpoint present in the *T6-9* (*043-1*; Longley 1961) reciprocal translocation. Recombination frequencies between breakpoint and *pl1* translated to 1.5 cM with a 95% confidence interval of 1.1 cM. A *B-I/B-I* stock and isogenic *B′/B′* derivative used for the *b1* paramutation tests are of mixed parentage with plant

color genotypes *B1-I*; *Pl1-Rhoades* (*Pl-Rh*); *r-r*. The *rmr1-1*, *rmr2-1* lines (Hollick and Chandler 2001) and *mop1-1*, *mop1-2* lines (Dorweiler *et al.* 2000) are as previously described. *R-r:standard* (*R-r*), *R-stippled* (*R-st*), and *r-g* haplotypes used for *r1* paramutation tests derive from color-converted W22 inbred lines as previously described (Dorweiler *et al.* 2000).

Stock constructions and complementation tests: Hand pollinations were used for all genetic crosses and detailed pedigree information is available upon request.

Genetic complementation tests between the ems97406 mutation and other mutations known to affect *Pl′* were conducted using +/ems97406 plants as pistillate parent. Staminate parent genotypes, number of crosses, and number of progeny plants with specific anther phenotypes are as follows: *rmr1-1/rmr1-1*, 4, 87 ACS 1–4, 2 ACS 5–6; *rmr2-1/rmr2-1*, 4, 88 ACS 1–4; *mop1-1/mop1-1*, 3, 56 ACS 1–4. Tests with the ems98225 mutation were conducted using ems98225 homozygotes as staminate parents. Pistillate parent genotypes, number of crosses, and number of progeny plants with specific anther phenotypes are as follows: *rmr1-1/+*, 3, 38 ACS 1–4; *rmr2-1/+*, 2, 25 ACS 1–4; *mop1-2/+*, 2, 31 ACS 1–4.

For both RNase protection analyses and *in vitro* transcription reactions, progeny sets were generated segregating 1:1 for *+/rmr6-1* and *rmr6-1/rmr6-1* siblings. Plants of these two genotypes were clearly identified by contrasting Pl′-like and Pl-Rh-like anther phenotypes, respectively. For *in vitro* transcription reactions, progeny sets also segregated *B1-I* alleles of *B′* state as each parent was heterozygous *B′/b1*. In these families, *+/rmr6-1* and *rmr6-1/rmr6-1* plants were further distinguished by contrasting light and dark plant colors, respectively. Materials used for the *rmr6-2* RNase protection assay came from a single F_2 family segregating 1:2:1 for *rmr6-2/rmr6-2*, *+/rmr6-2*, and *+/+* siblings. Plants of these two latter genotypes have Pl′-like color phenotypes and are indistinguishable.

Reversion of *Pl′* to *Pl-Rh* in *rmr6-1/rmr6-1* plants was shown by following paramutagenic activity of *Pl′* states carried on the *T6-9 (043-1)* translocation chromosome following segregation from a single *rmr6-1* homozygote (Figure 3; Table 4). A family homozygous for the *T Pl′* chromosome and segregating 1:1 for *+/rmr6-1* and *rmr6-1/rmr6-1* genotypes provided the individual for these reversion tests. Pollen from this ACS 7 individual was shared between isogenic *Pl-Rh/Pl-Rh* and *Pl′/Pl′* testers (W23/A632). Progeny of the *Pl-Rh/Pl-Rh* testcross that had Pl-Rh-like anther phenotypes (ACS 7) were subsequently crossed to another *Pl-Rh/Pl-Rh* tester (W23/CO159). Construction of the *T Pl′* stock segregating the *rmr6-1* mutation was similar to that described for the *rmr6-2* stock used in the *pl1* paramutation tests: the *T Pl′* line was crossed with an *rmr6-1/rmr6-1*; *Pl′/Pl′* individual derived from a family segregating 1:1 for *+/rmr6-1* (Pl′-like anthers) and *rmr6-1/rmr6-1* (Pl-Rh-like anthers) genotypes and subsequent *T Pl′/T Pl′*; *+/rmr6-1* (Pl′-like anthers) and *T Pl′/T Pl′* ; *rmr6-1/rmr6-1* (Pl-Rh-like anthers) F_2 individuals were intercrossed to establish the segregating families. Reversion of *Pl′* to *Pl-Rh* in *rmr6-2/rmr6-2* plants (Table 5) was shown using a similar strategy to that listed above for *rmr6-1*. Details of this construction are included as part of the following description for *pl1*-induced paramutation tests.

For *pl1*-induced paramutation tests using *rmr6-2* alleles, *Pl-Rh* and *Pl′* states were combined in *rmr6-2* homozygotes through crosses between *+/rmr6-2* individuals: *Pl-Rh/T Pl-Rh*; *+/rmr6-2* crossed with *T Pl′/T Pl′*; *+/rmr6-2* (Figure 4A). Progeny plants with fully colored anthers (ACS 7; *rmr6-2/rmr6-2*) that display ~50% pollen abortion (*Pl-Rh/T Pl′*) were used to pollinate color-converted A619 *Pl-Rh* testers (Figure 4B). Both *rmr6-2* parental lineages derive from an initial cross between our *T Pl′* stock and a single *rmr6-2* homozygote produced by sib mating two original M_2 *rmr6-2* homozygotes. Homozygous *T Pl′* individuals were selected from a single F_2 family [individual kernels homozygous for the translocation chromosome have a diagnostic mutant endosperm phenotype due to a 2.3-cM genetic linkage between the *T6-9 (043-1)* breakpoint and a mutant *waxy1* allele] and sib crosses were made between individuals with a Pl′ anther phenotype (either *+/rmr6-2* or *+/+*) and those with a Pl-Rh-like anther phenotype (*rmr6-2/rmr6-2*) to establish families segregating 1:1 for *rmr6-2/rmr6-2* and *+/rmr6-2* genotypes. This segregating line, preserved for three generations using sib matings, provided the *T Pl′/T Pl′*; *+/rmr6-2* parent used in the acquisition of the paramutagenicity test (Figure 4A). From the same segregating line, a nonparamutagenic *Pl-Rh* revertant was isolated following outcross of a single *rmr6-2/rmr6-2* plant to Coe's W23 full-color stock (*B-I/B-I*; *Pl-Rh/Pl-Rh*; *R-r/R-r*); several progeny with ACS 7 anther phenotypes were recovered. Reversion of *Pl′* to a nonparamutagenic *Pl-Rh* state in individual 02-490-8 was confirmed by analyzing progeny derived from reciprocal backcrosses to Coe's W23 full-color stock (Table 5); 43/47 progeny, both fully fertile and semisterile types, had ACS 7 anther phenotypes. These ACS 7 progeny provided the *Pl-Rh/T Pl-Rh*; *+/rmr6-2* parental plants used in the acquisition of paramutagenicity tests (Figure 4A).

For *pl1*-induced paramutation tests using *rmr6-1* alleles, *Pl-Rh* and *Pl′* states were combined in *rmr6-1* homozygotes through crosses between individuals heterozygous for *rmr6-1*: *T Pl-Rh/pl1-A619*; *+/rmr6-1* crossed with *Pl′/Pl′*; *+/rmr6-1* (Figure 5A). Progeny plants with fully colored anthers (ACS 7; *rmr6-1/rmr6-1*) that display ~50% pollen abortion (*T Pl-Rh/Pl′*) were used to pollinate color-converted A619 *Pl-Rh/Pl-Rh* testers (Figure 5B). A plant of *pl1-A619/pl1-A619*; *rmr6-1/rmr6-1* genotype was crossed to a *T Pl-Rh/T Pl-Rh* stock derived from a *T Pl-Rh* reversion event described above to generate the *T Pl-Rh/pl1-A619*; *+/rmr6-1* parent, and the *Pl′/Pl′*; *+/rmr6-1* parent was provided from a family segregating 1:1 for *+/rmr6-1* and *rmr6-1/rmr6-1* plants. The *pl1-A619/pl1-A619*; *rmr6-1/rmr6-1* plant derived from selfing an F_2 plant molecularly genotyped as being homozygous for a *pl1-A619* RFLP polymorphism and shown by testcrosses to *+/rmr6-1* plants to be homozygous for *rmr6-1*.

To test the role of *Rmr6* in *b1* paramutation, isogenic *B-I* and *B′* stocks (*B1-I*; *Pl1-Rh*) were first pollinated by a single *rmr6-1/rmr6-1*; *b1-W23/b1-W23*; *Pl1-Rh/Pl1-Rh* plant. Intercrossing the resulting F_1 plants gave rise to progenies in which there was segregation of Pl-Rh-like and Pl′-like plants (Figure 6A). Among these Pl-Rh-like plants, the following *b1* genotypes occurred at a 1:1:1:1 frequency: *b1-W23/b1-W23*; *b1-W23/B′*; *b1-W23/B-I*; *B-I/B′*. Blind testcrosses of plants displaying dark plant colors (*rmr6-1/rmr6-1*) were made to *b1-CO159/b1-CO159* pistillate testers (*Pl-Rh/Pl-Rh*; CO159/W23 stock). Actual *b1* genotypes of the staminate parents were assigned *ex post facto* on the basis of the segregation of plant color types among resulting testcross progenies.

To test the role of *Rmr6* in *r1* paramutation, near isogenic *R-r/R-r* and *R-st/R-st* W22 lines were first pollinated by a single *+/rmr6-1* plant homozygous for recessive *r-r*. F_1 progeny were backcrossed using a single recurrent staminate parent (*+/rmr6-1*; *r-r/r-r*) and subsequent progeny sets were screened for segregation of *rmr6-1/rmr6-1* types. BC_1 plants were molecularly genotyped for *Pl1-Rhoades vs. Pl1-W22* alleles using Southern blot hybridization analysis as previously described (Hollick *et al.* 1995) and only those with *Pl1-Rhoades/Pl1-Rhoades* genotypes were used in subsequent crosses. Crosses were made between *R-st/r-r* BC_1 plants with Pl′ anther phenotypes (either *+/rmr6-1* or *+/+*) and *R-r/r-r* BC_1 plants with a Pl-Rh-like anther phenotype (*rmr6-1/rmr6-1*). Two progeny sets of these crosses segregating 1:1 for Pl′-like (*+/rmr6-1*) and Pl-Rh-like (*rmr6-1/rmr6-1*) types were evaluated for

R-r pigmenting activity through individual testcrosses to recessive *r1* W23 *r-g/r-g* pistillate stocks.

***Rmr6* mapping:** *B-A* mapping of *rmr6-1* and *rmr6-2* was performed as outlined by Beckett (1978). Specifically for mapping mutations affecting *Pl′*, the *TB-1La* translocation chromosome was introgressed into stocks homozygous for the *Pl1-Rhoades* allele and maintained in heterozygous state. For mapping, *Pl′/Pl′*; *+/rmr6-1* and *Pl′/Pl′*; *+/rmr6-2* plants were pollinated by *Pl′/Pl-Rh*; *TB-1La* heterozygotes. *B* centromere nondisjunction occurring at the mitotic division giving rise to the sperm cells provides the opportunity to uncover recessive mutations transmitted through female meiosis (Beckett 1978). Resulting progeny were screened for *1L* hypoploid individuals (characterized by early maturing, short, and tiny plants; Lee 1997) displaying an ACS 7 phenotype, indicative of a segmental monosomy uncovering mutant *rmr6* alleles. Both *+/rmr6-1* and *+/rmr6-2* individuals were used as pistillate parents and, among their respective progeny, 3/9 and 4/7 hypoploid plants had ACS 7 phenotypes.

Cosegregation mapping of *rmr6-1* with molecular markers was initiated by crossing an *rmr6-1/rmr6-1*; *Pl′/Pl′* plant to a color-converted A632 line (*Pl′/Pl′*, >93% A632). F_1 progeny were self-pollinated, and 2-week-old F_2 seedlings were screened for Pl-Rh-like pigmentation. Leaf tissue was collected from mutant seedlings, mapping parents, and F_1 plants, and genomic DNA was extracted using the DNeasy 96 plant kit (QIAGEN, Valencia, CA). Undiluted genomic DNA was used as template for PCR amplification (AmpliTaq Gold DNA Polymerase, Applied Biosystems, Foster City, CA) of microsatellite marker *umc1035*, located in bin 1.06 of chromosome *1L* (5′-CTGGCATGATCACGCTATGTATG-3′; 5′-TAACATCAG CAGGTTTGCTCATTC-3′; http://www.maizegdb.org/cgi-bin/displayssrrecord.cgi?id=174092). PCR products were electrophoresed in 3.5% agarose gels (Agarose MS; Roche, Mannheim, Germany), visualized by ethidium bromide staining, and imaged using an AlphaInnotech Image Capture system (Alpha Innotech, San Leandro, CA). A *umc1035* size polymorphism between the mapping parents was used for cosegregation analysis. Among 62 F_2 *rmr6-1/rmr6-1* seedlings, six recombination events were detected between *rmr6* and *umc1035*. These results place the *rmr6* locus ~3 cM from *umc1035* and confine the position of *rmr6* to bin 1.06.

***r1* activity assay:** Pigmentation of testcross *r-g/r-g/R-r* kernel samples was measured with an Agtron reflectometer as described (Alleman and Kermicle 1993) and presented as relative pigmentation values (Kermicle *et al.* 1995).

Pollen scoring: Pollen samples from freshly extruded anthers were examined in the field with the aid of a 50× pocket microscope (Edmund Scientific, Tonawanda, NY). Plants either homozygous for the *T6-9 (043-1)* reciprocal translocation or not carrying the translocation pair have ~95–100% plump and opaque pollen grains whereas plants heterozygous for the translocation display ~50% collapsed or translucent pollen grains due to segmental chromosome deficiencies (Patterson 1994).

Anther pigmentation: ACS refers to specific pigment patterns (Hollick *et al.* 1995). For each plant, a single ACS value was assigned on the basis of visual examination of freshly extruded anthers.

Molecular expression analyses: Materials and methods used for nucleic acid purifications and RNase protection analyses are described in Hollick *et al.* (2000) except that three entire florets from the leading edge of anthesis on the tassel were used as sources of RNA for each sample. All tissues were harvested at ~12:30 pm. Husk tissues used as sources of nuclei for *in vitro* transcription reactions were prepared as described in Hollick *et al.* (2000). Nuclei isolations and transcription reactions (Dorweiler *et al.* 2000) were carried out with 50 μCi of radiolabeled UTP per reaction. Slot blots were prepared using a slot-blot apparatus (Bio-Rad, Hercules, CA). Four micrograms of linearized and denatured plasmids per slot were placed on prewetted Magna nylon membranes (GE Osmonics, Minnetonka, MN) and UV treated to covalently attach. The *pl1* plasmid (pJH7; Hollick *et al.* 2000), *b1* plasmid (315-bp cDNA of *b1*; Selinger and Chandler 1999), *a1* clone (Patterson *et al.* 1993), and both *actin1* and *ubiquitin2* clones (Dorweiler *et al.* 2000) have been previously described. pBS is a pBluescript II (KS+) cloning plasmid (Stratagene, La Jolla, CA). Slot blots were prehybridized, hybridized with heat-denatured nuclear RNA at 42°, and washed at a final stringency of 0.01% SSC, 0.05% SDS at 65° (Chandler *et al.* 1989). Hybridizations were visualized, quantified, and normalized as described in Dorweiler *et al.* (2000) using ImageQuant software (Amersham Biosciences, Piscataway, NJ).

***pl1* molecular genotyping:** The phi031 primer set: 5′-GCAACAGGTTACATGAGCTGACGA-3′; 5′-CCAGCGTGCT GTTCCAGTAGTT-3′ (http://www.maizegdb.org/cgi-bin/displayssrrecord.cgi?id=111049; Chin *et al.* 1996) was used to distinguish *Pl1-Rhoades* and *pl1-A632* alleles (187- and 223-bp products, respectively). Genomic DNA was isolated from seedling leaves (Voelker *et al.* 1997) with the following modifications: following the first isopropanol precipitation step, nucleic acids were resuspended in 400 μl of TE, extracted with a 1:1 volume of phenol/chloroform (1:1) and a 1:1 volume of chloroform, and reprecipitated with 0.3 m NaOAc and 95% EtOH. Precipitated DNA was rinsed with 70% EtOH and resuspended in 100 μl TE and quantified using Hoechst 33258 dye binding measured with a TD-360 fluorometer (Turner Designs, Sunnyvale, CA). Quantified DNA was diluted to 50 ng/μl prior to PCR analysis as described above. Southern blot analyses (Hollick *et al.* 1995) were used to genotype *Pl1-Rhoades* and *Pl1-W22* alleles in individuals used for the *r1* paramutation stock constructions.

RESULTS

EMS mutations define a novel *trans*-acting *rmr* locus: Because *Pl′/Pl′* seedlings are weakly pigmented (Hollick *et al.* 1995), recessive mutations that enhance pigmentation are readily identified in M_2 progenies derived from EMS-treated pollen (Dorweiler *et al.* 2000; Hollick and Chandler 2001). Two mutations, ems97406 and ems98225, were found in separate M_2 progenies segregating 1 of 38 and 3 of 26 dark-pigmented Pl-Rh-like seedlings, respectively. Correspondingly, anthers of mature plants had dark-pigmented Pl-Rh-like phenotypes (Figure 1A). The two mutations appeared allelic as they failed to genetically complement each other (16 of 43 progeny from two crosses of +/ems97406 plants by ems98225/ems98225 plants had Pl-Rh-like, ACS 7, anther phenotypes). Subsequent mapping experiments, using *B-A* translocation stocks to create segmental monosomics (Beckett 1978), confirmed that both mutations are found on the long arm of chromosome *1* (see materials and methods). Cosegregation analysis with molecular markers further positions ems97406 to bin 1.06 (see materials and methods). Both ems97406 and ems98225 genetically complement previously described mutations at *rmr1*, *rmr2*, and *mop1* (see materials and methods),

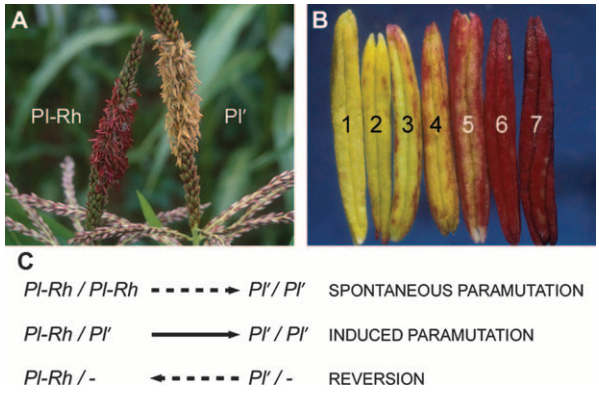

FIGURE 1.—Dynamic states of the *Pl1-Rhoades* allele. (A) Isogenic siblings displaying Pl-Rh and Pl′ anther phenotypes conferred by *Pl-Rh* and *Pl′* states of the *Pl1-Rhoades* allele, respectively. (B) Anther pigment phenotype examples corresponding to 1–7 graded ACS. (C) Generalized behaviors of *Pl-Rh* and *Pl′* regulatory states (HOLLICK *et al.* 1995; HOLLICK and CHANDLER 1998). Solid arrow indicates invariant changes while dashed arrows indicate changes occurring at ~10% frequency.

demonstrating these two mutations define the novel maize locus *rmr6*. The two mutant alleles, ems97406 and ems98225, are designated *rmr6-1* and *rmr6-2*, respectively.

***rmr6* mutations specifically affect *Pl1-Rhoades* alleles:** Pigment intensification seen in *rmr6-1* and *rmr6-2* homozygotes could be due to modifications of a rate-limiting step for either regulatory or biosynthetic pathways for anthocyanin production. The *rmr6-1* mutation was found to act specifically on *Pl1-Rhoades*-type *pl1* alleles in recessive Mendelian fashion by inspecting an F_2 family for segregation of distinct *pl1* alleles and mutant anthocyanin phenotypes (Table 1). A single *rmr6-1* homozygote was crossed to an A632 inbred individual and a single F_1 plant was self-pollinated to generate this F_2 family. The *pl1* genotype of each F_2 individual was determined using a set of simple sequence repeat primers (*phi031*; CHIN *et al.* 1996) that amplify allele-specific tetranucleotide repeat sequence polymorphisms from the first introns of *Pl1-Rhoades* (187 nt) and *pl1-A632* (223 nt). The mutant anthocyanin phenotype (ACS 7) was exclusively found among plants carrying a *Pl1-Rhoades* allele and appeared to segregate in an ~1:3 manner within the combined *Pl1-Rhoades/Pl1-Rhoades* and *Pl1-Rhoades/pl1-A632* classes (27:76; 26%; $\chi^2 = 0.043$; for H_o that difference from 1:3 segregation is due to random chance; no statistical difference), suggesting that *rmr6-1* acts as a recessive mutation. Among F_2 *Pl1-Rhoades* homozygotes, the ACS 7 phenotype also segregated in an ~1:3 fashion (9:22; 29%; $\chi^2 = 0.202$; for H_o that difference from 1:3 segregation is due to random chance; no statistical difference), confirming that the *rmr6* locus is genetically unlinked to *pl1*. None of the 23 *pl1-A632* homozygotes had increased pigmentation (0:23; 0%; $\chi^2 = 5.75$; for H_o that difference from 1:3 segregation is due to random chance; $P < 0.05$), indicating that *Rmr6* functions specifically maintain the repressed state of *Pl′* and are not involved in general suppression of either the genetic regulation or biosynthetic components of the anthocyanin pathway.

***Rmr6* maintains transcriptional repression of paramutant *Pl′* and *B′* states:** Both paramutant *Pl1-Rhoades* and *B1-Intense* allelic states (*Pl′* and *B′*, respectively) are associated with reduced transcriptional activity at the corresponding loci (PATTERSON *et al.* 1993; HOLLICK *et al.* 2000). Comparative RNase protection and nuclear run-on transcription analyses of *rmr6-1* homozygotes and *+/rmr6-1* siblings show that *Rmr6* function maintains low levels of *pl1* RNA largely by, if not exclusively, transcriptional repression (Figure 2). Using total floret RNA, we found that *rmr6-1* homozygotes had ~15-fold higher *pl1* RNA levels than *+/rmr6-1* siblings whereas *rmr6-2* homozygotes had ~18-fold higher levels than normal siblings with either *+/rmr6-2* or *+/+* genotypes (Figure 2, A and B). These RNA abundance differences are significantly higher than those observed between standard *Pl-Rh/Pl-Rh* and *Pl′/Pl′* genotypes (an ~9-fold difference; Figure 2B). *Rmr6* thus maintains low *pl1* RNA levels associated with the *Pl′* state. To discern whether *Rmr6*-dependent effects on *pl1* RNA levels involved transcription itself, we compared levels of radiolabeled RNA species transcribed in nuclei isolated from husks of *rmr6-1/rmr6-1* and *+/rmr6-1* genotypes (Figure 2, C

TABLE 1

F_2 segregation analysis (F_1 genotype: *pl1-A632/Pl′*; *Rmr6-A632/rmr6-1*)

	No. of F_2 progeny with specific anther phenotypes[b]				
		Pl1-Rhoades anther color scores			
F_2 progeny *pl1* genotype[a]	A632	1–4	5–6	7	Fraction of F_2 progeny[c]
Pl1-Rh/Pl1-Rh	0	22	0	9	31/126 (25)
pl1-A632/Pl1-Rh	0	53	1	18	72/126 (57)
pl1-A632/pl1-A632	23	0	0	0	23/126 (18)

[a] *pl1* genotype determined with codominant simple sequence repeat polymorphisms within *pl1* intron 1 (MATERIALS AND METHODS).
[b] A632 anther phenotype described in HOLLICK and CHANDLER (2001).
[c] The percentage of F_2 progeny is given in parentheses.

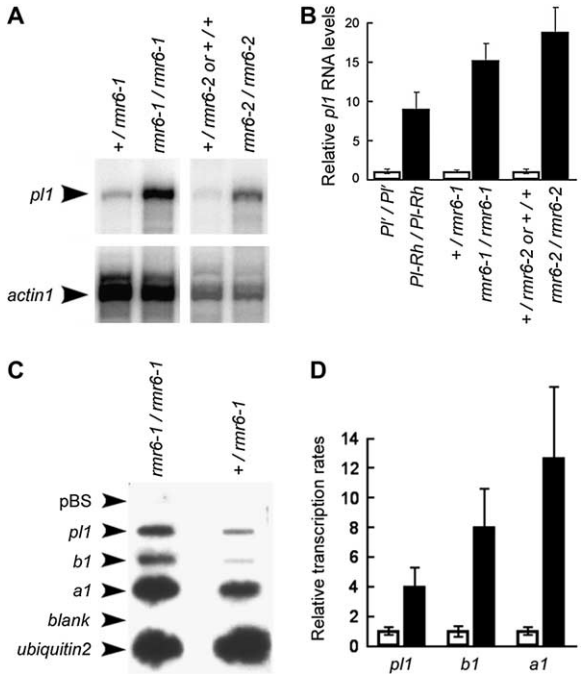

FIGURE 2.—*pl1* expression analyses. (A) RNase protection comparisons of *pl1* and *actin1* RNA levels from floret tissues of the indicated genotypes. (B) Histogram representation of average *pl1* RNA levels relative to *actin1* RNA levels (±SE) in the indicated genotypes ($n = 2, 2, 3, 4, 2, 2$ for genotypes *Pl′/Pl′*, *Pl-Rh/Pl-Rh*, *+/rmr6-1*, *rmr6-1/rmr6-1*, *+/rmr6-2* or *+/+*, and *rmr6-2/rmr6-2*, respectively). Measurements are normalized with data for nonmutant types set at unit value. (C) Slot-blot hybridization comparison of *in vitro* radiolabeled RNA species produced using nuclei isolated from husk tissues of the indicated genotypes. Plasmid DNA (pBS) is included as a negative control. (D) Histogram representation of average transcription rates of the indicated genes measured relative to *ubiquitin2* (±SE). Measurements for *rmr6-1/rmr6-1* genotypes (solid bars; $n = 5$) are displayed relative to data of *+/rmr6-1* genotypes (open bars; $n = 5$) set at unit value.

and D). Relative to levels of labeled *ubiquitin2* RNA, *pl1* RNA levels were higher in nuclei from *rmr6-1* homozygotes, indicating that synthesis of *pl1* transcripts within this tissue was, on average, ~4-fold greater than in *+/rmr6-1* siblings. Although additional effects on post-transcriptional regulation of *pl1* RNA levels are not excluded, it is clear that *Rmr6* is required to maintain transcriptional repression associated with the *Pl′* state. Because the tested nuclei came from plants also containing a paramutant *B′* allele, we found that *b1* transcription was also increased ~8-fold in *rmr6-1/rmr6-1* genotypes (Figure 2, C and D), a difference similar in magnitude to prior comparisons between standard *B-I/B-I* and *B′/B′* genotypes (PATTERSON *et al.* 1993). Consistent with the fact that B1 and PL1 proteins are required for transcriptional induction of the biosynthetic *anthocyaninless1* (*a1*) gene (encoding a dihydroflavonol reductase; O'REILLY *et al.* 1985), *a1* transcription was increased ~12-fold in *rmr6-1/rmr6-1* genotypes (Figure 2, C and D). These molecular expression assays confirm that *Rmr6* is genetically required to maintain transcriptional repression associated with paramutant *Pl′* and *B′* states.

***Rmr6* maintains the paramutagenic *Pl′* state:** Although previous tests established a strong inverse correlation between *Pl1-Rhoades* RNA levels and paramutagenic strength (HOLLICK *et al.* 1995), analyses of *mop1*, *rmr1*, and *rmr2* mutations illustrate that reacquisition of *Pl-Rh*-like RNA expression levels does not necessarily lead to loss of heritable paramutagenic activity. Most *Pl1-Rhoades* alleles inherited in the *Pl′* state and subsequently transmitted from *mop1-1*, *rmr1-1*, or *rmr2-1* homozygotes possess strong paramutagenic activity (DORWEILER *et al.* 2000; HOLLICK and CHANDLER 2001). Thus the type of repression mechanism acting to limit somatic *Pl1-Rhoades* expression is not necessarily the same as the mechanism responsible for defining meiotically heritable paramutagenicity. Given that *Rmr6* acts to maintain transcription-based repression, we could now ask whether heritable paramutagenic activity was necessarily or inseparably related to somatic reduction of transcription.

A series of genetic crosses and progeny analyses helped clarify the functional role of *Rmr6* in *pl1* paramutation. Heritable maintenance of paramutagenic activities was the focus of initial tests. Passage of *Pl′* states through plants homozygous for *rmr6* mutations was predicted to have one of the following possible outcomes: (1) paramutagenicity would be maintained so all transmitted *Pl1-Rhoades* alleles remained in the *Pl′* state, (2) paramutagenicity would be lost so all transmitted alleles were indistinguishable from *Pl-Rh*, (3) paramutagenicity would be partially affected such that transmitted alleles collectively represented a continuum of activities between fully paramutagenic *Pl′* and nonparamutagenic *Pl-Rh* (similar to the behavior of *Pl1-Rhoades* alleles transmitted from plants displaying ACS 5 and ACS 6 phenotypes; HOLLICK *et al.* 1995), or (4) transmitted *Pl1-Rhoades* alleles would not only have a *Pl-Rh*-like identity but also be immune to subsequent changes to *Pl′* states. To address these possibilities, pollen from individual *rmr6-1/rmr6-1* plants was distributed to pairs of isogenic pistillate parents with contrasting *Pl1-Rhoades* states. We ensured that the *rmr6-1* homozygotes used had received at least one *Pl1-Rhoades* allele in *Pl′* state from the previous generation either by self-pollinating a *+/rmr6-1* heterozygote having a Pl′ phenotype or by sib crossing a *+/rmr6-1* plant having a Pl′ phenotype with a *rmr6-1* homozygote having a Pl-Rh-like phenotype. Results of the *Pl′/Pl′* testcrosses (Table 2) show that *Pl1-Rhoades* alleles transmitted from *rmr6-1* homozygotes are fully susceptible to subsequent paramutation as all progeny from such crosses have clear Pl′ phenotypes (ACS 1–4). Results of *Pl-Rh/Pl-Rh* testcrosses represent a continuum of paramutagenic activity among *Pl1-Rhoades* alleles transmitted from *rmr6-1* homozygotes, in accord with prediction 3 above. While most *Pl1-Rhoades* alleles remained fully paramutagenic (testcross progeny with ACS 1–4), others displayed evidence of weaker

TABLE 2

Paramutagenicity testcross results: *Pl1-Rhoades* tester × *Pl′/Pl′*; *rmr6-1/rmr6-1*

Testcross[a]	Tester *Pl1-Rhoades* state[b]	No. of progeny with specific anther color score						
		1	2	3	4	5	6	7
99-277 × *99-250-7*	*Pl-Rh/Pl-Rh* (A632)	0	10	3	1	0	0	3
99-277 × *99-250-16*	*Pl-Rh/Pl-Rh* (A632)	0	9	4	2	1	1	2
00-1028 × *00-1166-1*	*Pl-Rh/Pl-Rh* (W23/A632)	1	1	4	1	0	0	2
99-277 × *99-250-7*	*Pl′/Pl′* (A632)	5	13	1	0	0	0	0
99-277 × *99-250-16*	*Pl′/Pl′* (A632)	0	17	1	0	0	0	0
00-1029 × *00-1166-1*	*Pl′/Pl′* (W23/A632)	6	7	2	1	0	0	0
Totals	*Pl-Rh/Pl-Rh*	1	20	11	4	1	1	7
	Pl′/Pl′	11	37	4	1	0	0	0

[a] Italic numbers identify specific *rmr6-1* homozygotes (ACS 7 anthers) from F_2 (99-250) and F_3 (00-1166) families.
[b] Inbred genetic backgrounds of individual *Pl1-Rhoades* stocks are indicated in parentheses (see MATERIALS AND METHODS for details).

paramutagenic action following transmission from *rmr6-1* homozygotes (testcross progeny with ACS 5–6). Furthermore, the maintenance of paramutagenicity is completely impaired in some *rmr6-1/rmr6-1* plants as progeny with ACS 7 phenotypes are obtained (Table 2). Thus, passage of *Pl′* states through *rmr6-1/rmr6-1* sporophytes affected paramutagenic activity such that some transmitted *Pl1-Rhoades* alleles were now indistinguishable from a nonparamutagenic *Pl-Rh* state.

The frequency of *Pl′*-to-*Pl-Rh* reversions transmitted from *Pl′/Pl′*; *rmr6-1/rmr6-1* plants was estimated from 25 separate testcrosses using two standard *Pl-Rh/Pl-Rh* stocks and 17 *rmr6-1/rmr6-1* individuals. All but one of these *rmr6-1* homozygotes gave individual progeny with an ACS 7 phenotype, indicating that a certain percentage (ranging from 7–100%) of *Pl1-Rhoades* alleles were transmitted in nonparamutagenic states indistinguishable from *Pl-Rh* (Table 3). In total, average reversion frequency

TABLE 3

Loss of *Pl′* paramutagenicity seen in *Pl-Rh/Pl-Rh* × *Pl′/Pl′*; *rmr6-1/rmr6-1* testcrosses

Staminate parent[a]	Pistillate tester	No. of testcrosses	No. of progeny with specific anther color scores						
			1	2	3	4	5	6	7[b]
98-273-17 (F_2)	W23	2	0	8	3	1	4	2	20 (53)
98-354 (F_2)	W23	2	2	17	10	4	1	1	9 (20)
98-1339-4 (F_3)	W23	1	1	12	6	1	0	0	6 (23)
98-1339-8 (F_3)	W23	1	2	6	4	0	0	0	12 (50)
98-1339-12 (F_3)	W23	1	0	6	8	0	0	0	1 (7)
98-1340-1 (F_3)	W23	1	0	2	4	0	0	3	15 (62)
98-1342-9 (F_3)	W23	1	0	5	4	0	0	0	14 (61)
99-141-4 (F_4)	W23	1	0	8	2	1	1	0	10 (45)
99-142-24 (F_4)	W23	1	0	0	1	1	2	0	11 (73)
99-315-1 (F_4)	W23	1	0	0	0	0	0	0	14 (100)
97-740-7 (M_2)	W23/CO159	1	0	1	0	0	0	0	6 (86)
98-151-13 (F_2)	W23/CO159	2	3	13	5	3	4	0	5 (15)
98-354 (F_2)	W23/CO159	4	19	34	10	9	5	0	9 (10)
99-142-24 (F_4)	W23/CO159	1	0	5	1	0	0	2	10 (56)
99-217-7 (F_4)	W23/CO159	2	8	17	0	1	0	1	0 (0)
99-250-7 (F_2)	W23/CO159	1	0	3	0	0	0	0	17 (85)
99-250-24 (F_2)	W23/CO159	2	7	11	1	4	0	0	10 (30)
Totals		25	42	148	59	25	17	9	169 (36)

[a] Pedigree origins of *rmr6-1* homozygotes are indicated in parentheses: M_2 is the family in which *rmr6-1* homozygotes were first found, F_2 indicates families in which Pl-Rh phenotypes were recovered following self-pollination of *Rmr6/rmr6-1* plants with Pl′-like phenotypes, F_3 and F_4 indicate families generated by intercrossing heterozygous (*Rmr6/rmr6-1*) individuals with homozygous *rmr6-1* F_2 or F_3 siblings, respectively.
[b] The percentage of testcross progeny with ACS 7 is given in parentheses.

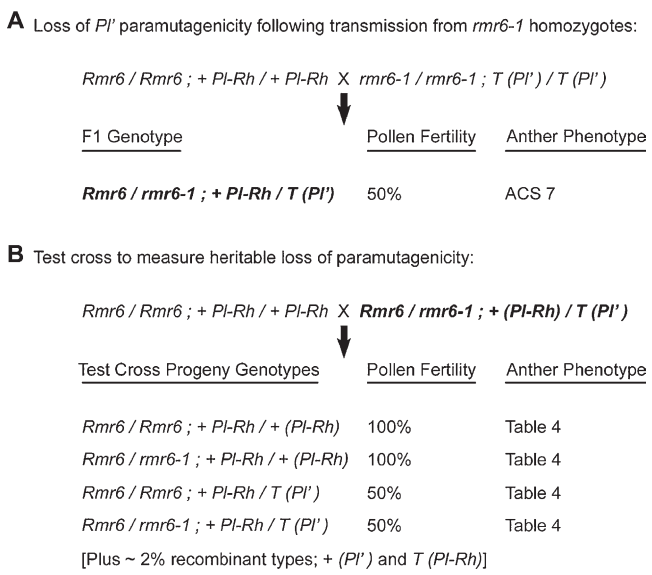

FIGURE 3.—Crossing scheme testing reversions of *Pl′* to *Pl-Rh* states. (A) Cross used to examine paramutagenic properties of *Pl1-Rhoades* alleles transmitted from *rmr6-1* homozygotes. Detailed information of parents and F_1 anther phenotypes is found in Table 2 for testcross 00-1028 × 00-1166-1. Parentheses denote uncertainty regarding paramutagenic properties of a particular *Pl1-Rhoades* allele. Boldface type indicates the genotype having an ACS 7 phenotype used in the subsequent testcrosses. (B) Individual F_1 plants (boldface type) having fully colored anthers were testcrossed to determine if the apparent loss of paramutagenicity was heritable. Segregating genotypes and associated pollen fertility phenotypes are listed. Anther phenotype data are presented in Table 4.

TABLE 4

Testcross results measuring heritable loss of *Pl′* paramutagenicity following transmission from *rmr6-1/rmr6-1* plants

Testcross[a]	Progeny structural genotype	No. of progeny with specific anther color scores		
		1–4	5–6	7
01-698-1 × 01-532	*T*/+	5	0	0
01-698-7 × 01-532	*T*/+	5	1	0
01-532 × *01-698-1*	+/*T*	3	1	3
01-532 × *01-698-7*	+/*T*	7	5	3
01-698-1 × 01-532	+/+	5	0	1
01-698-7 × 01-532	+/+	1	0	8
01-532 × *01-698-1*	+/+	0	0	5
01-532 × *01-698-7*	+/+	0	0	7
Totals	*T*/+	10	1	0
	+/*T*	10	6	6
	+/+	6	0	21

See Figure 3 for experimental design.

[a] Reciprocal crosses with a W23/CO159, *Pl-Rh/Pl-Rh* stock (01-532) in which the pistillate parent is listed first are indicated. Italic numbers represent individual F_1 plants (see Figure 3A) having an ACS 7 anther phenotype and ~50% aborted pollen grains.

was 36%. Reversion events were independent of prior allelic history as similar frequencies were obtained from *rmr6-1* homozygotes generated in M_2, F_2, F_3, or F_4 families. In every case, at least one *Pl1-Rhoades* allele in *rmr6-1/rmr6-1* plants was inherited from the previous generation in *Pl′* condition. The fact that nonparamutagenic *Pl-Rh* states were transmitted from *rmr6-1/rmr6-1* plants present in both M_2 and F_2 families in which both parental alleles were contributed in *Pl′* states demonstrates the requirement of *Rmr6* to faithfully maintain meiotically heritable information specifying paramutagenic behavior. Consistent with this interpretation, nine of the *rmr6-1* homozygotes also transmitted *Pl1-Rhoades* alleles displaying intermediate levels of paramutagenic action as evidenced by testcross progeny having ACS 5 and ACS 6 phenotypes.

Although *Pl1-Rhoades* alleles of *Pl′* state could revert to nonparamutagenic *Pl-Rh* states in *rmr6-1* homozygotes, this reversion was not always stable. Using genetic linkage (~1.5 cM; MATERIALS AND METHODS) to a *T6-9* translocation breakpoint, specific *Pl1-Rhoades* alleles in reference *Pl′* state were followed through *rmr6-1* homozygotes and two subsequent testcrosses to *Pl-Rh/Pl-Rh* plants. It is important to point out that *Pl1-Rhoades* alleles carried on all translocation chromosomes tested to date display normal paramutation properties (J. HOLLICK, unpublished results). Linkage between the translocation breakpoint "*T*" and reference *Pl1-Rhoades* allele is displayed as *T Pl′*. Figure 3 describes the crossing scheme in detail. When *T Pl′* passed through a *rmr6-1* homozygote, two of nine testcross progeny had ACS 7 phenotypes, suggesting that, as before, *Pl′* had reverted to nonparamutagenic *Pl-Rh* state (Figure 3A). These testcross progeny (*Pl-Rh/T Pl-Rh*) display significant pollen abortion (~50%) because chromosome segregations from translocation heterozygotes lead to a high percentage of aneuploid spores unable to develop functional male gametophytes (PATTERSON 1994). Results of a subsequent testcross assessed the status of *Pl1-Rhoades* alleles segregating from these initial ACS 7 plants (Figure 3B). To our surprise, we found that many *Pl1-Rhoades* alleles that had apparently reverted to the *Pl-Rh* state in the previous generation were strongly paramutagenic, while alleles found on normal homologs mostly retained nonparamutagenic *Pl-Rh* states (Table 4). Exceptions were found in both cases indicating that (1) *Pl′* could revert to a meiotically stable nonparamutagenic *Pl-Rh* state on the *T6-9* chromosome and (2) *Pl-Rh* could undergo heritable changes to paramutagenic *Pl′* state at high frequency despite all genetic and phenotypic indications that these *Pl1-Rhoades* alleles were in *Pl-Rh* state during the preceding generation. Because these last testcrosses were carried out reciprocally, we observed that both reestablishment of *Pl′* state on the translocation chromosome and spontaneous establishment of *Pl′* on the normal

TABLE 5

Testcross results measuring heritable loss of *Pl′* paramutagenicity following transmission from *rmr6-2/rmr6-2* plants

Testcross[a]	Progeny structural genotype	No. of progeny with specific anther color scores		
		1–4	5–6	7
02-490-8 × 02-470	*T*/+	1	2	5
02-470 × *02-490-8*	+/*T*	0	0	6
02-313 × *02-490-8*	+/*T*	0	0	9
02-234 × *02-490-3*	+/*T*	0	0	11
02-470 × *02-490-10*	+/*T*	2	0	8
02-313 × *02-490-13*	+/*T*	1	0	7
02-490-8 × 02-470	+/+	0	0	10
02-470 × *02-490-8*	+/+	1	0	4
02-313 × *02-490-8*	+/+	0	0	9
02-234 × *02-490-3*	+/+	0	0	5
02-470 × *02-490-10*	+/+	1	0	7
02-313 × *02-490-13*	+/+	0	0	8
Totals	*T*/+	1	2	5
	+/*T*	3	0	41
	+/+	2	0	43

Experimental design is similar to that described in Figure 3 for *rmr6-1*.

[a] Reciprocal crosses with a W23 *Pl-Rh/Pl-Rh* stock (02-234, 02-313, and 02-470) are indicated in which the pistillate parent is listed first. Italic numbers represent individual F_1 plants from family 02-490 (MATERIALS AND METHODS) having an ACS 7 anther phenotype (*rmr6-2/rmr6-2*) and ~50% aborted pollen grains (+/*T*).

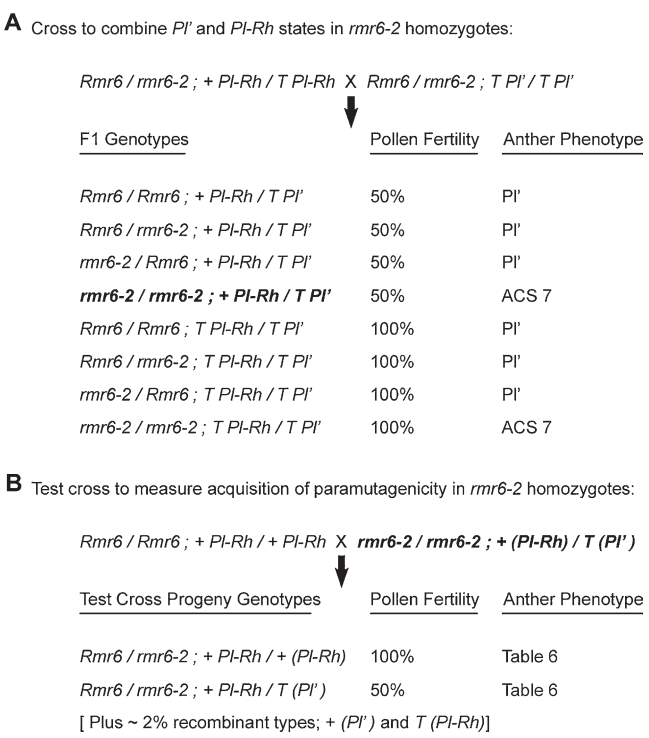

FIGURE 4.—Crossing scheme testing acquisition of paramutagenicity in *rmr6-2* homozygotes. (A) Cross used to introduce *Pl′* and *Pl-Rh* states in *rmr6-2* homozygotes. Segregant F_1 genotypes along with associated pollen and anther phenotypes are listed. The particular genotype used for subsequent testcrosses is in boldface type. (B) Testcross used to measure paramutagenicity of *Pl1-Rhoades* alleles following exposure of *Pl-Rh* to *Pl′* states in *rmr6-2* homozygotes. Boldface parental genotype corresponds to boldface segregant genotype found in A. Parentheses indicate uncertainty regarding paramutagenic properties of a given *Pl1-Rhoades* allele. Segregant genotypes and associated pollen fertility phenotypes are listed. Anther phenotype data are presented in Table 6.

chromosome occurred more frequently through female (pistillate), rather than male (staminate), transmission.

Reversions of *Pl′* to *Pl-RH* taking place in parallel lineages utilizing *rmr6-2* homozygotes appeared more stable (Table 5). This finding, together with *pl1* expression data (Figure 2, A and B), suggests that the *rmr6-2* allele represents a more severe functional defect relative to *rmr6-1*. Virtually all revertant *Pl-Rh* states obtained from *T Pl′/T Pl′*; *rmr6-2/rmr6-2* plants remained nonparamutagenic (Table 5). In this experiment, plants carrying the translocation chromosome were used as the pistillate parent in only one cross; therefore, it was not possible to assess whether there was parental transmission bias of *T Pl′ vs. T Pl-Rh* types. Although the frequency of spontaneous paramutation observed among the two different *Pl-Rh/Pl-Rh* testers (W23/CO159 *vs.* W23 backgrounds) used in the *rmr6-1* and *rmr6-2* tests is similar (<0.001; J. HOLLICK, unpublished results), it is possible that *Pl1-Rhoades* alleles maintained in these two lineages are differentially susceptible to low levels of paramutagenicity.

***Rmr6* is needed to heritably acquire a *Pl′* paramutagenic state:** The fact that *Pl′* states can revert to a stable *Pl-Rh* state in plants homozygous for *rmr6* mutant alleles obscures the role of *Rmr6* in inducing paramutation in *trans* (Figure 1C). To address this issue, we tracked paramutagenic behaviors of two *Pl1-Rhoades* alleles of contrasting *Pl′* and *Pl-Rh* states following segregation from *T Pl′/Pl-Rh*; *rmr6-2/rmr6-2* plants. Figure 4 describes the series of crosses. The *T Pl′* parents of these *T Pl′/Pl-Rh* ; *rmr6-2/rmr6-2* plants were heterozygous for a normal *Rmr6* allele to ensure transmission of a *Pl′* state. As in previous experiments, *Pl′* reverted to nonparamutagenic *Pl-Rh* state at high frequency following transmission from *rmr6-2* homozygotes (Table 6). Overall, *Pl-Rh* states carried by normal chromosomes appeared unchanged. Three of 86 Pl′-like testcross progeny plants having no significant pollen abortion either could reflect rare cases in which *Pl-Rh* had changed to paramutagenic *Pl′* state in *rmr6-2* homozygotes or, alternatively, could represent the expected ~1.5% of cases in which recombination placed the reference *Pl1-Rhoades* allele of *Pl′* state onto a structurally normal chromosome *6*. In either case, *Rmr6* is clearly required for efficient acquisition of a heritable paramutagenic state in *T Pl′/Pl-Rh* heterozygotes.

TABLE 6

Testcross results measuring acquisition of paramutagenicity by *Pl-Rh* in *rmr6-2/rmr6-2*; + *Pl-Rh/T Pl′* plants

Staminate testcross parent	Progeny structural genotype	No. of progeny with specific anther color scores						
		1	2	3	4	5	6	7
03-343-6	+/*T*	1	2	0	0	0	0	0
03-343-7	+/*T*	0	4	2	2	0	0	3
03-344-9	+/*T*	0	0	3	2	0	0	2
03-344-14	+/*T*	0	1	0	2	1	4	3
03-345-9	+/*T*	1	2	3	0	1	0	2
03-346-15	+/*T*	0	0	0	0	0	2	8
03-346-11	+/*T*	0	3	0	0	3	1	0
03-347-1	+/*T*	0	1	1	0	2	0	1
03-343-6	+/+	0	0	0	0	0	0	15
03-343-7	+/+	1	1	0	0	0	0	6
03-344-9	+/+	0	0	1	0	0	0	11
03-344-14	+/+	0	0	0	0	0	0	8
03-345-9	+/+	0	0	0	0	0	0	8
03-346-15	+/+	0	0	0	0	0	0	10
03-346-11	+/+	0	0	0	0	0	0	11
03-347-1	+/+	0	0	0	0	0	0	14
Totals	+/*T*	2	13	9	6	7	7	19
	+/+	1	1	1	0	0	0	83

Crossing scheme is presented in Figure 4. A619 *Pl-Rh/Pl-Rh* stocks were used for all testcrosses.

Similar tests performed with the *rmr6-1* allele had slightly different results (Figure 5 and Table 7). In contrast to the *rmr6-2* test, the reference *Pl1-Rhoades* allele of the *Pl′* state was carried on a structurally normal chromosome. As before, *Pl′* reverted back to *Pl-Rh* at lower frequency in *rmr6-1* homozygotes (Table 7) relative to *rmr6-2* homozygotes (Table 6); however, in this comparison, an identical *Pl-Rh/Pl-Rh* tester (A619 background) was used. While most *Pl-Rh* states on *T6-9* chromosomes remained unchanged in *T Pl-Rh/Pl′*; *rmr6-1/rmr6-1* plants, ~40% acquired a paramutagenic *Pl′* state (12 of 31 +/*T* testcross progeny genotypes had ACS 1–4 phenotypes; Table 7). While acquisition of paramutagenicity in *trans* appears to be prohibited in *rmr6-2* homozygotes, in *rmr6-1* homozygotes it is impaired but not prevented.

***Rmr6* is needed to acquire a heritable paramutant *B′* state:** In *B-I/B′* heterozygotes, the strongly expressed *B-I* state invariably changes to a transcriptionally repressed *B′* state (Patterson *et al.* 1993). To ask whether *B-I* would acquire a *B′* state in the absence of *Rmr6* function, *B-I* and *B′* states were combined in *rmr6-1* homozygotes and then evaluated for effects on plant pigmentation following transmission to recessive *b1* allele testers (Figure 6). Dark plant pigment phenotypes of the three *B′/B-I*; *rmr6-1/rmr6-1* plants tested were similar to that displayed by *B-I/B-I* genotypes. Although the two *B1-I* states in our experiment (*B-I* and *B′*) were on unmarked chromosomes, the near perfect 1:1 segregation of B-I-like and B′-like plant pigment types among testcross progeny (Table 8; Figure 7) strongly suggests that *B-I* is unable to attain a meiotically heritable *B′* state in the absence of full *Rmr6* function. On the basis of our experimental design (Figure 6; materials and methods), sibling *rmr6-1* homozygotes with either *B′/b1-W23* or *B-I/b1-W23* genotypes were also testcrossed. On the basis of the segregation of either colorless (*b1-CO159/b1-W23*) and light-colored progeny (*b1-CO159/B′*) or colorless and dark-colored progeny (*b1-CO159/B-I*), the *b1* genotypes of these plants were assigned *ex post facto*. Although plant pigment phenotypes of *rmr6-1/rmr6-1*; *B′/b1-W23* plants were similar to *rmr6-1/rmr6-1*; *B-I/b1-W23* plants (dark), *B′* never reverted to a heritable *B-I* state in 26 examples; all 26 *b1-CO159/B1-I* testcross progeny from three *B′/b1-W23* plants had a light plant pigment phenotype typical of *B′/B′* plants. The occurrence of B-I-like types segregating from *B-I/B′*; *rmr6-1/rmr6-1* plants is therefore highly unlikely to be due to reversion of *B′* to *B-I* states. Thus *Rmr6* is required for heritable acquisition of a *B′* paramutant state and the maintenance of transcriptionally repressed *B′* states, yet it appears not to be required for meiotic maintenance of a previously established paramutagenic *B′* state.

***Rmr6* is required to acquire a heritable paramutant *R-r′* state:** The seed component of *r1* haplotype *R-r:standard* also undergoes heritable changes in activity states (reviewed in Chandler *et al.* 2000). *R-r* changes to weaker expression states (*R-r′*) following exposure to structurally dissimilar, yet strongly paramutagenic, *r1* haplotypes like *R-stippled* (*R-st*) (Brink 1956). Standard

A Cross to combine *Pl′* and *Pl-Rh* states in *rmr6-1* homozygotes:

Rmr6 / rmr6-1 ; T Pl-Rh / + pl1-A619 X *Rmr6 / rmr6-1 ; + Pl′ / + Pl′*

F1 Genotypes	Pollen Fertility	Anther Phenotype
Rmr6 / Rmr6 ; T Pl-Rh / + Pl′	50%	Pl′
Rmr6 / rmr6-1 ; T Pl-Rh / + Pl′	50%	Pl′
rmr6-1 / Rmr6 ; T Pl-Rh / + Pl′	50%	Pl′
rmr6-1 / rmr6-1 ; T Pl-Rh / + Pl′	50%	ACS 7
Rmr6 / Rmr6 ; + pl1-A619 / + Pl′	100%	Pl′
Rmr6 / rmr6-1 ; + pl1-A619 / + Pl′	100%	Pl′
rmr6-1 / Rmr6 ; + pl1-A619 / + Pl′	100%	Pl′
rmr6-1 / rmr6-1 ; + pl1-A619 / + Pl′	100%	ACS 7

B Test cross to measure acquisition of paramutagenicity in *rmr6-1* homozygotes:

Rmr6 / Rmr6 ; + Pl-Rh / + Pl-Rh X ***rmr6-1 / rmr6-1 ; T (Pl-Rh) / + (Pl′)***

Test Cross Progeny Genotypes	Pollen Fertility	Anther Phenotype
Rmr6 / rmr6-1 ; + Pl-Rh / T (Pl-Rh)	50%	Table 7
Rmr6 / rmr6-1 ; + Pl-Rh / + (Pl′)	100%	Table 7

[Plus ~ 2% recombinant types; *T (Pl′)* and *+ (Pl-Rh)*]

Figure 5.—Crossing scheme testing acquisition of paramutagenicity in *rmr6-1* homozygotes. (A) Cross used to introduce *Pl′* and *Pl-Rh* states in *rmr6-1* homozygotes. Segregant F_1 genotypes along with associated pollen and anther phenotypes are listed. The particular genotype used for subsequent testcrosses is in boldface type. (B) Testcross used to measure paramutagenicity of *Pl1-Rhoades* alleles following exposure of *Pl-Rh* to *Pl′* states in *rmr6-1* homozygotes. Boldface parental genotype corresponds to boldface segregant genotype found in A. Parentheses indicate uncertainty regarding paramutagenic properties of a given *Pl1-Rhoades* allele. Segregant genotypes and associated pollen fertility phenotypes are listed. Anther phenotype data are presented in Table 7.

measure of *r1* action in these cases is relative pigmentation of the triploid kernel aleurone tissue following male transmission to recessive *r1* testers. To address whether *R-r* could adopt a heritable *R-r′* state in the absence of *Rmr6* function, *R-r* action was measured following exposure to *R-st* in *rmr6-1* homozygotes and in *+ / rmr6-1* heterozygous siblings. Figure 8 lists genotypes and corresponding kernel phenotypes of individuals used in the analysis. As control, *R-r* action was also measured following exposure to nonparamutagenic *r-r* haplotypes in sibling plants with similar *rmr6* genotypes. Two *r1* paramutation crosses (Figure 8A) were made and at least two individuals from each of the four relevant F_1 genotypes (genotypes in boldface type in Figure 8A) were testcrossed to recessive *r-g/ r-g* testers (Figure 8B). Mottled kernels (*r-g/ r-g/ R-r*) from testcross ears were cleaned of chaff and bulk samples evaluated for pigmentation in a reflectometer. Averages of mean reflectance measurements from each kernel sample are presented in Table 9. From these data it is clear that *R-r* does not change to a *R-r′*-like expression state following exposure to *R-st* in *rmr6-1* homozygotes. In contrast, *R-r* adopts a significantly reduced expression state following exposure to *R-st* in *+ / rmr6-1* heterozygous siblings. As is characteristic of *R-r* paramutation by *R-st*, aleurone pigmentation was reduced to a lower level in the lineage having the lower nonparamutant value (Styles and Brink 1966; H_o that the two average values from the 11,734 and 11,738 progenies differ from each other due to random chance; $t = 5.74$; $P < 0.01$; two sample *t*-test with 5 d.f.). In both cases, however, reduction of *R-r* action is consistent with heritable acquisition of a paramutant *R-r′* state.

***Rmr6* maintains the *R-r′* paramutant state:** When *Rmr6* is present, *R-r′* reverts partially toward the nonparamutagenic *R-r* state in *R-r′ / R-r* homozygotes (Brink 1964). Self-mating an F_1 *R-st/R-r*; *+ / rmr6-1* plant (Figure 8A) generated *R-r′*-carrying plants in which maintenance of *R-r′* in the absence of normal *Rmr6* function could be evaluated (Table 10). Using the same testcross protocol as before, it is clear that *R-r:standard* remained in paramutant *R-r′* state when transmitted from plants with normal *Rmr6* function but regained meiotically heritable pigmenting actions similar to nonparamutant *R-r* when transmitted from *rmr6-1* homozygotes. Remarkably, the extent of recovery in *R-r′ / R-st* heterozygotes matched that of *R-r′ / R-r′* homozygotes. Both stand in contrast to sibling *R-r′ / R-st Rmr6*-containing plants, which show low levels of pigmentation characteristic of F_2 *R-r′ / R-st* plants (Mikula 1961). Thus *Rmr6* is required for maintenance of paramutant *R-r:standard* as well as *Pl′* states.

DISCUSSION

Molecular expression and genetic segregation analyses with *rmr6* mutations show that normal *Rmr6* function maintains transcriptional repression of *Pl′* and *B′* relative to *Pl-Rh* and *B-I* reference states and is required for acquisition of meiotically heritable paramutant states at the *pl1*, *b1*, and *r1* loci. Pedigree analyses following specific *Pl1-Rhoades* alleles further illustrate that *Rmr6* is required to stably maintain meiotically heritable *Pl′* states; *Pl′* fully reverts to a transmissible nonparamutagenic *Pl-Rh* state in over one-third of gametes derived from *rmr6-1* homozygotes and nearly two-thirds of gametes transmitted from *rmr6-2* homozygotes. *R-r′* similarly reverts to meiotically heritable nonparamutant *R-r* states. Among *Pl-Rh* revertants arising in *rmr6-1* homozygotes, however, a *Pl′* state is often restored in the next generation, suggesting that persistence of heritable information confers instability to *Pl-Rh* states. The *Rmr6*-encoded factor thus defines heritable patterns of gene regulation as a common component of the maize paramutation mechanism by maintaining meiotically heritable epigenetic alterations affecting transcriptional regulation.

TABLE 7

Testcross results measuring acquisition of paramutagenicity by *Pl-Rh* in *rmr6-1/rmr6-1*; *T Pl-Rh/+ Pl′* plants

Staminate testcross parent	Progeny structural genotype	No. of progeny with specific anther color scores						
		1	2	3	4	5	6	7
03-1202-3	+/*T*	0	0	0	1	0	2	0
03-1202-9	+/*T*	2	2	0	1	0	0	5
03-1202-12	+/*T*	0	1	0	1	0	0	8
03-1202-17	+/*T*	0	2	0	2	1	0	3
03-1202-3	+/+	0	4	0	0	1	0	1
03-1202-9	+/+	6	0	0	0	0	1	1
03-1202-12	+/+	0	0	0	1	1	2	3
03-1202-17	+/+	5	5	0	0	0	0	0
Totals	+/*T*	2	5	0	5	1	2	16
	+/+	11	9	0	1	2	3	5

Crossing scheme is presented in Figure 5. A619 *Pl-Rh/Pl-Rh* stocks were used for all testcrosses.

***Rmr6* function:** Only specific alleles are affected by *Rmr6* action. Conceptually analogous to classical position effects (Muller 1930; Shultz 1936) in which the chromosomal environment can expose genes to heterochromatin-based influences, only certain alleles or transgene insertions exhibit paramutation or paramutation-like behaviors (reviewed in Chandler and Stam 2004). Because *Mop1* (Dorweiler *et al.* 2000), *Rmr1*, *Rmr2* (Hollick and Chandler 2001), and *Rmr6* functions act specifically on alleles subject to paramutation, it will be interesting to know if paramutation is a general characteristic of other genomic targets of *Rmr6* action.

Rmr6 is most similar to *Mop1* (Dorweiler *et al.* 2000) in its requirement for induced paramutation at *r1*, *b1*, and *pl1* loci, somatic maintenance of the *B′* transcription state, and meiotic maintenance of *Pl′*. The fact that *pl1* RNA levels are elevated in *Pl′/Pl′*; *mop1-1/mop1-1* plants (Dorweiler *et al.* 2000) suggests that *Pl′* transcription is also affected by *Mop1* action. *Mop1* and *Rmr6* therefore appear to play parallel or epistatic, rather than redundant, roles in maintaining *B′* transcriptional states. However, the observations that *Pl′* can revert to the *Pl-Rh* state within a single generation of being homozygous for *rmr6* mutations while similar reversions occur only after *Pl′* states are carried through at least two successive sporophyte generations of being homozygous for *mop1* mutations (Dorweiler *et al.* 2000) highlight an intriguing distinction. Both *Rmr1* and *Rmr2* also act specifically on *Pl1-Rhoades* alleles to maintain repressed *pl1* RNA levels of *Pl′* states and are required to maintain its meiotically heritable paramutagenicity (Hollick and Chandler 2001). It remains unclear whether loss of paramutagenicity in plants homozygous for *rmr1* and *rmr2* mutations is similarly correlated with loss of transcriptional repression. Ongoing tests of *Rmr1* and *Rmr2* functions in *b1* and *r1* paramutation will determine whether these, like *Mop1* and *Rmr6*, also represent common components of the paramutation mechanism.

Functional nonequivalence in several respects suggests that *rmr6-1* is a partial loss-of-function allele. The *rmr6-2* mutation represents a more severe loss-of-function allele possibly equivalent to a null situation. Both *r1*- and *b1*-induced paramutations, however, were severely or totally impaired in *rmr6-1* homozygotes whereas *pl1*-induced paramutation was only partially inhibited. Similarly, reversions of *Pl′* to *Pl-Rh* states occurring in *rmr6-1* homozygotes were variable from one plant to another and were, in general, inherently less stable than those occurring in *rmr6-2* homozygotes. A parent-of-origin effect regarding *Pl-Rh* stability (Table 4) reveals additional variability manifest by partial activity of the *rmr6-1* allele. Collectively, these observations point to a quantitative nature of *Rmr6* action occurring during somatic maintenance of *Pl′* states.

The fact that *Rmr6*-dependent maintenance functions are required for meiotic inheritance of both *Pl′* and *R-r′* states illustrates its involvement in maintaining heritable epigenetic alterations affecting transcriptional control. In this regard, *Rmr6*-encoded activity resembles the function of Swi6p—the presumed *Drosophila melanogaster* heterochromatin protein 1 ortholog in *Schizosaccharomyces pombe*—which is required to maintain specific transcription states through meiosis (Nakayama *et al.* 2000). However, *Rmr6* effects, directly or indirectly, on cytosine methylation patterns are also considered. Although no regional cytosine methylation differences distinguish *Pl′* from *Pl-Rh* states (Hollick *et al.* 2000), it is possible that distant regulatory elements, similar to those found reversibly modified by cytosine methylation upstream of *B1-I* (Stam *et al.* 2002a,b), may have functional consequences on *Pl1-Rhoades* transcription. DNA methylation and/or chromatin alterations might determine differential interactions between

A Cross to combine *B′* and *B-I* states in *rmr6-1* homozygotes:

Rmr6 / rmr6-1 ; B′ / b1-W23 X *Rmr6 / rmr6-1 ; B-I / b1-W23*

F1 Genotypes	Plant Phenotype
Rmr6 / Rmr6 ; b1-W23 / b1-W23	colorless
Rmr6 / rmr6-1 ; b1-W23 / b1-W23	colorless
rmr6-1 / Rmr6 ; b1-W23 / b1-W23	colorless
rmr6-1 / rmr6-1 ; b1-W23 / b1-W23	colorless
Rmr6 / Rmr6 ; B′ / b1-W23	light
Rmr6 / rmr6-1 ; B′ / b1-W23	light
rmr6-1 / Rmr6 ; B′ / b1-W23	light
rmr6-1 / rmr6-1 ; B′ / b1-W23	dark
Rmr6 / Rmr6 ; B′ / B-I	light
Rmr6 / rmr6-1 ; B′ / B-I	light
rmr6-1 / Rmr6 ; B′ / B-I	light
(*rmr6-1 / rmr6-1 ; B′ / B-I*)	dark
Rmr6 / Rmr6 ; b1-W23 / B-I	dark
Rmr6 / rmr6-1 ; b1-W23 / B-I	dark
rmr6-1 / Rmr6 ; b1-W23 / B-I	dark
rmr6-1 / rmr6-1 ; b1-W23 / B-I	dark

B Test cross to measure acquisition of paramutagenicity in *rmr6-1* homozygotes:

Rmr6 / Rmr6 ; b1-CO159 / b1-CO159 X **(*rmr6-1 / rmr6-1 ; B′ / B-I*)**

Test Cross Progeny	Plant Phenotype
Rmr6 / rmr6-1 ; b1-CO159 / B-I	Table 8, Figure 7
Rmr6 / rmr6-1 ; b1-CO159 / B′	Table 8, Figure 7

FIGURE 6.—*b1* paramutation analysis pedigree. (A) Plants with the indicated *b1* and *rmr6* genotypes were crossed together to obtain the given set of F_1 progeny genotypes. Two progeny sets (12873 and 12874) were used in the analysis. Three general plant-color phenotypes [colorless, light (B′-like), and dark (similar to B-I)] characterize the indicated genotypes. Genotypes giving rise to a dark plant phenotypes are in boldface type with the relevant *B′/B-I* genotype in parentheses. (B) Progeny plants listed in A displaying dark plant phenotypes (boldface type) were testcrossed to *b1-CO159/b1-CO159* plants and assigned the actual *b1* genotypes on the basis of segregation of plant phenotypes among testcross progenies. Testcross progeny genotypes are listed only for the relevant *B′/B-I* parent (boldface type in parentheses). Corresponding progeny phenotypes for the three relevant testcrossed plants are listed in Table 8 and are shown in Figure 7.

enhancers, silencers, or insulators affecting loop-domain organizations affecting subsequent transcriptional activities similar to those recently documented in murine *Igf2 H19* parent-of-origin imprinting (MURRELL *et al.* 2004).

Genetic nature of paramutant states: Paramutant states share two general properties: they reflect a meiotically inherited muted expression pattern relative to reference nonparamutant states, and they facilitate paramutations in *trans* (paramutagenicity). Paramutation simply describes the process of adopting a paramutant state. This process, by definition, takes place during one sporophytic generation and is manifest in the following. In this study, paramutant *R-r:standard* and

TABLE 8

Evaluation of *b1* paramutation in *rmr6-1/rmr6-1*; *B-I/B′* plants through crosses to *Rmr6 b1* testers

Staminate parent	No. of progeny with specific plant phenotypes		
	B-I	B′	$\chi^{2\,a}$
02-513-4	32	38	0.17
02-513-52	40	34	0.49
02-719-33	7	13	0.9
Totals	79	85	0.22

A stock homozygous for a null *b1* allele (*b1-CO159*) was used as pistillate parents for testcrosses of *rmr6-1* homozygotes in which *B-I* and *B′* states were combined (see Figure 6 and MATERIALS AND METHODS for details).

[a] A χ^2 test was applied to the null hypothesis that the difference from an expected 1:1 segregation of B-I and B′ types is due to random chance. None of the χ^2 values were statistically significant ($P \gg 0.05$).

B1-I states were evaluated only in regards to pigmenting function following sexual transmission whereas paramutant *Pl1-Rhoades* states were defined by heritable paramutagenic properties. When paramutagenicity is measured by reduced *pl1* expression patterns following testcrosses to plants homozygous for nonparamutant states, the pigmenting function and paramutagenicity properties are inseparable. In plants homozygous for *mop1*, *rmr1*, *rmr2*, or *rmr6* mutations, however, it is obviously useful to discriminate between phenotypes suggestive of nonparamutant states *vs.* experimental evidence derived from inheritance tests. Spontaneous examples emphasize that the paramutation behavior is an endogenous property of these unique alleles and invites the concept that paramutagenicity reflects a permissive environment in which spontaneous paramutation is favored (STYLES and BRINK 1969). In previous studies of *b1* and *pl1* paramutation, somatic sectors of muted plant

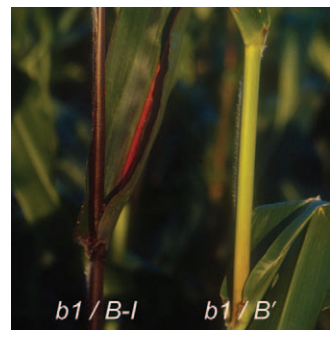

FIGURE 7.—Transmission of *B-I* and *B′* states in the absence of *Rmr6* function. The two sibling plants represent color phenotypes displayed by the indicated *b1* progeny genotypes resulting from *Rmr6/Rmr6*; *b1-CO159/b1-CO159* × *rmr6-1/rmr6-1*; *B-I/B′* testcrosses (Figure 6B; Table 8).

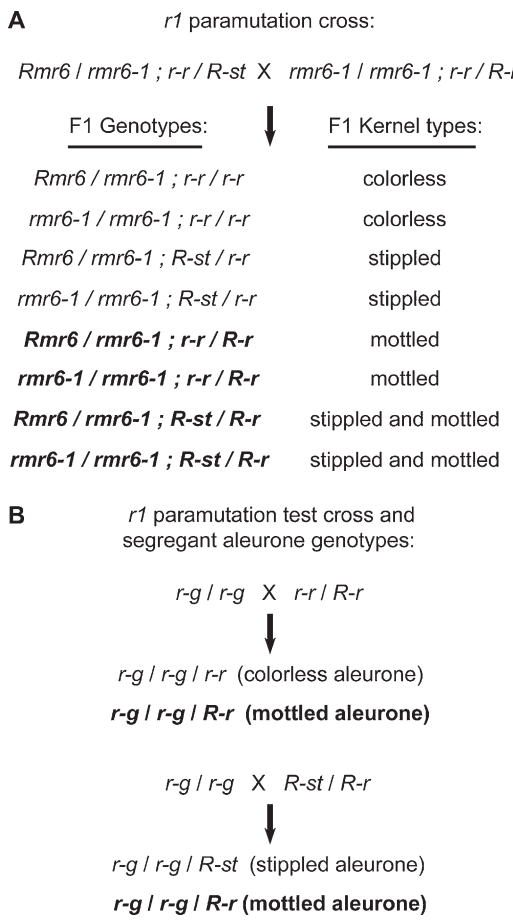

FIGURE 8.—*r1* paramutation analysis pedigree. (A) Plants with indicated *r1* and *rmr6* genotypes were crossed together to obtain the given set of F_1 progeny genotypes. Two progeny sets (11734 and 11738) were used in this analysis. Diagnostic kernel phenotypes are indicated for given genotypic classes. Genotypes used as staminate parents for subsequent testcrosses are in boldface type. (B) *r1* genotypes of testcross parents are given along with segregant triploid endosperm genotypes and corresponding phenotypes of resulting kernels. Kernel classes used to measure *R-r* expression (Table 9) are in boldface type.

color in *B-I/B-I*; *Pl-Rh/Pl-Rh* plants presage the appearance of paramutant states in the immediately subsequent generation (COE 1966; WALBOT 2001). Hence paramutation is an inherent property of *Pl1-Rhoades* alleles that appears sensitive to allelic interactions potentiated by *Mop1*, *Rmr1*, *Rmr2*, and *Rmr6* activities.

Maintenance *vs.* establishment of paramutation: Given that *Rmr6* somatically maintains *Pl′* and *R-r′* paramutant states, the role of *Rmr6* in establishment of a paramutant state is in question. Without somatic maintenance, establishment cannot be detected. Somatic maintenance is required for meiotic transmission of a somatically adopted paramutant state and for paramutagenicity. The fact that *B′* does not revert to the *B-I* state in *rmr6-1* homozygotes indicates that transmission of ~50% *B-I* states from *B-I/B′* individuals reflects failure of *B-I* to acquire a heritable *B′* state. This is surprising, given that ~40% of *Pl1-Rhoades* alleles in *Pl-Rh* states changed to *Pl′* in *rmr6-1/rmr6-1*; *Pl′/T Pl-Rh* plants. If *Rmr6* acted similarly at *b1* and *pl1*, we would predict that some *b1* paramutation would occur in *rmr6-1/rmr6-1*; *B-I/B′* plants. Similarly, why should *B′* not revert to *B-I* in *rmr6-1* homozygotes? One possibility is that new *B′* states induced in *B-I/B′* heterozygotes are not somatically maintained and hence not transmitted in *B′* state from *rmr6-1/rmr6-1* plants. PATTERSON and CHANDLER (1995) showed that newly formed *B′* states from *B-I/B′* plants are distinguished from the inducing *B′* state in terms of pigmenting potential and STAM *et al.* (2002a) showed that cytosine demethylation of the upstream repeats associated with the *B′* state lags approximately one generation from the original *B-I* to *B′* induction event occurring in *B-I/B′* plants. Thus previously established paramutant *B′* states may persist in *rmr6-1* and *mop1-1* homozygotes, while somatically formed *B′* states may not. In light of this possibility, it remains unclear as to whether *Rmr6* or *Mop1* are required for the actual inductive event necessary for establishment or more simply are required to somatically maintain labile paramutant states. These two functions, however, need not be mutually exclusive. Regardless, this apparent discrepancy of *Rmr6* function in *B-I/B′ vs. R-r/R-st* and *Pl-Rh/Pl′* genotypes underscores the unique features of these alleles that differentially utilize common components of the basic paramutation mechanism.

Transcriptional repression and paramutagenic activity: *Rmr6* action maintains the relatively low *Pl′* transcription rates in husk tissues. The approximately fourfold increase in average transcription rate found in *rmr6-1* homozygotes is slightly greater than differences between *Pl′* and *Pl-Rh* states in *Rmr6/Rmr6* materials (approximately threefold; HOLLICK *et al.* 2000). Consistent with this greater transcription rate measured in husk tissues, there is an approximately twofold greater level of *pl1* RNA in mutant floret tissues (*Pl′/Pl′*; *rmr6-1/rmr6-1*) relative to *Pl-Rh* reference states (*Pl-Rh/Pl-Rh*; *Rmr6/Rmr6*). Despite this hyperexpression of *pl1*, a significant fraction of *Pl1-Rhoades* alleles are transmitted in paramutagenic *Pl′* states. Thus neither pigment nor *pl1* molecular expression phenotypes found in plants homozygous for mutant *rmr6* alleles are a reliable indicator of subsequent paramutagenic activity. While heritable loss of paramutagenic activity occurs only in plants with *Pl-Rh*-like levels of *pl1* expression (HOLLICK and CHANDLER 1998, 2001; DORWEILER *et al.* 2000), such RNA levels or transcriptional activities are not sufficient to stabilize a *Pl-Rh* state through meiosis nor fully prevent induced changes of *Pl-Rh* to *Pl′* occurring in *Pl-Rh/Pl′* genotypes. These observations point to regulatory features upstream of transcriptional activation, such as those affecting chromatin or nuclear architecture, as the target of *Rmr6* action as well as the source of heritable paramutagenic activity.

TABLE 9

***r1* paramutation analysis**

Progeny set	Reflectance measurements for testcrossed *r-g/r-g/R-r* kernels from specific staminate genotypes[a]				Statistical significance[b]
	r-r/R-r		*R-st/R-r*		
	+/rmr6-1	*rmr6-1/rmr6-1*	*+/rmr6-1*	*rmr6-1/rmr6-1*	
11734	80.6 ± 3.0 (2)	79.6 ± 2.5 (3)	58.5 ± 7.7 (6)	*79.8 ± 3.5 (4)*	$P < 0.01$
11738	76.3 ± 4.0 (6)	78.0 ± 4.6 (4)	34.5 ± 7.8 (8)	*73.9 ± 2.1 (5)*	$P \ll 0.01$

[a] Reflectance measurements indicate relative *R-r* expression through its effects on kernel pigmentation. Average measurement values ±SD$^+$ are presented for the total number of testcross individuals shown in parentheses. Measurements indicating absence of *r1* paramutation are in italics.

[b] A two-sample *t*-test was applied to the null hypothesis that differences between average measurements derived from *R-st/R-r*; *+/rmr6-1* and *R-st/R-r*; *rmr6-1/rmr6-1* genotypes are due to random chance.

The nature of paramutagenic action: While our results clearly implicate that *Rmr6* functions in the acquisition of paramutant states at multiple loci, its specific role is unknown. For example, in a *Pl-Rh/Pl′* plant, *Rmr6* could be required to adopt a meiotically heritable paramutagenic *Pl′* state, to mediate paramutagenicity from the *Pl′* state, to maintain paramutagenicity of the *Pl′* state, or some combination thereof. It is difficult to discriminate among these conceptually distinct functions since the assays for stable paramutational changes are spatially and temporally separated from site and timing of *Rmr6* function. On the basis of the observed reversions of *Pl′* to *Pl-Rh* in *Pl-Rh/Pl′*; *rmr6-2/rmr6-2* plants and *R-r′* to *R-r* in *R-st/R-r′*; *rmr6-1/rmr6-1* plants, the most parsimonious inference is that *Rmr6* action maintains paramutagenicity in somatic lineages. Our results with the *rmr6-1* mutation indicate that paramutagenicity, a property measured after meiosis, can be uncoupled to some extent from transcriptional regulatory activities measured in the preceding sporophytic generation. Even in cases where experimental evidence suggested heritable loss of paramutagenicity, our ability to follow specific *Pl1-Rhoades* alleles through subsequent generations showed that many of these revertant *Pl-Rh*-like states retained a latent mark allowing them to regain full, or partial, paramutagenic activity following the next meiotic segregation. Taken together, these results suggest that paramutagenic activity transmitted at meiosis is based on preexisting epigenetic marks that may, or may not, have altered transcriptional activities affecting changes in pigmentation. *Rmr6* function serves to define and maintain this epigenetic mark.

An emerging genetic mechanism for meiotically heritable change: So far, four functions—encoded by *Mop1*, *Rmr1*, *Rmr2*, and *Rmr6*—that are required to maintain reduced RNA expression typical of the *Pl′* paramutant state have been described. Five other loci are currently under investigation (J. Hollick, unpublished results). Thus, at least nine genetic components defined by mutations are required for some aspect of *pl1* paramutation. As specific sequences responsible for *p1* and *b1* parmutation are now identified (Sidorenko and Peterson 2001; Stam *et al.* 2002a,b), these mutations should help illuminate functionally relevant molecular alterations associated with paramutational change. Identification of sequences responsible for *pl1* paramutation will be similarly important in understanding functional changes responsible for meiotically heritable alterations of transcriptional regulation. Discovery of other genomic targets of *Mop1* and *Rmr* actions promises to clarify additional roles of paramutation in higher eukaryotes.

TABLE 10

Reflectance measurements for testcrossed *r-g/r-g/R-r* kernels from specific staminate genotypes

Staminate *r1* genotypes	Staminate *rmr6* genotypes[a]		Statistical significance[b]
	Rmr6/−	*rmr6-1/rmr6-1*	
R-r′/R-r′	48.5 ± 8.8 (8)	70.2 ± 1.4 (3)	$P < 0.05$
R-st/R-r′	35.6 ± 4.2 (7)	68.2 ± 7.8 (3)	$P < 0.05$

Reflectance measurements indicate relative *R-r* expression through its effects on kernel pigmentation. Average measurement values ± SD$^+$ are presented for the total number of testcross individuals shown in parentheses.

[a] *Rmr6/−* designation represents both *Rmr6/rmr6-1* and *Rmr6/Rmr6* genotypes.

[b] A two-sample *t*-test was applied to the null hypothesis that the differences between average measurements derived from *Rmr6* and *rmr6-1/rmr6-1* genotypes are due to random chance.

Technical and field support from Patrick Brown, Ben VanDusen, Loan Nguyen, Galina Ishkhanova, Ruba Khenaisser, and Bill Pickle is gratefully acknowledged. Thanks go to Stephen Gross and Sharon Amacher for critical reviews. We are indebted to the Maize Genetics Cooperation Stock Center (Urbana, IL), United States Department of Agriculture North Central Plant Introduction Station (Ames, IA), and Vicki Chandler (University of Arizona, Tucson) for providing germplasm. This work was supported by the United States Department of Agriculture National Research Initiative awards 99-35301-7753 and 2001-35301-10641 to J.B.H. and additional funding from Syngenta.

LITERATURE CITED

Alleman, M., and J. L. Kermicle, 1993 Somatic variegation and germinal mutability reflect the position of transposable element *Dissociation* within the maize *R* gene. Genetics **135:** 189–203.

Beckett, J. B., 1978 B-A translocations in maize. I. Use in locating genes to chromosome arms. J. Hered. **69:** 27–36.

Brink, R. A., 1956 A genetic change associated with the *R* locus in maize which is directed and potentially reversible. Genetics **41:** 872–879.

Brink, R. A., 1958 Paramutation at the *R* locus in maize. Cold Spring Harbor Symp. Quant. Biol. **23:** 379–391.

Brink, R. A., 1964 Genetic repression of *R* action in maize, pp. 183–230 in *Role of Chromosomes in Development*, edited by M. Locke. Academic Press, New York.

Chandler, V. L., and M. Stam, 2004 Chromatin conversations: mechanisms and implications of paramutation. Nat. Rev. Genet. **5:** 532–544.

Chandler, V. L., J. P. Radicella, T. P. Robbins, J. Chen and D. Turks, 1989 Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of *B* utilizing *R* genomic sequences. Plant Cell **1:** 1175–1183.

Chandler, V. L., W. B. Eggleston and J. E. Dorweiler, 2000 Paramutation in maize. Plant Mol. Biol. **43:** 121–145.

Chandler, V. L., M. Stam and L. V. Sidorenko, 2002 Long-distance *cis* and *trans* interactions mediate paramutation. Adv. Genet. **46:** 215–234.

Chin, E. C., M. L. Senior, H. Shu and J. S. Smith, 1996 Maize simple repetitive DNA sequences: abundance and allele variation. Genome **39:** 866–873.

Coe, E. H., Jr., 1966 The properties, origin, and mechanism of conversion-type inheritance at the *B* locus in maize. Genetics **53:** 1035–1063.

Cone, K. C., S. M. Cocciolone, F. A. Burr and B. Burr, 1993 Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. Plant Cell **5:** 1795–1805.

Das, O. P., and J. Messing, 1994 Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. Genetics **136:** 1121–1141.

Dorweiler, J. E., C. C. Carey, K. M. Kubo, J. B. Hollick, J. L. Kermicle *et al.*, 2000 *mediator of paramutation1 (mop1)* is required for the establishment and maintenance of paramutation at multiple maize loci. Plant Cell **12:** 2101–2118.

Goff, S. A., K. C. Cone and V. L. Chandler, 1992 Functional analysis of the transcriptional activator encoded by the maize *b* gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev. **6:** 864–875.

Hagemann, R., 1969 Somatic conversion (paramutation) at the *Sulfurea* locus of *Lycopersicon esculentum* Mill. III. Studies with trisomics. Can. J. Genet. Cytol. **11:** 446–458.

Hollick, J. B., and V. L. Chandler, 1998 Epigenetic allelic states of a maize transcriptional regulatory locus exhibit overdominant gene action. Genetics **150:** 891–897.

Hollick, J. B., and V. L. Chandler, 2001 Genetic factors required to maintain repression of a paramutagenic maize *pl1* allele. Genetics **157:** 369–378.

Hollick, J. B., G. I. Patterson, E. H. Coe, Jr., K. C. Cone and V. L. Chandler, 1995 Allelic interactions heritably alter the activity of a metastable maize *pl* allele. Genetics **141:** 709–719.

Hollick, J. B., J. E. Dorweiler and V. L. Chandler, 1997 Paramutation and related allelic interactions. Trends Genet. **13:** 302–308.

Hollick, J. B., G. I. Patterson, I. M. Asmundsson and V. L. Chandler, 2000 Paramutation alters regulatory control of the maize *pl1* locus. Genetics **154:** 1827–1838.

Kermicle, J. L., 1970 Dependence of the *R*-mottled phenotype in maize on mode of sexual transmission. Genetics **66:** 69–85.

Kermicle, J. L., and M. Alleman, 1990 Gametic imprinting in relation to the angiosperm life cycle. Dev. Suppl., 9–14.

Kermicle, J. L., W. Eggleston and M. Alleman, 1995 Organization of paramutagenicity in *R-stippled* maize. Genetics **141:** 361–372.

Lee, E. A., 1997 B-A aneuploidy, pp. 315–316 in *Mutants of Maize*, edited by M. G. Neuffer, E. H. Coe and S. R. Wessler. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Longley, A. E., 1961 Breakage points for four corn translocation series and other corn chromosome aberrations. USDA-ARS Crops Res. Bull. **34–16:** 1–40.

Martienssen, R., 1996 Epigenetic phenomena: paramutation and gene silencing in plants. Curr. Biol. **6:** 810–813.

Matzke, M. A., A. J. M. Matzke and W. B. Eggleston, 1996 Paramutation and transgene silencing: A common response to invasive DNA? Trends Plant Sci. **1:** 382–388.

Matzke, M. A., W. Aufsatz, T. Kanno, M. F. Mette and A. J. Matzke, 2002 Homology-dependent gene silencing and host defense in plants. Adv. Genet. **46:** 235–275.

Mikula, B. C., 1961 Progressive conversion of *R*-locus expression in maize. Genetics **47:** 566–571.

Muller, H. J., 1930 Types of visible variations induced by X-rays in *Drosophila*. J. Genet. **22** (3): 299–337.

Murrell, A., S. Heeson and W. Reik, 2004 Physical contact between differentially methylated regions partitions the imprinted *Igf2* and *H19* genes into parent specific chromatin loops. Nat. Genet. **36:** 889–893.

Nakayama, J., A. J. Klar and S. I. Grewal, 2000 A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis. Cell **101:** 307–317.

O'Reilly, C., N. Shepherd, A. Pereira, Z. Schwarz-Sommer, I. Bertram *et al.*, 1985 Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements *En* and *Mu1*. EMBO J. **4:** 877–882.

Patterson, E. B., 1994 Translocations as genetic markers, pp. 361–363 in *The Maize Handbook*, edited by M. Freeling and V. Walbot. Springer-Verlag, New York.

Patterson, G. I., and V. L. Chandler, 1995 Timing of *b* locus paramutation. Maydica **40:** 35–41.

Patterson, G. I., C. J. Thorpe and V. L. Chandler, 1993 Paramutation, an allelic interaction, is associated with a stable and heritable reduction of transcription of the maize *b* regulatory gene. Genetics **135:** 881–894.

Selinger, D. A., and V. L. Chandler, 1999 A mutation in the *pale aleurone color1* gene identifies a novel regulator of the maize anthocyanin pathway. Plant Cell **11:** 5–14.

Sidorenko, L. V., and T. Peterson, 2001 Transgene-induced silencing identifies sequences involved in the establishment of paramutation of the maize *p1* gene. Plant Cell **13:** 319–335.

Shultz, J., 1936 Variegation in *Drosophila* and the inert chromosome regions. Proc. Natl. Acad. Sci. USA **22:** 27–33.

Stam, M., C. Belele, J. E. Dorweiler and V. L. Chandler, 2002a Differential chromatin structure within a tandem array 100 kb upstream of the maize *b1* locus is associated with paramutation. Genes Dev. **16:** 1906–1918.

Stam, M., C. Belele, W. Ramakrishna, J. E. Dorweiler, J. L. Bennetzen *et al.*, 2002b The regulatory regions required for *B′* paramutation and expression are located far upstream of the maize *b1* transcribed sequences. Genetics **162:** 917–930.

Styles, E. D., and R. A. Brink, 1966 The metastable nature of paramutable *R* allele in maize. I. Heritable enhancement in level of standard R^r action. Genetics **54:** 433–439.

Styles, E. D., and R. A. Brink, 1969 The metastable nature of paramutable *R* allele in maize. IV. Parallel enhancement of *R* action in heterozygotes with *r* and in hemizygotes. Genetics **61:** 801–811.

Voelker, R., J. Mendel-Hartvig and A. Barkan, 1997 Transposon-disruption of a maize nuclear gene, *tha1*, encoding a chloroplast SecA homologue: *in vivo* role of cp-SecA in thylakoid protein targeting. Genetics **145:** 467–478.

Walbot, V., 2001 Imprinting of *R-r*; paramutation of *B-I* and *Pl*, and epigenetic silencing of *MuDR/Mu* transposons in *Zea mays L.* are coordinately affected by inbred background. Genet. Res. **77:** 219–226.

Communicating editor: V. Sundaresan