Genetic Factors Required to Maintain Repression of a Paramutagenic Maize *pl1* Allele

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ABSTRACT

A genetic screen identified two novel gene functions required to maintain mitotically and meiotically heritable gene silencing associated with paramutation of the maize *purple plant 1* (*pl1*) locus. Paramutation at *pl1* leads to heritable alterations of *pl1* gene regulation; the *Pl-Rhoades* (*Pl-Rh*) allele, which typically confers strong pigmentation to juvenile and adult plant structures, changes to a lower expression state termed *Pl′-mahogany* (*Pl′*). Paramutation spontaneously occurs at low frequencies in *Pl-Rh* homozygotes but always occurs when *Pl-Rh* is heterozygous with *Pl′*. We identified four mutations that caused increased *Pl′* pigment levels. Allelism tests revealed that three mutations identified two new maize loci, *required to maintain repression 1* (*rmr1*) and *rmr2* and that the other mutation represents a new allele of the previously described *mediator of paramutation 1* (*mop1*) locus. RNA levels from *Pl′* are elevated in *rmr* mutants and genetic tests demonstrate that *Pl′* can heritably change back to *Pl-Rh* in *rmr* mutant individuals at variable frequencies. Pigment levels controlled by two *pl1* alleles that do not participate in paramutation are unaffected in *rmr* mutants. These results suggest that RMR functions are intimately involved in maintaining the repressed expression state of paramutant *Pl′* alleles. Despite strong effects on *Pl′* repression, *rmr* mutant plants have no gross developmental abnormalities even after several generations of inbreeding, implying that RMR1 and RMR2 functions are not generally required for developmental homeostasis.

EPIGENETIC control of gene expression plays a central role in both developmental processes and the maintenance of genome homeostasis. Much of our current understanding of these fundamental gene control mechanisms has resulted from studies of genes involved in color production (Russo *et al.* 1996; Wu and Morris 1999). Variations in pigment expression are easily detected and often occur without directly affecting viability. These attributes have facilitated the identification and study of X chromosome inactivation (Lyon 1961), position effect variegation (reviewed in Weiler and Wakimoto 1995), heterochromatin-induced inactivation (Dorer and Henikoff 1994; Henikoff *et al.* 1995), transvection (Jack and Judd 1979), pairing-sensitive silencing (reviewed in Pirrotta 1997), paramutation (reviewed in Chandler *et al.* 2000), homology-dependent gene silencing (Furner *et al.* 1998), cosuppression (reviewed in Jorgensen 1995; Pal-Bhadra *et al.* 1997), transposon regulation (reviewed in Federoff 1996 and in Martienssen 1996), quelling (Cogoni and Macino 1997), silencing of natural duplications (Ronchi *et al.* 1995; Todd and Vodkin 1996), imprinting (Kermicle 1978), and DNA methylation (Meyer *et al.* 1993). Genetic screens using pigment reporters have been particularly powerful in revealing the genes and mechanisms responsible for some of these types of epigenetic control (Reuter and Spierer 1992; Csink *et al.* 1994; Talbert *et al.* 1994; Cogoni and Macino 1997; Furner *et al.* 1998). Herein we describe results of a genetic screen using a pigment reporter gene to identify mutations that affect meiotically heritable gene silencing occurring as a result of paramutation in maize.

Paramutation results in meiotically heritable changes in gene expression. Paramutable alleles are susceptible to these changes while paramutagenic alleles induce these changes. The hallmark of paramutation is the observation that the expression of a paramutable allele is invariably reduced following exposure to a paramutagenic allele in a heterozygote. Additionally, paramutable alleles themselves become paramutagenic following exposure to a paramutagenic allele. Alleles that are neither susceptible to such changes nor induce these changes are referred to as "neutral." Four distinct examples of paramutation are known in maize (reviewed in Chandler *et al.* 2000) and all four occur among alleles of genes encoding transcriptional activators of the anthocyanin or phlobaphene genetic pathways. Thus, paramutation occurring at these loci results in reduced pigment production, a simple and visual indicator of gene expression.

Temporal and spatial patterns of anthocyanin pigment production are specified by overlapping expression of two transcriptional activators required for the

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transcriptional induction of genes encoding anthocyanin biosynthetic enzymes (reviewed in Dooner *et al.* 1991). These two transcriptional activators are encoded by two sets of functionally duplicate genes: the *booster 1* (*b1*) and *red 1* (*r1*) genes encode basic helix-loop-helix factors and the *colorless 1* (*c1*) and *purple plant 1* (*pl1*) genes encode myb-like factors (reviewed in Chandler *et al.* 2000). In our material, seedling and anther tissues are pigmented due to the combined action of the *r1* and *pl1* genes while other mature plant tissues are pigmented due to overlapping *b1* and *pl1* functions. The *Pl-Rhoades* (*Pl-Rh*) allele of the *pl1* locus is paramutable and can change to a paramutagenic state referred to as *Pl′-mahogany* (*Pl′*; Hollick *et al.* 1995). *Pl-Rh* normally conditions strong pigmentation to aerial tissues of the maize plant, but paramutation of *Pl-Rh* leads to reduced *pl1* RNA levels and *pl1* transcription that are visualized by reductions in pigment throughout the plant (Hollick *et al.* 2000). The paramutant state (*Pl′*) is very stable; no changes of *Pl′* to a fully expressed *Pl-Rh* state are seen in *Pl′/Pl′* or *Pl′/Pl-Rh* plants (Hollick *et al.* 1995). However, *Pl′* can change back to *Pl-Rh* when *Pl′* is heterozygous with a neutral *pl1* allele or when carried over a deletion (Hollick and Chandler 1998).

Several known modifier loci affect maize plant pigmentation. Recessive mutations at both *anthocyanin 3* (*a3*) and *mediator of paramutation 1* (*mop1*) loci lead to elevated levels of pigment in juvenile and adult plant structures (Lindstrom 1935; Dorweiler *et al.* 2000). The action of A3 appears to be independent of any effects on *pl1* alleles (C. C. Carey, W. VanHeeckeren and V. L. Chandler, unpublished results) but MOP1 acts specifically to maintain the repressed expression of both paramutant *b1* and *pl1* alleles (Dorweiler *et al.* 2000). Mutations in *mop1* lead to derepression of *Pl′* and allow some *Pl-Rh* alleles to be recovered upon outcrossing (Dorweiler *et al.* 2000). Thus MOP1 plays a central role in maintaining the repression of paramutant alleles.

Herein we describe a seedling-based genetic screen that identified two novel maize loci, *required to maintain repression 1* (*rmr1*) and *rmr2*, whose functions are required to maintain heritable repression of *Pl′*. Anthocyanin pigment and *pl1* RNA levels are elevated in all plants that are homozygous for either *rmr1* or *rmr2* mutations and a significant fraction of the gametes produced from these plants carry nonparamutagenic *Pl-Rh* alleles. In contrast to mutations in *mop1*, mutations in *rmr1* and *rmr2* do not cause gross developmental abnormalities. Together with the identification of *mop1* we are beginning to dissect the genetic requirements for paramutation in higher plants.

MATERIALS AND METHODS

Genetic stocks: All stocks contain dominant, functional alleles for all genes required for the production of anthocyanin pigments in seedling and anther tissues unless otherwise noted. Pistillate parents used for the mutagenesis experiment had the following *pl1* and *r1* genotypes: *Pl′*; *R-r* or *R-r/r-g*. Staminate parents for the mutagenesis experiment were originally obtained from the Maize Cooperation Stock Center (Urbana, IL; accession no. 611A: *Pl-Rh sm1*; *R-r*). The *salmon silks 1* (*sm1*) locus is 10 cM distal to *pl1*. The recessive *sm1* allele (*sm1*) confers salmon-colored silks when homozygous and thus provides a linked morphological marker to *Pl-Rh*. The *Pl′* testers used for initial crosses with putative mutants were obtained via spontaneous paramutation of *Pl-Rh* (Hollick *et al.* 1995). A632 (*pl-A632*; *R-r*), A619 (*pl-A619*; *r-g*), and W22 (*pl-W22*; *r-g*) inbred material was obtained from the USDA North Central Plant Introduction Station (Ames, IA). Additional W22 stocks (*pl-W22*; *R-r:standard*) were provided by Jerry Kermicle (University of Wisconsin, Madison). Five *Pl-Rh/Pl-Rh* tester stocks of different genetic backgrounds were used in crosses to determine whether or not mutant plants carried paramutagenic *Pl′* alleles. Material for *pl1* RNA measurements was produced by crossing plants homozygous for the given EMS-derived mutation by sibling plants heterozygous for the same mutations. Additional details of the specific genetic stocks used for these experiments are available upon request.

Pollen mutagenesis: Pollen pooled from multiple tassels was treated with EMS and applied to silks according to Neuffer and Coe (1978). Pistillate parents were derived from two related *Pl′/Pl′* families. Both families together yielded 345 ears with an average of 49 kernels per ear. Germination frequency was ~77% for the first 7500 M_1 seed planted. A total of 9000 M_1 seedlings were screened for dominant mutations affecting pigment production. Seedlings were grown in potting flats at an approximate density of 100 per square foot. Approximately 1000 M_1 plants were grown to maturity and self-pollinations of M_1 plants generated 495 M_2 families.

Seedling screens: M_2 families of 30 seeds each were germinated in unheated sand benches and grown under high-intensity lighting (1660 $\mu E/m^2$ sec using a 1:1 mixture of sodium vapor and metal halide lamps). Visible seedling phenotypes (Table 1) were noted between 14 and 18 days post-imbibition.

Genetic crosses and stock syntheses: Hand pollinations were used for all genetic crosses. Material for the *sm1*, *rmr1* cosegregation test was derived by crossing a single plant heterozygous for *ems136* and homozygous *sm1* to a plant homozygous for *ems235* but heterozygous for the recessive *sm1* allele. The following syntheses and analyses were used to generate material to test the effects of the EMS-derived mutations on neutral *pl1* alleles. Plants heterozygous for a given mutation (*Pl′* anthers) were crossed to both the A632 inbred line and a W22 line (obtained from J. Kermicle, University of Wisconsin, Madison) containing the weakly expressed *pl1* alleles *pl-A632* and *pl-W22*, respectively. The *pl-A632*, *pl-W22*, and *Pl-Rh* alleles are all distinct on the basis of restriction fragment length polymorphism (RFLP) analyses (J. Hollick, unpublished results). F_1 plants were either self-pollinated (A632 material) or backcrossed to plants homozygous for the given mutation (W22 material). F_2 (A632 material) and BC_1 (W22 material) progeny were grown to maturity. The *pl1* genotypes of all plants in the F_2 and BC_1 families were determined using RFLP gel blot analysis as previously described (Hollick *et al.* 1995). Anther phenotypes of all homozygous *pl-A632* F_2 plants were photographed for later comparison. All homozygous *pl-A632* F_2 plants were crossed to plants heterozygous for the given EMS-induced mutation to determine whether the F_2 plants were also homozygous for the given EMS-induced mutation. Two F_2 plants that were homozygous *pl-A632* by RFLP analysis and homozygous for *rmr1-1* by testcross analysis were identified and five F_2 plants that were homozygous *pl-A632* by RFLP analysis and homozygous for *rmr2-1* by testcross analysis were

TABLE 1

EMS-derived M_2 seedling mutations

Mutant class	No. of EMS-derived mutations	Frequency of EMS-derived mutations	Frequency of EMS-derived mutations (Neuffer 1978)
Chlorophyll deficient			
Albino	15	0.09	0.13
Lemon white	21	0.12	0.037
Yellow green	14	0.08	0.015
Pale green	16	0.09	0.015
Variable mutants			
Piebald	1	0.006	0.03
Striped	12	0.076	0.09
Premature chlorosis	7	0.04	NR
Cross banded	3	0.02	NR
Seedling lethals	37	0.21	0.12
Glossy	8	0.05	0.026
Dwarf	6	0.04	NR
Others	63	0.36	0.29

The number and frequency of visible mutations identified in the current M_2 screen are compared with the frequency of similar mutations identified from an earlier EMS pollen mutagenesis (Neuffer 1978). NR, none reported.

identified. Examination of the anther photographs did not indicate any pigment differences between A632 inbred anthers or any F_2 *pl-A632* plants regardless of whether or not they carried the EMS-induced mutations. The BC_1 plants that were *Pl′/pl-W22* were self-pollinated and also crossed to plants heterozygous for a given EMS-induced mutation to identify BC_1 plants that were also homozygous for EMS-induced mutations. Anther pigmentation of *pl-W22/pl-W22*; *rmr1-1/rmr1-1* and *pl-W22/pl-W22*; *rmr2-1/rmr2-1* plants was weak and not obviously different from the anther pigmentation of heterozygous siblings or grandparental W22 plants.

RNA measurements: RNA isolations from anther tissues and RNase-protection assays were performed as previously described (Hollick *et al.* 2000).

RESULTS

Pollen mutagenesis generated new maize mutations: The number of mutable loci affecting paramutation-based gene silencing is unknown. To identify such loci, we performed chemical mutagenesis using ethyl methanesulfonate (EMS) to produce a high frequency of new maize mutations. M_1 seed was obtained by applying EMS-treated pollen from *Pl-Rh/Pl-Rh* plants to the silks of receptive *Pl′/Pl′* ears (materials and methods). A total of 495 M_2 families were subsequently generated by self-pollination of M_1 plants to screen for recessive mutations. Small M_2 families (30 seeds each) were grown for 14–18 days (materials and methods) and then visually examined for germination frequencies and unusual morphological phenotypes. Table 1 outlines the frequency of mutant phenotypes identified. Observed mutation frequencies are similar to previous EMS-pollen mutageneses (Neuffer 1978), indicating that our chemical mutagenesis was highly efficient in producing new maize mutations.

Dominant mutations affecting *Pl′* expression were not found: Because the primary leaf sheath of *Pl′/Pl-Rh* seedlings is normally weakly pigmented (Hollick *et al.* 1995), dominant EMS-induced mutations that either release *Pl′* from a repressed expression state or prohibit the establishment of paramutation might be expected to confer fully colored M_1 seedling phenotypes. Sixteen out of 9000 M_1 seedlings examined were fully colored. However, no flowering plants from these 16 fully colored seedlings had fully colored *Pl-Rh*-like anthers. This result indicates that our seedling screen for dominant mutations affecting anther pigmentation has an ~0.2% false-positive rate. In addition, none of the ~1000 M_1 plants grown to maturity had a *Pl-Rh* phenotype. Thus, no dominant mutations affecting either the establishment of *pl1* paramutation or the maintenance of *Pl′* repression were identified out of 10,000 M_1 plants tested.

Genetic screens identified recessive mutations affecting seedling and anther pigmentation: Recessive mutations that release *Pl′* from a repressed expression state might also be expected to confer fully colored seedling phenotypes (Figure 1). Our M_2 screen identified five families (nos. 60, 96, 136, 235, 240) that segregated fully colored seedlings to weakly colored seedlings in the following ratios (60, 4:23; 96, 1:29; 136, 6:24; 235, 3:25; 240, 8:16). In four of five families, fully colored seedlings gave rise to mature plants with fully pigmented *Pl-Rh*-like anthers (Figure 2A). Material from family 60 was dropped from further analyses because plants from fully colored seedlings in family 60 did not have fully pigmented anthers.

Genetic segregation tests indicated that the *Pl-Rh*-like phenotypes seen in three of the M_2 families (nos. 96,

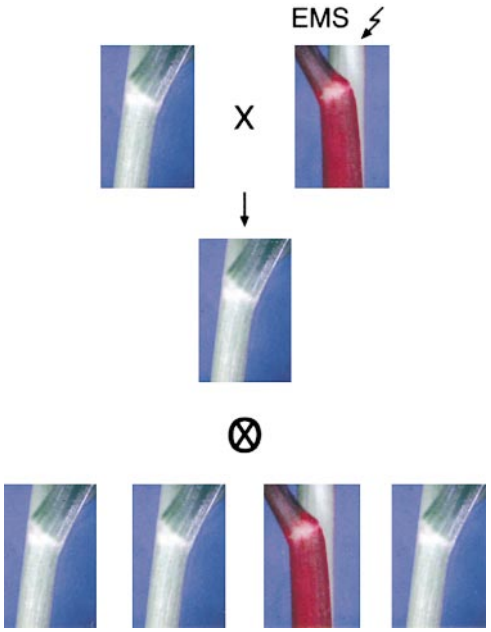

FIGURE 1.—Outline of genetic screen used to identify mutations affecting seedling pigmentation. *Pl-Rh* seedlings have fully colored first leaf sheaths (top right). *Pl′* seedlings have very weakly colored first leaf sheaths (top left). Pollen from *Pl-Rh* plants was treated with EMS and brushed on the silks of *Pl′* plants. All *Pl′*/*Pl-Rh* M_1 plants had a *Pl′* seedling phenotype (middle). M_1 plants were self-pollinated and M_2 families were screened for *Pl-Rh*-like seedlings. (Bottom) An M_2 family segregating 3:1 for *Pl′* and *Pl-Rh*-like seedlings.

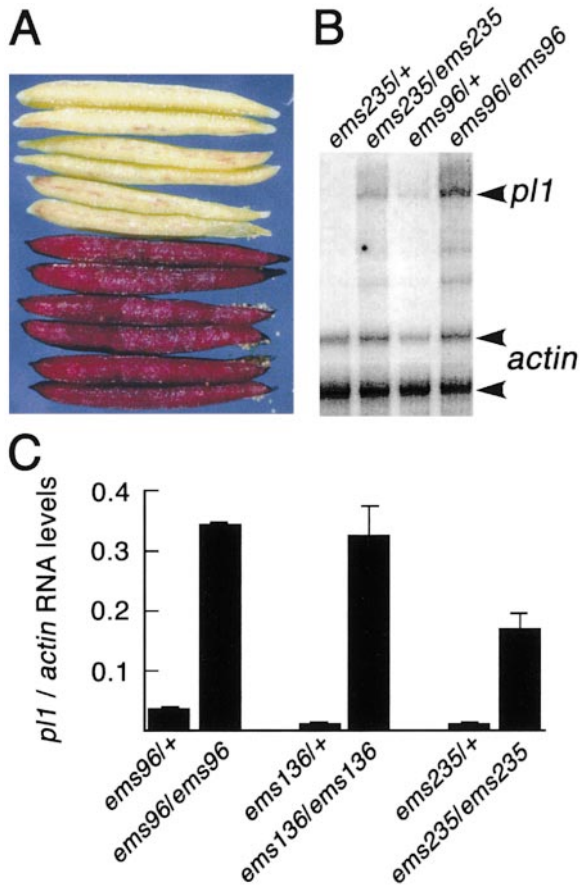

FIGURE 2.—EMS-derived mutations affect anther pigment and *pl1* RNA levels. (A) Anther phenotypes of plants that are heterozygous (top three variegated anthers) and homozygous (bottom three fully colored anthers) for the *ems235* allele. The difference between anther pigment phenotypes was identical for both the *ems96* and *ems136* alleles (not shown). (B) RNase-protection assay measuring the levels of *pl1* and *actin* RNA found in anther tissues of plants with the indicated genotypes. (C) Histogram of RNase-protection results for *pl1* RNA levels measured relative to *actin* RNA for the indicated genotypes. Bars represent average measurements for each genotype and crossbars above each bar indicate the standard error. The number of samples measured for each genotype is as follows: *ems96*/+, $n = 2$; *ems96*/*ems96*, $n = 2$; *ems136*/+, $n = 3$; *ems136*/*ems136*, $n = 4$; *ems235*/+, $n = 5$; *ems235*/*ems235*, $n = 6$.

136, and 235) were heritable as single-locus recessive traits. Plants with fully colored anthers derived from the three M_2 families were first crossed with *Pl′*/*Pl′* plants to complement the putative recessive mutations. All F_1 plants from these crosses had a clear *Pl′* phenotype (family 96, 35/35; family 136, 36/36; family 235, 36/36), indicating that the putative mutations affecting seedling and anther color are recessive. F_1 plants from families 96, 136, and 235 were self-pollinated to recover the fully colored seedling and anther trait. In all three cases, fully colored anther phenotypes were recovered at roughly a 1:3 ratio in F_2 families (Table 2), consistent with the interpretation that *Pl-Rh*-like phenotypes are due to single-locus recessive mutations. As expected, subsequent crosses between mutant plants and heterozygous siblings gave rise to families in which the mutant and normal phenotypes approximated a 1:1 ratio (family 96, 231:272; family 136, 100:88; family 235, 111:125).

Complementation tests define three loci: Genetic complementation was tested in pairwise combinations among the four EMS-induced mutations and also with the recessive *a3-Styles* allele (STYLES and COE 1986) and the recessive *mop1-1* allele (DORWEILER *et al.* 2000). Specific EMS-induced mutations were designated according to the family in which they were identified: *ems96*, *ems136*, *ems235*, and *ems240*. For each mutation, plants heterozygous for a given mutation (*Pl′* anthers or lightly colored plant in the case of *a3-Styles* heterozygotes) were individually crossed by plants homozygous for a different allele (*Pl-Rh*-like anthers or darkly colored plants in the case of *a3-Styles* homozygotes). Anther pigment phenotypes of progeny from these crosses were quantified (Table 3) on a 1–7 graded anther color score (ACS). ACS 7 describes the fully colored *Pl-Rh* phenotype, ACS 1–4 represent a stable *Pl′* state, and ACS 5 and 6 correspond to a typically metastable *Pl′* state (HOLLICK *et al.* 1995). On the basis of these results, mutations *ems136* and *ems235* fail to complement and thus define a single locus; *ems240* is allelic to *mop1*; *ems96* defines a third locus. Further description of the *ems240* allele, designated *mop1-2EMS*, is provided in DORWEILER *et al.* (2000).

EMS-derived mutations allow increased expression

TABLE 2

Inheritance of EMS-induced mutations

EMS allele	*Pl-Rh* anthers	*Pl′* anthers	Frequency of plants with *Pl-Rh* anthers
ems96	8	33	0.24
ems136	9	52	0.17
ems235	46	170	0.27

EMS-induced factors affecting *Pl′* are inherited as single gene recessive mutations. The number and frequency of plants with fully colored anthers are indicated for the following number of F_2 families: *ems96*, three families; *ems136*, four families; *ems235*, six families.

TABLE 3

Complementation tests

Alleles tested	Ears examined	No. of plants with given anther color score: 1–4	5 or 6	7
ems96				
a3-Styles	4	112	0	0
mop1-1	1	13	0	0
ems136	3	47	0	0
ems235	5	75	0	0
ems240	4	78	0	0
ems136				
a3-Styles	2	29	1	0
mop1-1	1	16	0	0
ems235	3	24	0	26
ems235				
a3-Styles	2	36	2	0
mop1-1	1	14	0	0
ems240	2	23	1	0
ems240				
mop1-1	1	7	0	11

Complementation tests identify three unique loci. For each allele listed, the underlying series of alleles were tested for complementation. The number of ears sampled for each test are indicated along with the total numbers of mature plants with a given anther color score derived from the seeds off those ears.

of *pl1* RNA: We used RNase-protection experiments to compare *pl1* RNA levels from anthers of *Pl′/Pl′* plants either homozyogus or heterozygous for the *ems136*, *ems235*, or *ems96* alleles. Using *actin* RNA as a control, *pl1* RNA levels were 26-, 14-, and 9-fold greater in homozygous *ems136*, *ems235*, and *ems96* mutants *vs.* heterozygous siblings, respectively (Figure 2, B and C). These results indicate that the normal functions of these genes identified by mutations are required to maintain repression of *pl1* RNA accumulation.

Genes affecting repression of *Pl′* encode *trans*-acting factors: Both *mop1* and the locus defined by the *ems96* allele genetically map to chromosome *2* (J. Dorweiler and V. Chandler, unpublished results; J. Hollick, unpublished results) whereas the *pl1* locus is found on chromosome *6*. Alleles that define the other locus (*ems136* and *ems235*) failed to cosegregate with a genetic marker closely linked to the *pl1* locus. The *salmon silks 1* (*sm1*) locus, located 10 cM distal to *pl1*, normally conditions yellow maize silks but plants that are homozygous for the recessive *sm1* allele (*sm1*) have salmon-colored silks. Genetic crosses (materials and methods) were used to ask whether the fully colored anther phenotype found in homozygous mutant plants cosegregated with the recessive *sm1* allele. A total of 6/15 (40%) segregant plants with fully colored anthers were *sm1/sm1* and 9/21 (42%) plants with weakly colored anthers were *sm1/sm1*. The lack of strong cosegregation between the fully colored anther phenotype and recessive alleles of the *sm1* locus indicates that the locus defined by the *ems136* and *ems235* alleles is distinct from the *pl1* locus. Thus all three loci identified in our genetic screen define *trans*-acting genetic factors affecting *pl1* RNA accumulation in *Pl′/Pl′* plants.

All three loci identified by mutation encode factors required to maintain heritable repression of *Pl′*: The fully colored phenotypes of seedlings and anthers, together with the increases in *pl1* RNA seen when the EMS-derived mutations are homozygous, suggested that *Pl′* may have changed to a *Pl-Rh* state. Plants homozygous for *mop1-1* sometimes show heritable changes of *Pl′* to *Pl-Rh* (Dorweiler *et al.* 2000). To test mutations at the other two loci, mutant plants with fully colored anthers were crossed to a series of *Pl-Rh/Pl-Rh* testers and the anther phenotypes of the progeny were quantified. If *Pl′* heritably changes to *Pl-Rh* in plants homozygous for the *ems136*, *ems235*, or *ems96* alleles, then fully colored *Pl-Rh* phenotypes should be found in the resulting progeny. If *Pl′* does not heritably change to *Pl-Rh*, then only *Pl′* phenotypes would be found (Hollick *et al.* 1995). Results of these crosses (Table 4) indicate that while *Pl′* can be transmitted, *Pl′* often changes to a meiotically heritable *Pl-Rh* state in plants homozygous for the *ems136*, *ems235*, or *ems96* alleles.

We have designated these new loci "*rmr*" for *required to maintain repression* to reflect the necessity of *rmr* functions for maintaining *Pl′* in a mitotically and meiotically heritable repressed state. The *ems136* and *ems235* alleles together define the *rmr1* locus and are designated *rmr1-1* and *rmr1-2*, respectively. The *ems96* allele defines the *rmr2* locus and is designated *rmr2-1*.

Pl′ most frequently changed to a nonparamutagenic (*Pl-Rh*) state in plants homozygous for either the *rmr1-1* or *rmr1-2* alleles (Table 4). Approximately 70% of crosses between *Pl-Rh/Pl-Rh* testers and *Pl′/Pl′* plants homozygous for either the *rmr1-1* or *rmr1-2* allele produced at least one progeny plant having a *Pl-Rh* anther phenotype compared to only 22% of similar testcrosses with *Pl′/Pl′* plants homozygous for the *rmr2-1* allele. This difference may partially relate to observations showing that different *Pl-Rh/Pl-Rh* testers themselves have different frequencies of spontaneous paramutation; *Pl-*

TABLE 4

Pl′* changes to *Pl-Rh*: *Pl′/Pl′*; *ems/ems* × *Pl-Rh/Pl-Rh* and *Pl-Rh/Pl-Rh* × *Pl′/Pl′*; *ems/ems

Allele tested	Ears examined	No. of plants with given anther color score							Frequency of *Pl-Rh* types
		1	2	3	4	5	6	7	
ems136	21 (15/21)	40	113	100	38	26	22	61	0.15
ems235	9 (6/9)	4	33	46	26	8	13	17	0.12
ems96	40 (9/40)	267	390	84	46	13	0	80	0.09

Genetic crosses listed at the top of the table were used to determine whether or not *Pl′* alleles could change to a nonparamutagenic *Pl-Rh* state in plants that were homozygous for the given EMS-derived alleles. The total number of ears sampled is given along with the fraction of ears that gave rise to plants with fully colored anthers. The total numbers of progeny with a given anther color score are listed along with the frequency of progeny having a *Pl-Rh* phenotype (ACS 7).

Rh can spontaneously change to *Pl′* in the absence of *Pl′* (Hollick *et al.* 1995). To control for such differences, *Pl-Rh/Pl-Rh* pollen collected from a single plant was used to pollinate *Pl′/Pl′*; *rmr1-1/rmr1-1* and *Pl′/Pl′*; *rmr2-1/rmr2-1* plants. Both sets of crosses produced at least one progeny plant with a *Pl-Rh* phenotype, indicating that both *rmr1-1* and *rmr2-1* mutations can allow meiotically heritable derepression of *Pl′*. However, proportionally more *Pl-Rh*-like progeny were derived from crosses made with homozygous *rmr1-1* plants (Table 5). In addition, progeny plants had higher anther color scores from the *rmr1-1* crosses relative to the *rmr2-1* crosses, suggesting that *Pl′* alleles are, in general, less paramutagenic when transmitted through plants homozygous for *rmr1-1* as compared to *rmr2-1* (Tables 4 and 5).

Given that *Pl′* could sometimes change to a meiotically heritable *Pl-Rh* state in either homozygous *rmr1-1* or *rmr2-1* plants, we asked whether such newly changed *Pl′* alleles, formally designated *Pl(′)*, were distinguishable from a naive *Pl-Rh* allele. It was possible that *Pl(′)* might retain residual paramutagenic activity relative to *Pl-Rh*. A cosegregation test using linked *sm1* markers was used to show that a *Pl(′)* allele transmitted from a either a *rmr1-1* or *rmr2-1* homozygote was indistinguishable from naive *Pl-Rh* in terms of its paramutagenicity (Figure 3). Thus, both RMR1 and RMR2 functions contribute to the meiotically heritable maintenance of the paramutagenic *Pl′* state. In the absence of RMR1 or RMR2 function, *Pl′* always has a somatic expression phenotype indistinguishable from *Pl-Rh* but this does not ensure that *Pl′* always changes to a meiotically heritable *Pl-Rh* state.

***rmr* mutations do not affect the expression of other *pl1* alleles:** We addressed the possibility that *rmr* functions generally affect all *pl1* alleles by combining neutral *pl1* alleles with either the *rmr1-1* or *rmr2-1* alleles (materials and methods) and examining the anther phenotypes. The neutral *pl-A632* and *pl-W22* alleles confer weak, sunlight-dependent pigmentation to the anthers. Anthers of plants homozygous for the *pl-A632* or *pl-W22* alleles had visibly identical levels of pigmentation regardless of whether the plants were homozygous for a *rmr* mutation (Figure 4; materials and methods), implying that RMR1 and RMR2 functions specifically affect the expression of paramutant *Pl′* alleles.

Heritable changes of *Pl′* to *Pl-Rh* in *rmr1* mutants are unaffected by mode of sexual transmission: Given that male and female gametes are differentially imprinted at a few loci in plants (Kermicle and Alleman 1990; Vielle-Calzada *et al.* 2000) at different times and locations during development, we wondered whether meiot-

TABLE 5

Pl′* changes to *Pl-Rh* using a single pollen source: *Pl′/Pl′*; *rmr/rmr* × *Pl-Rh/Pl-Rh

Allele tested	Ears examined	No. of plants with given anther color score							Frequency of *Pl-Rh* Types
		1	2	3	4	5	6	7	
rmr1-1	4 (2/4)	4	21	10	24	7	0	9	0.12
rmr2-1	4 (1/4)	24	20	5	5	2	0	1	0.02

The *Pl′* state can change to *Pl-Rh* in plants homozygous for mutations in *rmr* loci. Genetic crosses using a shared source of *Pl-Rh/Pl-Rh* pollen were made as indicated at the top of the table with female parents homozygous for the given *rmr* alleles. The total number of ears sampled is given along with the fraction of ears that gave rise to plants with fully colored anthers. The total numbers of progeny with a given anther color score are listed along with the frequency of progeny with a *Pl-Rh* phenotype (ACS 7).

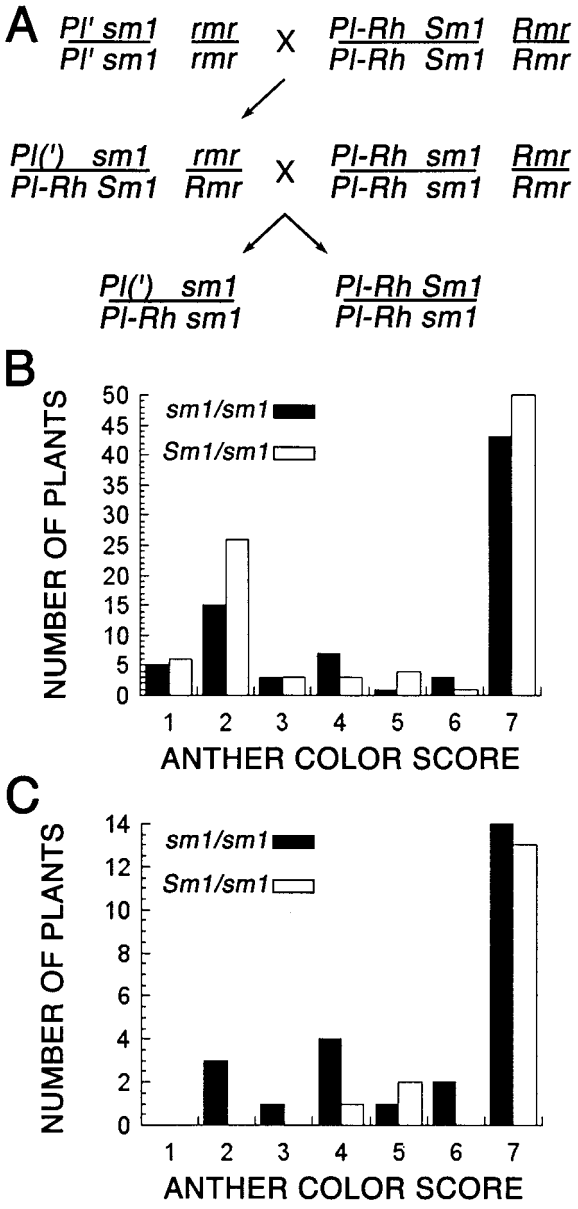

FIGURE 3.—*Pl'* can change to *Pl-Rh* in plants that are homozygous for *rmr* mutations. (A) Pedigree outlining two genetic crosses used to show that plants homozygous for the *rmr* mutations can transmit nonparamutagenic *Pl-Rh* alleles. *Pl(')* is used to identify a *pl1* allele that should have been *Pl'* in the first generation but is not paramutagenic; the *Pl(')/Pl-Rh* plants in the second cross have fully colored anthers. (B) Results of crosses initiated with plants that were homozygous for the *rmr1-1* allele. Histogram represents the number of plants with a given anther color score that were either *sm1/sm1* (salmon-colored silks; solid bars) or *Sm1/sm1* (yellow silks; open bars). (C) Results of crosses initiated with plants that were homozygous for the *rmr2-1* allele. Histogram is similar to that shown in B.

ically heritable changes of *Pl'* to *Pl-Rh* occurred with equal frequencies in the two separate somatic cell lineages or whether there were gametophyte-specific effects. This possibility was addressed using reciprocal crosses between *Pl-Rh/Pl-Rh* testers and plants homozygous for the *rmr1-1* allele. Pollen from *Pl-Rh/Pl-Rh* testers was placed on receptive silks of *Pl'/Pl'*; *rmr1-1/rmr1-1* plants and vice versa. Plants derived from seven reciprocal crosses were grown to maturity and the anther color scores determined (Table 6). When all the data are combined, the same frequency of *Pl-Rh* progeny was observed when *Pl'* was transmitted through either female or male gametes. When each reciprocal cross was examined, three sets of families had very similar frequencies. Frequencies for the remaining four sets of families varied 1.5- to 2-fold but there was no sex-specific trend. The frequencies of *Pl'* to *Pl-Rh* changes observed between individual *rmr1-1/rmr1-1* plants could vary as much as 6-fold but the frequencies observed within each pair of reciprocal crosses varied no more than 2-fold. Thus, the frequency of heritable *Pl'* to *Pl-Rh* changes in *rmr1-1* homozygotes appears to be intrinsic to each individual sporophyte and is not differentially affected by female *vs.* male gametophyte development.

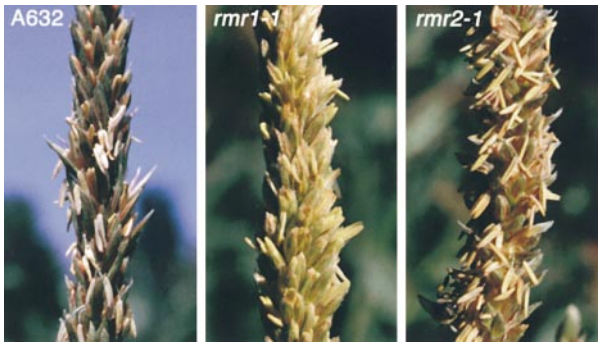

FIGURE 4.—Anther phenotypes of plants that are homozygous for the *pl-A632* allele. Photographs are of tassels from an A632 plant (left), a plant homozygous for the *rmr1-1* allele (center), and a plant homozygous for the *rmr2-1* allele (right).

***rmr1* and *rmr2* mutations do not appear to affect plant development:** Although mutations in *mop1* can lead to a wide range of developmentally abnormal phenotypes (DORWEILER *et al.* 2000), we have not observed grossly abnormal phenotypes in homozygous *rmr1* and *rmr2* mutant plants. We began this series of observations by first generating F_1 individuals between A632 inbred plants and plants homozygous for either *rmr1-1*, *rmr1-2*, or *rmr2-1* and then by examining the F_2 homozygous mutants derived from self-pollination. This strategy was adopted to reduce the number of other unlinked EMS-induced mutations that might potentially affect plant morphology. Of the 200 *rmr* F_2 plants segregating for each *rmr* mutation, all plants that had a fully colored anther phenotype were otherwise similar in stature, morphology, and flowering time to sibling plants. Similar uniform phenotypes were observed among F_2 plants generated from F_1 plants made by crossing the *rmr* mutations to either the A619 or W22 inbred lines.

Further generations of inbreeding did not produce abnormal plants. Several *rmr* F_2 plants (50% A632 back-

TABLE 6

Transmission of *Pl-Rh* from *rmr1-1* plants

Reciprocal parent crosses	No. of progeny plants with given anther color scores							Frequency of *Pl-Rh* types
	1	2	3	4	5	6	7	
98-720-10 × 98-649-1	0	5	3	3	2	0	4	0.24
98-649-1 × 98-720-10	1	1	2	4	1	2	6	0.35
98-720-19 × 98-645-1	0	2	9	2	3	1	3	0.15
98-645-1 × 98-720-19	0	1	2	5	5	1	6	0.3
98-720-22 × 98-648-3	0	0	1	2	2	2	14	0.67
98-648-3 × 98-720-22	0	2	1	0	3	1	12	0.63
98-721-4 × 98-648-1	0	3	2	4	0	0	9	0.5
98-648-1 × 98-721-4	0	2	0	6	3	2	6	0.32
98-721-12 × 98-646-4	0	0	3	0	1	1	13	0.72
98-646-4 × 98-721-12	0	0	0	1	2	1	11	0.73
98-721-17 × 98-645-2	0	2	4	2	0	5	9	0.41
98-645-2 × 98-721-17	0	0	0	1	2	1	18	0.82
98-721-18 × 98-648-4	0	1	0	1	0	0	17	0.89
98-648-4 × 98-721-18	0	0	0	1	2	0	9	0.75
Total for *rmr1-1* females	0	13	22	18	8	9	87	0.55
Total for *rmr1-1* males	1	6	5	18	20	10	82	0.58

Results of reciprocal crosses between seven plants within a single family of *rmr1-1* individuals and four different *Pl-Rh/Pl-Rh* testers. Specific pedigree numbers are used to define each set of crosses. Pedigree number of the *rmr1-1* homozygote used in each cross is underlined. The female and male parents are listed first and second, respectively. The total numbers of progeny with a given anther color score are listed along with the frequency of plants with a *Pl-Rh* phenotype (ACS 7).

ground) with fully colored anthers that had a *Pl-Rh/Pl-Rh* RFLP genotype were self-pollinated and also outcrossed to *Pl-Rh/Pl-Rh* testers. All F_3 plants from the self-pollinations had fully colored anthers but were otherwise similar in all other respects to testcross progeny. F_4 plants derived by self-pollination of homozygous *rmr1-1* F_3 plants were also normal in appearance and similar in all respects to the previous F_3 plants. A single F_3 plant homozygous for *rmr2-1* was crossed to a *rmr2-1* heterozygote from a separate lineage to generate a family where *rmr2-1* homozygotes and heterozygotes could be compared. Aside from differences in anther pigmentation, *rmr2-1* homozygotes were indistinguishable from their heterozygous siblings. Although it remains possible that prolonged exposure of the genome to defects in RMR1 and RMR2 functions could have pleiotropic consequences, we currently have no indications that they are required for proper plant development, at least in a 50% A632 background.

DISCUSSION

Using a seedling-based genetic screen, we identified three maize loci (*mop1*, *rmr1*, and *rmr2*) whose *trans*-acting functions are required to maintain gene silencing that occurs as the result of paramutation at the *pl1* locus. In all plants homozygous for mutations in *mop1*, *rmr1*, or *rmr2*, *Pl′* is expressed at a high level indistinguishable from *Pl-Rh* (Dorweiler *et al.* 2000; this report). While the reduced expression state (*Pl′*) is most frequently restored upon outcrossing to nonmutant plants, an increased expression state indistinguishable from *Pl-Rh* can be meiotically heritable. Because *pl1* paramutation is associated with changes in *pl1* transcription (Hollick *et al.* 2000), our working model is that RMR1, RMR2, and MOP1 functions are involved with the maintenance of specific chromatin structures that prohibit high levels of *pl1* transcription.

Additional *rmr* loci are clearly involved in *Pl′* repression. Our reported genetic screen represents only 10% theoretical saturation. This estimate is based on the apparent mutation rate of one detectable hit per gene per 1000 genomes screened and the roughly 95% probability that 5000 screened genomes would yield at least one detectable mutation in every given gene. These numbers suggest that 6–10 such *rmr*-like loci exist in maize. Indeed, 4 additional *rmr* loci have already been identified in ongoing genetic screens (J. Hollick, unpublished results).

Paramutation silencing at the *pl1* locus not only leads to repression of *Pl-Rh* expression, it also affects the way in which the *Pl-Rh* allele is regulated (Hollick *et al.* 2000). *Pl-Rh* expression is light insensitive while *Pl′* expression is light dependent. As most *pl1* alleles are regulated by light cues, the observations that *rmr1* and *rmr2* mutations have no effect on *pl-A632* or *pl-W22* suggest that RMR functions do not mediate light induction. Rather, we hypothesize that RMR functions are used in maintaining a specific regulatory state unique to *Pl′*. The *Pl-Rh* state then must actively prevent or avoid RMR action. Two general and nonexclusive modes of action are considered: (1) *rmr* RNA or proteins are compo-

nents of a heritable chromatin structure that affects *pl1* transcription, and (2) RMR functions facilitate alterations or maintenance of a heritable chromatin structure. Previous work has shown that paramutant *pl1* and *r1* alleles require continued allelic interactions with another paramutant or paramutagenic partner (Styles and Brink 1968; Hollick and Chandler 1998). It is possible that RMR functions actually mediate these allelic interactions through homology searching or specifying internuclear positions.

It is not known whether the *rmr* mutations described represent complete loss-of-function alleles. In fact, the observation that *pl1* RNA levels are significantly lower in homozygous *rmr1-2* plants *vs. rmr1-1* plants hints that the two alleles have different levels of activity. Nonetheless, mutations at both the *rmr1* and *rmr2* loci are completely recessive, suggesting that neither the RMR1 nor RMR2 functions that affect *Pl′* repression are dosage sensitive as are many of the genes required to mediate examples of position effect variegation seen in Drosophila (reviewed in Weiler and Wakimoto 1995).

Despite similar effects on *pl1* RNA levels, the frequency of meiotically heritable *Pl′* to *Pl-Rh* changes is clearly different in the *rmr1* mutants *vs. rmr2-1* mutants. Even in *rmr1-2* homozygotes where the relative level of *pl1* RNA is lower than in *rmr2-1* homozygotes, *Pl′* changes to a *Pl-Rh* state more frequently in homozygous *rmr1-2* plants. This set of results implies that derepression of *Pl′* in somatic tissues is, by itself, insufficient to allow a meiotically heritable change of *Pl′* to *Pl-Rh*. In addition to *Pl′* heritably changing to *Pl-Rh* more frequently in *rmr1* mutant plants, those alleles that remain *Pl′* upon transmission appear to be less paramutagenic than those transmitted from *rmr2-1* mutant plants. One interpretation is that RMR1 and RMR2 functions are distinct in terms of their role(s) in maintaining meiotically heritable repression of *Pl′*. Alternatively, the *rmr2-1* allele may encode a partially active function with respect to maintaining the meiotically heritable *Pl′* state.

Although *Pl′* heritably changes to *Pl-Rh* at different frequencies between individual *rmr1-1/rmr1-1* plants, similar frequencies were observed independent of female *vs.* male transmission in several reciprocal crosses (Table 6). In these families, the frequency of meiotically heritable changes of *Pl′* to *Pl-Rh* was established early in development prior to the point that cell lineages diverged to specify the lateral *vs.* apical inflorescence meristems. Frequencies of heritable *Pl′* to *Pl-Rh* changes were distinct, however, between each set of reciprocal crosses. This observation implies that different frequencies can be established and maintained through most of sporophyte development with relatively high fidelity. It remains unclear as to whether *Pl′* actually changes to a meiotically heritable *Pl-Rh* state during early development or whether the changes are only preset early.

RMR1 and RMR2 functions do not appear to be involved in general gene control mechanisms required for development. To date, plants homozygous for the *rmr1-1*, *rmr1-2*, or *rmr2-1* alleles have been morphologically and developmentally indistinguishable from their heterozygous siblings. This absence of morphological defects, even after three generations of selfing, suggests that *rmr1* and *rmr2* are unlikely to be maize orthologues of the Arabidopsis *ddm1* or *met1* genes. Mutations of *ddm1*, a SWI2/SNF-2-like gene (Jeddeloh *et al.* 1999), and dominant inhibitors of *met1*, a DNA methyl transferase enzyme, have broad and cumulative effects on Arabidopsis development (Finnegan *et al.* 1996; Kakutani *et al.* 1996; Ronemus *et al.* 1996). Because mutations in *mop1* also appear to have effects on plant development (Dorweiler *et al.* 2000), it appears that the *rmr1* and *rmr2* loci define a class of genetic functions distinct from MOP1. Alternatively, RMR1 and RMR2 may participate in developmental pathways similar to MOP1 but have redundant developmental functions. Double mutant combinations are currently being synthesized to test this possibility.

Several other genes required to maintain transgene silencing in Arabidopsis have been recently described. Some of these genes, such as *sgs1*, *sgs2*, and *sgs3* (*suppressor of gene silencing*) and *sde1*, *sde2*, *sde3*, and *sde4* (*silencing defective*), are required for post-transcriptional silencing (Elmayan *et al.* 1998; Dalmay *et al.* 2000; Mourrain *et al.* 2000) while others, *hog1* (*homology-dependent gene silencing 1*), *sil1*, *sil2* (*silencing*), and *mom1* (*Morpheus' molecule 1*), are required for transcriptional-based silencing (Furner *et al.* 1998; Amedeo *et al.* 2000). Given that paramutation at the *b1* and *pl1* loci affect heritable states of transcriptional control (Patterson *et al.* 1993; Hollick *et al.* 2000), the *rmr* genes could potentially be related to this latter class of Arabidopsis genes. It is also possible that RMR functions are specific for paramutation interactions, which to this point have not been described in Arabidopsis.

Our initial genetic dissection of the components required to maintain *Pl′* repression lays the foundation for further studies to understand the broad effects of RMR action and represents an initial step toward a molecular characterization of the epigenetic mechanism(s) responsible for paramutation in higher plants.

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