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Multiple *Trans*-Sensing Interactions Affect Meiotically Heritable Epigenetic States at the Maize *pl1* Locus

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ABSTRACT

Interactions between specific maize *purple plant1* (*pl1*) alleles result in heritable changes of gene regulation that are manifested as differences in anthocyanin pigmentation. Transcriptionally repressed states of *Pl1-Rhoades* alleles (termed *Pl′*) are remarkably stable and invariably facilitate heritable changes of highly expressed states (termed *Pl-Rh*) in *Pl′/Pl-Rh* plants. However, *Pl′* can revert to *Pl-Rh* when hemizygous, when heterozygous with *pl1* alleles other than *Pl1-Rhoades*, or in the absence of *trans*-acting factors required to maintain repressed states. *Cis*-linked features of *Pl1-Rhoades* responsible for these *trans*-sensing behaviors remain unknown. Here, genetic tests of a *pl1* allelic series identify two potentially separate *cis*-linked features: one facilitating repression of *Pl-Rh* and another stabilizing *Pl′* in *trans*. Neither function is affected in ethyl-methanesulfonate-induced *Pl1-Rhoades* derivatives that produce truncated PL1 peptides, indicating that PL1 is unlikely to mediate *trans* interactions. Both functions, however, are impaired in a spontaneous *Pl1-Rhoades* derivative that fails to produce detectable *pl1* RNA. *Pl′*-like states can also repress expression of a *pl1-W22* allele, but this repression is not meiotically heritable. As the *Pl′* state is not associated with unique small RNA species representing the *pl1*-coding region, the available data suggest that interactions between elements required for transcription underlie *Pl1-Rhoades* epigenetic behaviors.

TRANS-sensing behaviors represent exceptional forms of gene control (Henikoff and Comai 1998), often involving transfer of regulatory information between chromosomes. Paramutation is an example of a *trans*-sensing allelic interaction resulting in meiotically heritable epigenetic changes in gene regulation. Specifically, epigenetic regulatory states of one allele can facilitate heritable changes of homologous alleles in *trans*, resulting in a departure from Mendelian inheritance. This type of behavior is well documented for specific alleles of the *Zea mays* (maize) *red color1* (*r1*) (Brink 1956), *booster1* (*b1*) (Coe 1959), *pericarp1* (*p1*) (Das and Messing 1994), and *purple plant1* (*pl1*) (Hollick *et al.* 1995) loci. Each of these loci encode tissue-specific transcriptional activators of flavonoid biosynthetic enzyme genes (Dooner *et al.* 1991). However, paramutation is restricted to neither maize nor regulators of pigment biosynthesis as paramutation-like interactions exist in other eukaryotes (reviewed in Chandler and Stam 2004).

While most documented examples of paramutation represent interhomolog events causing acquisition of repressive states, the term also encompasses reversals from repressed to active states. For certain examples, interactions between distinct alleles of a given locus can result in enhanced pigment phenotypes not expected from simple dominance (Styles and Brink 1969; Hollick and Chandler 1998). Such allelic relationships typify overdominance, in which the heterozygote phenotype exceeds that of a homozygote (East 1936; Shull 1948). Overdominance may contribute to heterosis (East 1936; Shull 1948), also known as hybrid vigor, which has been critical for successful maize-breeding programs (Duvick 2001). While the underlying biology of heterosis is unknown, *trans*-sensing behaviors (Birchler *et al.* 2003) or epigenetic complementation similar to that observed at *r1* and *pl1* (Kermicle and Alleman 1990; Hollick and Chandler 1998) may play a role.

Specific alleles of *pl1*, such as *Pl1-Rhoades*, can exhibit epigenetically distinct activity states. All *pl1* alleles encode R2R3-type MYB-domain proteins (Cone *et al.* 1993a) that function in concert with either B or R basic helix-loop-helix proteins to activate anthocyanin biosynthetic genes (Goff *et al.* 1992). Using pigmentation as a proxy for *pl1* action, most *pl1* alleles found in the U. S. Corn Belt inbred lines display weak, light-dependent expression and are collectively referred to as sun-red types (Cone *et al.* 1993b). However, a highly expressed state of *Pl1-Rhoades*, referred to as *Pl-Rh*, confers robust, light-independent pigmentation to plant tissues and anthers (Cone *et al.* 1993b). *Pl-Rh* is unstable and can spontaneously change to a transcriptionally repressed state, referred to as *Pl′*, which confers relatively weaker

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and light-dependent anthocyanin pigmentation (Hollick *et al.* 1995, 2000). Activity states of *Pl1-Rhoades* are quantified using a 1–7 graded *a*nther *c*olor *s*core (ACS) (Hollick *et al.* 1995), a visual assay of anther pigmentation patterns that mirrors abundance of *pl1* transcripts (Hollick *et al.* 2000). *Pl′* invariably facilitates change of *Pl-Rh* to *Pl′* in a *Pl′/Pl-Rh* heterozygote, a hallmark of paramutation (Hollick *et al.* 1995). Both *Pl1-Rhoades* alleles from a *Pl′/Pl-Rh* heterozygote are transmitted in a heritable *Pl′* state. The ability of *Pl′* to facilitate change of *Pl-Rh* is referred to as paramutagenic activity or paramutagenicity; thus *Pl′* (ACS 1–4) is operationally a fully paramutagenic state, while *Pl-Rh* (ACS 7) is a nonparamutagenic state. *Pl1-Rhoades* alleles transmitted from plants with intermediate color scores (ACS 5 and 6) do not facilitate changes of *Pl-Rh* to *Pl′* with 100% efficiency, resulting in a broad distribution of ACS phenotypes in testcross progeny sets. Either the ACS 5 and 6 classes represent metastable states with weak paramutagenicity (Hollick *et al.* 1995) or a significant portion of gametes from these plants carry nonparamutagenic *Pl-Rh* states. Maintenance of *Pl′* is dependent upon *trans*-acting factors, including those encoded by *required to maintain repression1* (*rmr1*), *rmr2* (Hollick and Chandler 2001), *rmr6* (Hollick *et al.* 2005), and *mediator of paramutation1* (*mop1*) (Dorweiler *et al.* 2000) loci. Identification of MOP1 as the presumptive maize ortholog of the Arabidopsis RNA-dependent RNA polymerase RDR2 (Alleman *et al.* 2006; Woodhouse *et al.* 2006b) implicates a role for small RNAs in maintaining *Pl′* states.

Alleles displaying paramutation are exceptional; thus most alleles are incapable of acquiring paramutagenicity (reviewed in Chandler and Stam 2004) and are designated "neutral" (Hollick *et al.* 1995). Previous studies show that *Pl′* is unstable when heterozygous with neutral alleles and, as an example of overdominance, can revert to a fully expressed *Pl-Rh* state (Hollick *et al.* 1995; Hollick and Chandler 1998). *Pl′* is also unstable in hemizygous configuration (Hollick and Chandler 1998; J. B. Hollick, unpublished results), suggesting that reversion of *Pl′* to *Pl-Rh* is a passive process. The stability of *Pl′* may be sensitive to either gene dosage or direct homolog interactions. A simple model predicts that neutral alleles lack specific DNA features that stabilize paramutant states in *trans* (Hollick and Chandler 1998).

An unresolved issue is whether *pl1* RNA or PL1 protein mediates the above-mentioned *trans*-sensing behaviors. To address these issues, we isolated loss-of-function *Pl1-Rhoades* derivatives and assayed their allelic interactions with *Pl′* and *Pl-Rh* states. Two ethyl methanesulfonate (EMS)-induced derivatives are predicted to encode truncated PL1 proteins, yet they still acquire paramutagenic activity and maintain the *Pl′* state in *trans*, demonstrating that the PL1 protein is unlikely to mediate these *trans*-sensing behaviors. One spontaneous *Pl1-Rhoades* derivative fails both to acquire paramutagenicity and to maintain *Pl′* states in *trans* and thus genetically defines a *cis*-linked feature critical for *pl1* allelic interactions. This derivative coincidently does not produce detectable *pl1* RNA, suggesting that either transcriptional regulatory elements or the *pl1* RNA itself is responsible for these *trans*-sensing behaviors. We similarly assayed nine additional *pl1* alleles from maize inbred lines for their *trans*-interaction properties. Although no tested *pl1* allele acquires paramutagenicity, two functional classes are apparent: one that maintains the *Pl′* state in *pl1/Pl′* heterozygotes and one that facilitates *Pl′* reversion to a nonparamutagenic *Pl-Rh* state at high frequency. These data show that the two *trans*-sensing behaviors of paramutagenicity and *Pl′* stability are functionally distinct. Consistent with an RNA interference (RNAi)-type role for the *pl1* RNA itself, paramutagenic *pl1* alleles transiently repress expression of *pl1-W22*. However, sense-oriented small RNAs homologous to the *pl1*-coding region are found in plants homozygous for *Pl-Rh*, *Pl′*, and neutral *pl1* alleles from both functional classes. Absence of an obvious *Pl′*-specific small RNA species leads us to hypothesize that *trans*-sensing between *pl1* alleles occurs through *cis*-linked elements affecting transcription.

MATERIALS AND METHODS

Genetic nomenclature: Following standard conventions (http://www.maizegdb.org/maize_nomenclature.php), maize loci are designated by lowercase italics (*i.e.*, *pl1*). Specific recessive alleles are designated with a hyphen, followed by a descriptor of the allele, usually the inbred line from which the allele originated (*i.e.*, *pl1-B73*). Dominant alleles begin with uppercase letters (*e.g.*, *Pl1-Rhoades*). Loss-of-function derivative alleles of *Pl1-Rhoades* are noted with a lowercase prefix (*e.g.*, *pl1-Rhoades*). Translocation breakpoints are indicated with a "*T*" and paramutagenic states with a prime symbol (′). Plant phenotypes displayed by particular states of *Pl1-Rhoades* are written in nonitalic text (*i.e.*, Pl′). All diploid genotypes are presented with pistillate (female)-derived alleles preceding staminate (male)-derived alleles.

Germplasm: All stocks are homozygous for alleles encoding functional enzymes of the anthocyanin biosynthetic pathway and each contains either *R-r*-like haplotypes conferring kernel aleurone color in combination with dominant *colored aleurone1* (*c1*) alleles or *r-r*-like haplotypes (nonfunctional for seed color only) unless indicated otherwise. Specific coding regions of both *R-r* and *r-r* haplotypes are expressed in somatic tissues and confer pigment to seedling sheaths and anthers in combination with functional PL1 protein. Three *Pl-Rh/Pl-Rh*-converted inbred lines (W23, A619, and A632) have been previously described (Hollick *et al.* 2005). The *Pl1-Rhoades* allele in each line spontaneously changes to *Pl′* at distinct frequencies (A619, ~1/5000; W23, ~1/1500; A632, ~1/10). All other *Pl1-Rhoades* lines have been previously described (Hollick *et al.* 1995; Hollick and Chandler 1998). Other inbred lines and/or *pl1* alleles were obtained from the following sources: the North Central Regional Plant Introduction Station, U. S. Department of Agriculture-Agricultural Research Station (USDA-ARS), Ames, Iowa (A619, B73, Mo17); the Maize Genetics Cooperation Stock Center, USDA-ARS, University of Illinois, Urbana, Illinois (KWF, *Pl1-Blotched*); Arnel Hallauer,

Iowa State University, Ames, Iowa (4Co63); Jerry Kermicle, University of Wisconsin, Madison, Wisconsin (W22 *R-r:standard* converted); and Inna Golubovskaya, University of California, Berkeley, California (KYS). *pl1-CO159* was maintained as described (Cone *et al.* 1993a; Hollick and Chandler 1998). Lines used for mutagenesis are described in Hollick and Chandler (2001) and where indicated below. The *T6-9 (043-1)* interchange (referred to here as *T Pl′*) was used to identify chromosomes carrying *Pl1-Rhoades* of the *Pl′* state as previously described (Hollick *et al.* 2005). On the basis of recombination frequencies and new linkage relationships, *Pl1-Rhoades* is 1.5 cM (±1.1 cM) distal to the *6L* breakpoint and 5.3 cM from the *waxy1* (*wx1*) locus on chromosome *9S*. The breakpoint acts as a dominant semisterility locus in translocation heterozygotes (Patterson 1994). *T Pl′ / T Pl′* plants used in this study derive from a single *T Pl′* homozygote. All other chromosomes are structurally normal unless indicated otherwise and are noted as "+" where appropriate.

Genetic analyses: Hand pollinations were used for all genetic crosses. For genetic tests of individual *pl1* alleles, inbred plants or plants homozygous for *pl1-Rhoades* or *Pl1-Blotched* alleles were crossed by *T Pl′* homozygotes. In the sole exception to this scheme, a W22 inbred plant was crossed by a *pl1-Rhoades(ems9710)/T Pl′* pollen source. F_1 heterozygotes (*pl1/T Pl′*) were crossed with *Pl-Rh/Pl-Rh* testers (either W23 or A619 converted lines, depending on current availability at time of flowering, were used) and *Pl1-Rhoades* expression was visually assessed for each progeny individual as described (Hollick *et al.* 1995). Individuals were subsequently scored for pollen sterility (Hollick *et al.* 2005) to determine the structural genotype (+/*T*, ~50% sterile or +/+, fully fertile). For tests of *pl1-B73*, *pl1-Mo17*, and *pl1-4Co63*, the specific *T Pl′ / T Pl′* plants used for the assays came from F_2 generations derived from prior crosses of *T Pl′ /T Pl′* plants to the respective inbred lines (B73, Mo17, 4Co63). Given tight linkage of a recessive *wx1* mutation to *Pl′*, selection of kernels with opaque, nonstarchy endosperms assisted in selection of *T Pl′ /T Pl′* F_2 individuals. These F_2 plants derived from opaque kernels (*T Pl′ / T Pl′*) were used in the genetic assays for the cognate inbred *pl1* allele. All *pl1* alleles not directly derived from *Pl1-Rhoades*, with the possible exception of *pl1-W22* (see results), displayed no paramutagenic activity. The *pl1* alleles tested, the specific genetic background of the *Pl-Rh/Pl-Rh* tester used, and the number of fully fertile ACS 7 progeny individuals resulting from *pl1/T Pl′* × *Pl-Rh/Pl-Rh* crosses are as follows: *Pl1-Blotched* (W23, 18), *pl1-KYS* (W23, 10), *pl1-4Co63* (A619, 18), *pl1-B73* (A619, 5), *pl1-KWF* (A619, 20), *pl1-Mo17* (A619, 16), and *pl1-CO159* (W23, 54). Test of the *pl1-A619* allele (W23 *Pl-Rh/ Pl-Rh* tester) resulted in 15 ACS 7 and 1 ACS 6 fully fertile testcross progeny individuals.

Mutagenesis: Both *Mutator* (*Mu*) lines and EMS pollen mutagenesis were used to generate loss-of-function *Pl1-Rhoades* derivatives. One derivative, *pl1-Rhoades(mum9515)*, was isolated as follows. Pistillate parents (*pl1-CO159/pl1-CO159*; *c1/c1*; *r-r/r-r*) were pollinated by plants (*Pl1-Rhoades/Pl1-Rhoades*; *C1/C1*; *R-r/r-g*) containing hypomethylated *Mu* elements. Colored (*r-r/r-r/R-r*) kernels were subsequently planted in greenhouse sand benches and seedlings lacking sheath color—putatively containing a loss-of-function *Pl1-Rhoades* derivative—were rescued and grown to maturity. Plants carrying putative *pl1-Rhoades* alleles were screened for fully fertile pollen to eliminate maternal haploids and then tested by RFLP analysis (see below) to identify any potential *pl1-CO159/pl1-CO159* contaminants. A screen of ~20,000 seedlings derived from *Mu* stocks described in Dorweiler *et al.* (2000) recovered 14 individuals with putative nonfunctional *pl1-Rhoades* alleles. Eight of these plants survived, but only one new allele, *pl1-Rhoades(mum9515)*, was recovered. The *pl1-CO159/pl1-Rhoades(mum9515)* M_1 individual was backcrossed to a *pl1-CO159/pl1-CO159* plant and only colorless progeny seedlings (7 of 7) were found, indicating that the pigment deficiency maps to the *pl1* locus. A *pl1-CO159/pl1-Rhoades(mum9515)* plant was crossed to a *Pl′ / Pl′* individual and a RFLP genotyped *Pl′ /pl1-Rhoades(mum9515)* F_1 was crossed to a *Pl-Rh/Pl-Rh* tester. All testcross progeny (20 of 20) had colored anthers, indicating that *pl1-Rhoades(mum9515)* is a recessive allele.

Twelve loss-of-function *Pl1-Rhoades* derivatives were recovered from EMS treatments. Pollen from *Pl-Rh/Pl-Rh*; *C1/C1*; *R-r/R-r* plants (W23 background; Hollick and Chandler 1998) was treated with EMS as described (Neuffer and Coe 1978) and applied to receptive ears of *pl1-CO159/pl1-CO159*; *c1/c1*; *r-r/r-r* plants. Approximately 14,000 colored kernels (*r-r/r-r/R-r*) were planted and screened for colorless seedlings. Colorless seedlings were grown to maturity and absence of anther color was confirmed. Pollen was verified to be fully fertile to avoid selection of maternal haploids. EMS-derived alleles recovered include *pl1-Rhoades(ems9703)*, *ems9704*, *ems9707*, *ems9710*, *ems9711*, *ems9714*, *ems9715*, *ems9716*, *ems9718*, *ems9725*, *ems9727*, and *ems9729*. *pl1-CO159/pl1-Rhoades(ems)* individuals (with the exception of *ems9729*) were also crossed to *Pl-Rh/Pl-Rh* (W23) plants. All progeny from this cross have colored anthers, indicating that the colorless phenotypes observed in the genetic screen were not caused by dominant mutations. Plants homozygous for *pl1-Rhoades(ems9703)*, *ems9711*, and *ems9718* were backcrossed to *pl1-CO159/pl1-CO159*; *r-r/r-r* plants and all progeny were colorless (*ems9703*, 9/9; *ems9711*, 34/34; *ems9718*, 32/32), confirming that the colorless phenotypes are due to recessive loss-of-function lesions at the *pl1* locus. Two additional testcrosses using *pl1-CO159* similarly showed that the loss-of-function lesion associated with the *pl1-Rhoades(ems9710)* allele maps to the *pl1* locus (see results).

Molecular analyses: Genomic DNA was extracted from leaves as described (Voelker *et al.* 1997). A 3′ 1.1-kb *Xho*I fragment derived from *pl1-Tx303* (Cone *et al.* 1993a) and a 5′ 0.9-kb *Hin*dIII fragment (pJH1) (Hollick *et al.* 2000) were used to distinguish *pl1-CO159*, *Pl1-Rhoades*, and their derivatives by RFLP analysis. The *pl1-W22*, *pl1-CO159*, and *Pl1-Rhoades* alleles are distinguished by intron 2 simple sequence length polymorphisms detectable with PCR using primers phi031-F (5′-GCA ACA GGT TAC ATG AGC TGA CGA-3′) and phi031-R (5′-CCA GCG TGC TGT TCC AGT AGT T-3′) (Chin *et al.* 1996).

For sequencing, DNA was prepared from *pl1-Rhoades(ems9703)*, *ems9711*, *ems9718*, and *mum9515* homozygotes and the first two *pl1* exons were amplified using primers nc009-F (5′-CGA AAG TCG ATC GAG AGA CC-3′) and phi031-R (see above) (Chin *et al.* 1996) and high-fidelity $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA). Reaction conditions were 94° for 30 sec, 61° for 30 sec, and 72° for 1 min. Amplicons of at least two independent PCR reactions for each allele were extracted from 1.5% agarose gels using a QIAquick gel extraction kit (QIAGEN, Valencia, CA) and sequenced from both strands using nc009-F and phi031-R primers to achieve complete 2× coverage. Sequences of *pl1-Rhoades* alleles were deposited and assigned the following GenBank accession nos.: *pl1-Rhoades(ems9703)*, DQ394071; *pl1-Rhoades(ems9711)*, DQ379502; *pl1-Rhoades(ems9718)*, DQ470841; and *pl1-Rhoades(mum9515)*, DQ379499.

Small RNAs from either husk leaves or seedlings were analyzed by Northern blots. Husk leaves from silking ears of field-grown isogenic *Pl-Rh/Pl-Rh* and *Pl′ /Pl′* (A632) plants were harvested at mid-day. The cob, silks, shank, and the two most-exterior husk leaves were removed from each ear and the remaining husk leaves were lightly pulverized in a mortar using liquid nitrogen prior to the extraction procedure. Five 2-week-old seedlings homozygous for *pl1-CO159*, *pl1-B73*, or *pl1-W22* were pooled and lightly pulverized in a mortar using liquid nitrogen prior to the extraction procedure. Total RNA

was extracted from 3 g of husk or seedling tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA was resuspended in 500 μl 50% formamide, and 150 μl of 50% PEG 8000 was added to precipitate high-molecular-weight RNA. Following incubation on ice and centrifugation at 18,000 × *g*, supernatant was transferred to a new tube and low-molecular-weight (LMW) RNA was precipitated by adding 3 vol of 100% ethanol. LMW RNA was resuspended in 100 μl 50% formamide and quantified by spectrophotometric analysis at 260 nm. A total of 50 μg LMW RNA was separated at 700 V on 15% polyacrylamide gels with 0.33× TBE and 7 M urea. Gels were stained with ~1 μg/ml ethidium bromide in 0.5× TBE for 20 min. RNA was transferred to a Magnaprobe nylon membrane (GE Osmonics, Minnetonka, MN) using a Trans-Blot SD electroblotter (Bio-Rad Laboratories, Hercules, CA) at 10 V and 0.5 A for 60 min. Size standards are 25 pmol DNA oligos as follows: antisense 22 nt, phi031-R described above (Chin *et al.* 1996), and sense 24 nt, 5′-GAA CTA CTG GAA CAG CAC GCT GGG-3′. *pl1* RNA probes labeled with [α^{32}P]UTP were synthesized from a linearized plasmid containing 5′ sequences of *pl1* cDNA (pJH7) (Hollick *et al.* 2000) using either T7 or T3 RNA polymerase for sense and antisense probes, respectively. Probes were hydrolyzed in 100 mM sodium carbonate buffer, pH 10.2, for 30 min at 65° prior to hybridization at 40° and washing as described (Hamilton and Baulcombe 1999). Hybridized blots were exposed to Molecular Dynamics phosphor detection screens (GE Healthcare Bio-Sciences, Piscataway, NJ) for 72 hr prior to image acquisition using a Molecular Dynamics PhosphorImager 445SI.

For transcript analysis, RNA was extracted from florets as described (Hollick *et al.* 2000). cDNAs were generated using Omniscript reverse transcriptase kit (QIAGEN) and PCR amplified with *pl1*-specific primers, 5′-CAC GGC GAA GGC AAA TGG AG-3′ and 5′-CTG TTG CCG AGG AGC TTG TG-3′ as specified (Cocciolone and Cone 1993). Seedlings were grown in vermiculite and sand for 2 weeks. RNA was extracted from tissues above ground but below the first leaf. RNA from a single individual was harvested using TRIZOL reagent. A total of 10 μg RNA was treated with DNase I (Roche Applied Science, Indianapolis), primed with oligo$(dT)_{15}$ (Promega, Madison, WI), and reverse transcribed with SuperScript II (Invitrogen). Samples were PCR amplified using *pl1* primers (5′-ACC CTG CTG CTA GCT AGC TG-3′ and 5′-CTG TTG CCG AGG AGC TTG TG-3′) (Cone *et al.* 1993b) and 35 cycles with the following conditions: 95° for 30 sec, 60° for 1 min, and 72° for 1 min. The control *alanine aminotransferase* (*aat*) transcript was amplified as described (Woodhouse *et al.* 2006a) using 35 cycles. PCR amplicons were separated on 3% agarose gels, stained with ethidium bromide, and imaged.

RESULTS

Allele screens identify loss-of-function *Pl1-Rhoades* derivatives: To test whether *cis*-elements required for the acquisition and maintenance of paramutagenicity are related to *Pl1-Rhoades* expression, we isolated nonfunctional *Pl1-Rhoades* derivatives (collectively referred to as *pl1-Rhoades* alleles) and tested their ability to facilitate heritable change of *Pl-Rh* and to stabilize *Pl′* in *trans*. The *pl1-CO159* allele, which confers no pigment to either seedling sheaths or anthers (Cone *et al.* 1993a), was used as a reference null. Plants homozygous for *pl1-CO159* were crossed by *Pl1-Rhoades* homozygotes either treated with EMS or carrying *Mu* transposons. EMS mutagenesis generated 12 putative nonfunctional *pl1-Rhoades* alleles from ~14,000 M_1 seedlings. Six of these colorless M_1 individuals were selfed and M_2 progeny were RFLP genotyped to recover homozygotes for the derivative alleles *pl1-Rhoades(ems9703)*, *ems9707*, *ems9710*, *ems9711*, *ems9718*, and *ems9729* (materials and methods). RFLP analysis of the EMS derivative alleles indicates that they are structurally similar, if not identical, to *Pl1-Rhoades* (data not shown). An allele screen using a pollen parent carrying hypomethylated *Mu* transposons yielded *pl1-Rhoades(mum9515)*. Genetic crosses show that *pl1-Rhoades (mum9515)* is recessive (materials and methods). DNA from *pl1-Rhoades(mum9515)* homozygotes was examined by Southern blot hybridization with 5′ and 3′ *pl1* probes. *pl1-Rhoades(mum9515)* has RFLP patterns distinct from *pl1-CO159*, but has very similar, if not identical, restriction patterns to *Pl1-Rhoades* (Figure 1). This finding strongly suggests that no *Mu* elements have inserted within ~12 kb 5′ and 10 kb 3′ of the *Pl1-Rhoades* coding region.

Genetic tests of EMS- and *Mutator*-derived *Pl1-Rhoades* alleles show that only *pl1-Rhoades(mum9515)* is impaired in *trans*-sensing behaviors: To assay allelic interactions of *pl1-Rhoades* alleles, each allele was exposed to a reference *Pl1-Rhoades* allele of *Pl′* state carried on a *T6-9 (043-1)* interchange chromosome (referred to as *T Pl′*; Hollick *et al.* 2005) and then evaluated for paramutagenicity following meiotic transmission to stable *Pl-Rh/Pl-Rh* testers. Because the reference *Pl1-Rhoades* allele is linked (~1.5 cM) to the *6L* breakpoint, testcross progeny segregate 1:1 for fully fertile (*pl1-Rhoades/Pl-Rh*) and semisterile (*T Pl′/Pl-Rh*) plants. Anther pigmentation of testcross progeny measures paramutagenicity of both the tested *pl1-Rhoades* allele (fully fertile class) and the reference *Pl1-Rhoades* allele (semisterile class). The ACS of *pl1-Rhoades/Pl-Rh* progeny indicates the paramutagenicity of each *pl1-Rhoades* allele. Low ACS values (ACS 1–4) suggest that the *pl1-Rhoades* allele can still acquire paramutagenicity following exposure to *Pl′*, while high ACS values (ACS > 4) suggest that the *pl1-Rhoades* allele is defective in acquiring or transferring paramutagenicity. Using these interpretations, all but one derivative acquires paramutagenicity similar to the progenitor *Pl1-Rhoades* allele (Table 1). The *pl1-Rhoades (mum9515)* allele is completely nonparamutagenic as all 23 fully fertile progeny have ACS 7 phenotypes. All other derivatives behave identically to the progenitor in acquiring strong paramutagenicity. From the same testcross, ACS values of semisterile progeny indicate paramutagenicity of the reference *Pl1-Rhoades* allele. If *Pl′* retains paramutagenicity in *pl1-Rhoades/T Pl′* plants, all semisterile progeny will be Pl′-like (ACS 1–4), while loss of paramutagenicity will result in higher ACS values (ACS 5–7). The data show that all derivatives except *pl1-Rhoades(mum9515)* retain the ability to stabilize *Pl′* states in *trans* (Table 1). Among the 24 semisterile progeny derived from *pl1-Rhoades(mum9515)/T Pl′* heterozygotes, one was scored ACS 7 and 11 were scored ACS 5 or ACS 6. Hence, *pl1-Rhoades(mum9515)* behaves like previously

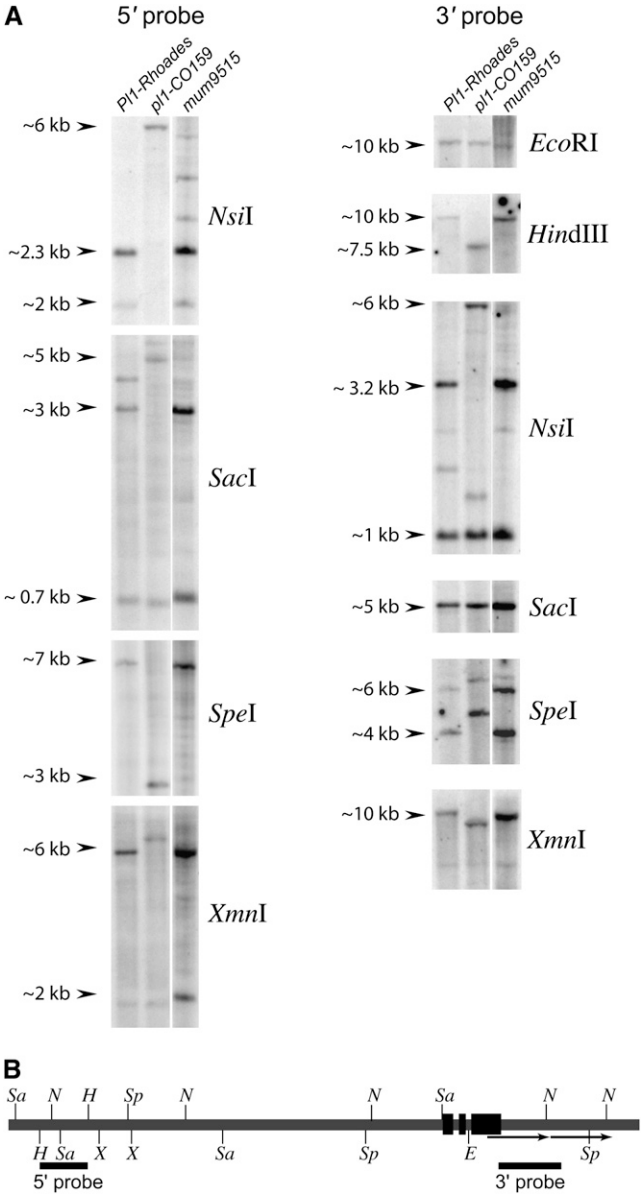

FIGURE 1.—*pl1-Rhoades* derivative *mum9515* is structurally identical to *Pl1-Rhoades*. (A) Southern blots were hybridized with either 5′ or 3′ *pl1*-specific probes (MATERIALS AND METHODS). Approximate sizes of hybridizing fragments are indicated. (B) Restriction map of *Pl1-Rhoades* and probe locations based on genomic sequence (GenBank accession no. L19494). Arrows below gene structure indicate a 3′ tandem duplication. Only restriction sites generating fragments hybridizing to either the 5′ or 3′ probe are shown. *E*, *Eco*RI; *H*, *Hin*dIII; *N*, *Nsi*I; *Sa*, *Sac*I; *Sp*, *Spe*I; *X*, *Xmn*I.

described neutral *pl1* alleles by facilitating *Pl′* reversion (HOLLICK *et al.* 1995; HOLLICK and CHANDLER 1998).

Paramutagenic activity of some *pl1-Rhoades* alleles does not require *cis*-encoded PL1 protein: As EMS treatment commonly results in guanine-to-adenine transitions (KOHALMI and KUNZ 1988) that can yield dysfunctional proteins, we suspected our paramutagenicity tests indicated that functional PL1 protein was neither required to transfer paramutagenicity nor

TABLE 1

Anther phenotypes of progeny from *pl1-Rhoades/T Pl′* × *Pl-Rh/Pl-Rh* crosses

pl1-Rhoades allele	Progeny structural genotype	No. of progeny individuals with indicated ACS						
		1	2	3	4	5	6	7
ems9703	+/+	1	10	8	0	0	0	0
ems9707	+/+	4	8	4	0	0	0	0
ems9710	+/+	3	9	5	0	0	0	0
ems9711	+/+	5	16	11	1	0	0	0
ems9718	+/+	4	17	4	0	0	0	0
ems9729	+/+	4	13	5	1	0	0	0
mum9515	+/+	0	0	0	0	0	0	23
ems9703	*T*/+	1	9	12	1	1	0	0
ems9707	*T*/+	1	8	9	0	0	0	0
ems9710	*T*/+	0	4	15	2	0	0	0
ems9711	*T*/+	1	8	7	0	0	0	0
ems9718	*T*/+	1	6	11	1	0	0	0
ems9729	*T*/+	5	14	4	1	0	0	0
mum9515	*T*/+	0	4	8	0	3	8	1

Results for each *pl1-Rhoades* allele tested are combined from two independent progeny ears derived using the *Pl-Rh/Pl-Rh* (W23) tester.

required to stabilize a *Pl′* state in *trans*. We sequenced N-terminal coding regions of several *pl1-Rhoades* alleles and discovered that *pl1-Rhoades(ems9718)* and *pl1-Rhoades (ems9711)* are not predicted to encode full-length PL1 proteins (Figure 2A). *pl1-Rhoades(ems9718)* has a guanine-to-adenine transition mutation at position +104, resulting in a nonsense mutation, and should encode a 16-amino-acid peptide. *pl1-Rhoades(ems9711)* has a guanine-to-adenine transition mutation at the intron 1 splice donor site (position +187), which should prevent the first intron from being properly spliced from the nascent RNA transcript. As predicted, reverse transcription PCR shows that the *pl1-Rhoades(ems9711)* mRNA is larger by the size of intron 1 (Figure 2B). The resulting protein should be truncated due to an in-frame termination codon within the unspliced intron, and thus *pl1-Rhoades(ems9711)* should encode a 46-amino-acid polypeptide. *pl1-Rhoades(ems9703)* contains a cytosine-to-thymine transition mutation within codon 90 (position +538), predicted to result in a nonconservative serine-to-leucine substitution. The S90L alteration occurs within a conserved α-helix of the MYB repeat shown in the PL1 functional paralogue, COLORED ALEURONE1, to be required for RED COLOR1-dependent transcriptional activation (GROTEWOLD *et al.* 2000), suggesting that this mutation in *pl1-Rhoades(ems9703)* should disrupt protein function. These results point to *cis*-linked features, independent of the encoded PL1 transcription factor, responsible for the *Pl1-Rhoades trans*-sensing behaviors.

***pl1-Rhoades(mum9515)* is defective in RNA expression:** We sequenced the genomic DNA corresponding to the N-terminal coding region of *pl1-Rhoades(mum9515)*

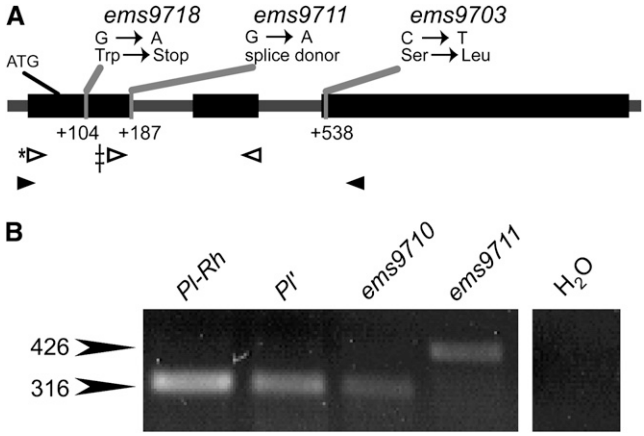

FIGURE 2.—*pl1-Rhoades* loss-of-function alleles. (A) Molecular lesions of *pl1-Rhoades(ems9718)*, *ems9711*, and *ems9703*. Exons (thick boxes), translation start site, and positions of lesions are relative to +1 as defined by CONE *et al.* (1993b). Solid arrows indicate positions of sequencing primers; open arrows indicate positions of primers used for RT–PCR reactions (*, the forward primer used for anther RNA RT–PCR; ‡, the forward primer used for seedling RT–PCR; see MATERIALS AND METHODS). (B) RT–PCR of *Pl1-Rhoades* and two derivative alleles from anther RNA. The notations 316 bp and 426 bp are the sizes of properly spliced and unspliced *pl1* transcript, respectively. H_2O designates the negative control in the PCR reaction.

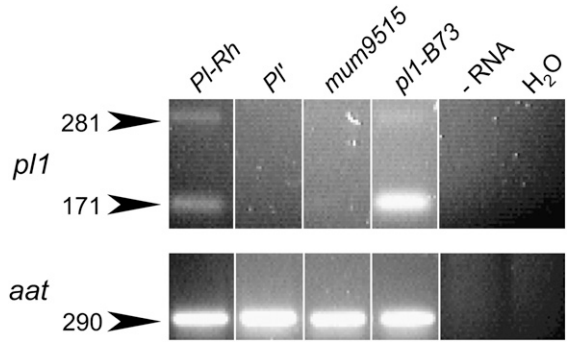

FIGURE 3.—*pl1-Rhoades(mum9515)* is an RNA null. Reverse transcriptase–PCR analysis of seedling RNA. *pl1*: spliced, 171 bp; unspliced, 281 bp. *aat*: spliced, 290 bp; unspliced, 454 bp (not shown). Negative controls: −RNA, full assay in absence of RNA; H_2O, no template in PCR stage only.

and found that it was identical to *Pl1-Rhoades*. We therefore wondered if the mutational lesion of *pl1-Rhoades(mum9515)* affected *pl1* mRNA expression. Using RT–PCR analysis, we detected polyadenylated *pl1* transcripts from seedlings homozygous for *Pl-Rh* and *pl1-B73*, but not for *Pl′* or *pl1-Rhoades(mum9515)* (Figure 3). Additionally, *pl1* RNA from *pl1-Rhoades(mum9515)* homozygotes is undetectable in anther florets using RNAse protection assays (J. B. HOLLICK, unpublished results). These results are consistent with a regulatory lesion prohibiting RNA expression of *pl1-Rhoades(mum9515)*. Plants homozygous for *pl1-Rhoades(mum9515)* are always colorless at the seedling stage, yet can sometimes have weakly pigmented anthers, consistent with a mutation affecting developmental regulation or expression levels. The observations that polyadenylated *pl1* RNA levels from *Pl′* states are found in anther florets but not in seedling tissue (Figure 2B) and that unspliced polyadenylated *pl1* RNA from *Pl-Rh* states are found in seedlings but not in anther florets (Figure 3) are consistent with the idea of tissue-specific regulatory differences affecting *pl1* mRNA expression. Thus, while the PL1 protein appears unimportant to either *trans*-sensing behavior, the lack of *pl1* RNA, or some feature required for its production, appears necessary for both paramutagenicity and stabilization of *Pl′* states.

Genetic analysis of a naturally occurring allelic series reveals functional diversity for *trans*-sensing behaviors: The finding that *cis*-elements of *Pl1-Rhoades* mediating *pl1* allelic interactions could be disrupted by mutations motivated a broader survey of *pl1* allelic variation represented among maize inbred lines. No *pl1* allele tested to date, aside from *Pl1-Rhoades*, can acquire paramutagenicity (HOLLICK *et al.* 1995, 2000; HOLLICK and CHANDLER 1998) but some neutral *pl1* alleles have different abilities to stabilize *Pl′* in *trans* (HOLLICK and CHANDLER 1998). We tested nine *pl1* alleles (*Pl1-Blotched*, *pl1-KYS*, *pl1-4Co63*, *pl1-B73*, *pl1-KWF*, *pl1-Mo17*, *pl1-W22*, *pl1-A619*, and *pl1-CO159*) in the same genetic assay described above. These alleles are resident in a diversity of inbred lines commonly used in our studies, which represent extreme early (KWF) and late (KYS) flowering lines, Reid Yellow Dent (B73) and Lancaster Sure Crop (Mo17 and A619) varieties, and well-studied anthocyanin lines (4Co63 and W22). Both *pl1-CO159* and *Pl1-Blotched*, an allele structurally identical to *Pl1-Rhoades* (COCCIOLONE and CONE 1993), have been evaluated in previous cosegregation assays (HOLLICK and CHANDLER 1998; HOLLICK *et al.* 2000). If a given *pl1* allele can acquire paramutagenicity upon exposure to *Pl′*, the fully fertile class of testcross progeny (*pl1/Pl-Rh*) has low ACS values (Pl′-like phenotypes). Conversely, inability to acquire paramutagenicity results in fully fertile progeny with fully colored anthers (ACS 7; Pl-Rh-like phenotypes). With one exception, results show that no fully fertile progeny have Pl′-like phenotypes, indicating that none of the tested *pl1* alleles acquire paramutagenicity (MATERIALS AND METHODS). This confirms previous results using both *Pl1-Blotched* and *pl1-CO159* (HOLLICK and CHANDLER 1998; HOLLICK *et al.* 2000). The exceptional ACS 2 individual from the fully fertile *pl1-W22/Pl1-Rhoades* progeny class likely represents a rare recombinant chromosome in which the reference *Pl1-Rhoades* allele of the *Pl′* state has become unlinked from the *T6-9* breakpoint [$\chi^2 = 0.6$ ($P > 0.05$; not significant) for the null hypothesis that the number of observed ACS 7 exceptions is greater than the number expected from recombination between the translocation breakpoint and *pl1*]. However, two additional fully fertile progeny are ACS 5 and ACS 6, suggesting that *pl1-W22* may

TABLE 2

Anther phenotypes of semisterile progeny from *pl1/T Pl′* × *Pl-Rh/Pl-Rh* crosses

pl1 allele	No. of progeny ears	Progeny structural genotype	No. of progeny individuals with indicated ACS						
			1	2	3	4	5	6	7
Pl1-Blotched	1	+/*T*	1	11	7	0	0	0	0
pl1-KYS	1	+/*T*	2	3	6	0	0	0	0
pl1-4Co63	1	+/*T*	1	6	2	3	2	0	2
pl1-B73	1	+/*T*	3	4	3	1	0	0	0
pl1-KWF	2	+/*T*	1	3	1	3	2	2	8
pl1-Mo17	1	+/*T*	1	6	1	3	1	0	1
pl1-W22	4	*T*/+	1	18	8	0	0	0	0
pl1-A619	2	*T*/+	0	2	3	5	2	5	1
pl1-CO159	6	*T*/+	1	5	17	7	4	7	11

acquire weak paramutagenicity. The specific *Pl-Rh/Pl-Rh* line (A619 inbred conversion) used for the *pl1-W22* test exhibits a very low level of spontaneous paramutation (MATERIALS AND METHODS).

As documented previously (HOLLICK *et al.* 1995; HOLLICK and CHANDLER 1998), we found that *pl1* alleles other than *Pl1-Rhoades* differ in their ability to maintain *Pl′* states in *trans*. If the tested *pl1* allele stabilizes *Pl′* in *pl1/T Pl′* F_1 plants, then semisterile testcross progeny (*T Pl′ /Pl-Rh*) are Pl′-like (ACS 1–4). In contrast, reversion of *Pl′* in *pl1/T Pl′* plants results in higher ACS values (ACS 5, 6, and 7) among semisterile (*T Pl′ /Pl-Rh* or *T Pl-Rh/Pl-Rh*) progeny. Results (Table 2) suggest that *Pl1-Blotched* and *pl1* alleles resident in KYS, B73, and W22 inbreds maintain paramutagenicity of *Pl′* in *pl1/T Pl′* plants as all semisterile testcross progeny individuals (*T Pl′ /Pl-Rh*) have Pl′-like phenotypes (ACS 1–4). In contrast, *pl1-4Co63*, *pl1-KWF*, *pl1-A619*, *pl1-CO159*, and *pl1-Mo17* appear to allow reversion of *Pl′* to *Pl-Rh* at high frequency. Of 16 semisterile testcross progeny from *pl1-4Co63/T Pl′* plants, 2 are ACS 7 (12.5%), and an additional 2 individuals are ACS 5. Similarly, 8 of 20 (40%) from *pl1-KWF/T Pl′* heterozygotes are ACS 7, and an additional 4 (20%) are ACS 5 or ACS 6. One of 13 (7.6%) from *pl1-Mo17/T Pl′* are ACS 7 and an additional one is ACS 5. One of 18 (5.5%) from *pl1-A619/T Pl′* are ACS 7, while an additional 7 (38%) are ACS 5 or ACS 6. Consistent with prior results (HOLLICK and CHANDLER 1998), 11 of 52 (21%) semisterile individuals from *pl1-CO159/T Pl′* parents are ACS 7 and an additional 11 individuals are ACS 5 or ACS 6. These high reversion frequencies stand in sharp contrast to results seen with *Pl1-Blotched*, *pl1-B73*, *pl1-KYS*, and *pl1-W22*, but they are not *a priori* due to allelic differences at the *pl1* locus. However, previous cosegregation data clearly showed that the chromosome region linked to *pl1-CO159* is responsible for facilitating *Pl′* reversion (HOLLICK and CHANDLER 1998) and that *6L* segmental deficiencies, including the *pl1* locus, allow similar reversions (HOLLICK and CHANDLER 1998; J. B. HOLLICK, unpublished results). We did not see any phenotypic reversion of *Pl′* to *Pl-Rh* in *pl1/T Pl′* plants used for our genetic assays, but we clearly observed that *Pl′* had lost meiotically heritable paramutagenicity while heterozygous with some *pl1* alleles (Table 2). We grew additional F_1 *pl1/T Pl′* plants and observed no pigmentation values greater than ACS 3 in the following genotypes: *pl1-CO159/T Pl′*, 61 plants; *pl1-KWF/T Pl′*, 39 plants; *pl1-A619/T Pl′*, 50 plants; *pl1-Mo17/T Pl′*, 64 plants; and *pl1-4Co63/T Pl′*, 40 plants. Thus, we found no evidence for unlinked dominant modifiers affecting somatic *Pl′* stability. Collectively, with the *pl1-Rhoades(mum9515)* results, these data suggest that functional diversity exists among naturally occurring *pl1* alleles with regards to stabilization of *Pl′* states in *trans*.

***pl1-W22* expression is repressed in *trans* by *pl1* paramutagenic states:** Because several inbred *pl1* alleles appear to stabilize *Pl′* states, we wondered if their expression might be affected in *pl1/Pl′* plants. This is not easily addressed with most neutral *pl1* alleles, given their already low levels of expression. The *pl1-W22* allele, however, confers strong, uniform pigmentation in the presence of light (Figure 4B). We could therefore ask whether *pl1-W22* expression was suppressed in heterozygous combination with paramutagenic states. To control for potential differences in *pl1* gene dosage, we compared anther phenotypes of sibling *pl1-W22/pl1-CO159* and *pl1-W22/T Pl′* genotypes derived from a cross between *pl1-W22/pl1-W22* and *pl1-CO159/T Pl′* parents (Figure 4A). A dosage effect is seen as *pl1-W22/pl1-CO159* plants have pink, rather than brick-red, anthers. However, *Pl′* appears to suppress expression of *pl1-W22* (Figure 4B, Table 3). Anthers of *pl1-W22/T Pl′* individuals have a characteristic Pl′-like phenotype in which a variegated pattern of strongly pigmented epidermal cells exists within a field of near-colorless cells. If *pl1-W22* and *Pl′* are codominant, then darkly colored cells should be surrounded by pink-colored cells. The observation that *Pl′* appears dominant to *pl1-W22* is consistent with the idea that *Pl′* can also suppress expression of alleles other than *Pl1-Rhoades*. We reexamined this apparent *trans*-suppression using a nonfunctional *pl1-Rhoades* derivative. By using a *pl1-Rhoades (ems9710)/T Pl′* pollen parent (Figure 4A) to confer a paramutagenic state to a pigment-defective allele, the phenotype of *pl1-W22/pl1-Rhoades(ems9710)′* progeny provides a clearer assessment of *pl1-W22* expression. Results show both *Pl′* and *pl1-Rhoades(ems9710)′* repress *pl1-W22* expression as *pl1-W22/T Pl′* individuals have Pl′-like anthers and *pl1-W22/pl1-Rhoades(ems9710)′* individuals have completely colorless anthers (Table 3, Figure 4B). Both data sets suggest that *pl1* paramutagenic states suppress *pl1-W22* expression in *trans*. *pl1-W22* does not, however, acquire paramutagenicity following exposure to *Pl′*, and subsequent crosses of two separate *pl1-W22/pl1-Rhoades(ems9710)′* heterozygotes with *pl1-CO159/pl1-CO159* testers show that pigmenting

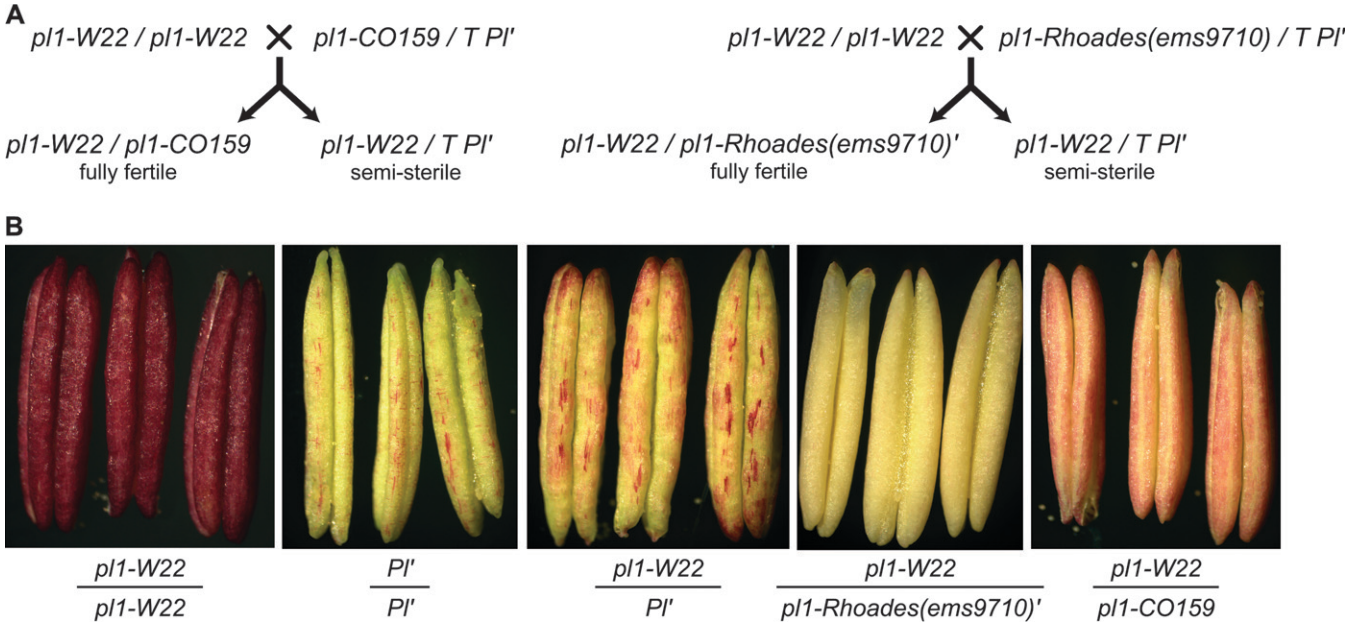

FIGURE 4.—*pl1-W22* is sensitive to dosage and paramutagenic *pl1* alleles. (A) Crosses, progeny, and diagnostic pollen phenotypes used to expose *pl1-W22* to both *Pl'* and *pl1-Rhoades(ems9710)'* states. (B) Representative anthers of the given genotypes collected from single mature florets at the beginning of dehiscence.

action is typically restored in *pl1-CO159/pl1-W22* and *pl1-W22/pl1-CO159* progeny [15 of 16 plants scored with pink anthers were subsequently genotyped as having *pl1-W22*, while 17 of 18 plants scored with colorless anthers were genotyped as having *pl1-Rhoades(ems9710)'*]. These results indicate that the repression of *pl1-W22* expression by *pl1* paramutant states is not meiotically heritable.

All tested *pl1* alleles produce sense-oriented small RNAs: The findings that *pl1-Rhoades(mum9515)* is defective in *pl1* RNA production and that *Pl'* states repress *pl1-W22* phenotypic expression are consistent with an RNAi-type allelic interaction involving *pl1* RNA. Small 21- to 24-nt RNAs, hallmarks of RNAi, can act in *trans* to both trigger destruction of homologous RNA transcripts and facilitate epigenetic change of homologous DNA sequences within the genome (reviewed in BRODERSEN and VOINNET 2006). If allelic interactions between *pl1* alleles are RNAi mediated, then small RNAs are predicted to correlate with *pl1* paramutagenic states. We compared small RNA profiles of the *Pl-Rh* and *Pl'* states in otherwise isogenic *Pl1-Rhoades/Pl1-Rhoades* plants (MATERIALS AND METHODS) using low-molecular-weight RNA isolated from mature husk leaves. Results show that both *Pl-Rh* and *Pl'* are associated with sense-oriented small RNAs >24 nt that hybridize with the *pl1*-specific probe, yet no antisense *pl1* small RNAs were detected (Figure 5A). We also found sense-oriented small RNAs in seedlings homozygous for *pl1-CO159*, *pl1-B73*, and *pl1-W22* (Figure 5B), indicating that these RNAs are not unique to *Pl1-Rhoades*. Thus, these detected small RNAs representing the *pl1*-coding sequence do not appear to be involved in *pl1 trans*-sensing behaviors.

TABLE 3

Progeny anther phenotypes from crosses to *pl1-W22/pl1-W22* pistillate parents

Staminate parent genotype	Progeny structural genotype	No. of progeny individuals with indicated anther phenotypes						
				Anther color score				
		Colorless	Pink	1	2	3	4	5
pl1-CO159/T Pl'	+/+	0	34	0	0	0	0	0
	+/*T*	0	0	10	18	6	1	1
pl1-Rhoades(ems9710)/T Pl'	+/+	30	0	0	0	0	0	0
	+/*T*	0	0	0	7	12	3	1

Data represent a single progeny ear for the *pl1-CO159/T Pl'* cross and two independent progeny ears for the *pl1-Rhoades(ems9710)/T Pl'* cross.

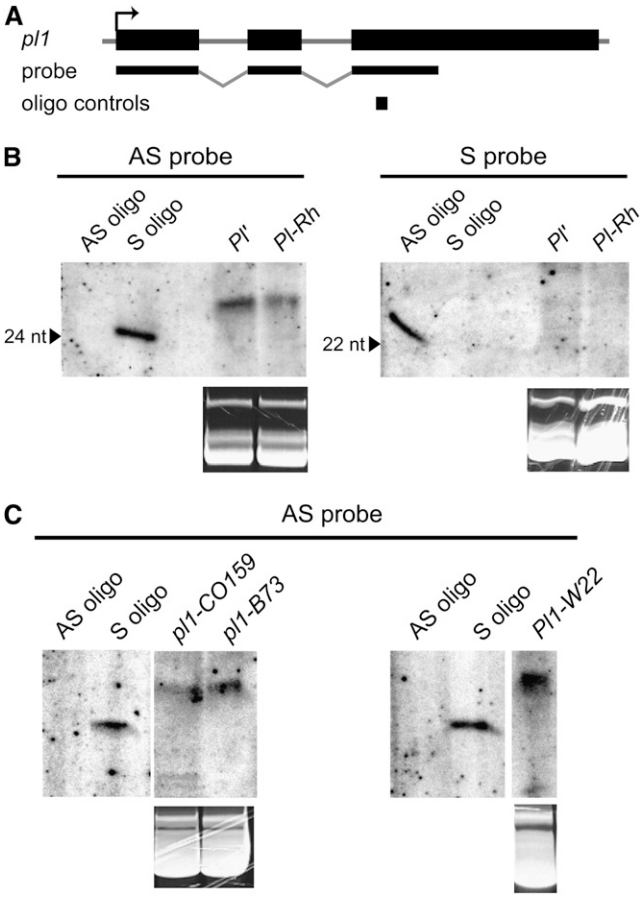

FIGURE 5.—All *pl1* alleles produce sense-oriented small RNAs. (A) Map of *pl1*-transcribed region, partial cDNA probe, and location of oligo controls. (B) Northern blot of husk tissue small RNAs from isogenic *Pl-Rh/Pl-Rh* and *Pl′/Pl′* plants. AS (antisense), S (sense). (C) Northern blot of seedling small RNAs from *pl1-CO159*, *pl1-B73*, and *pl1-W22* homozygotes. Ethidium-bromide-stained tRNA for the relevant samples is shown below blots in B and C as a loading control. See MATERIALS AND METHODS for additional details.

DISCUSSION

The dynamic relationships among *Pl1-Rhoades* paramutagenic states (*Pl′*), the nonparamutagenic state (*Pl-Rh*), and specific *pl1* alleles highlight the concept that different allele combinations can lead to different meiotically heritable changes in gene regulation. Interactions between *Pl′* and *Pl-Rh* result in the heritable change of *Pl-Rh* to *Pl′*, and *Pl′* itself is maintained through interactions with either *Pl1-Rhoades* or specific *pl1* alleles on the alternate homolog. The additional findings that certain *pl1* alleles appear to stabilize the *Pl′* state suggest a model in which genetically separable elements mediate distinct *trans*-sensing interactions responsible for paramutation and stabilization of paramutagenic states. Overdominant relationships between *Pl′* and other *pl1* alleles, documented here and in previous studies (HOLLICK *et al.* 1995; HOLLICK and CHANDLER 1998), provide further examples of single-locus heterosis, in which heterozygotes confer a phenotype measurably greater than that of either homozygote alone. The *pl1-Rhoades(mum9515)* mutation, in particular, defines a feature that normally prevents overdominance at the *pl1* locus.

One diffusible molecule that could potentially mediate *trans*-interactions at *pl1* is PL1 protein. As a transcription factor, PL1 protein might act as a heritable auto-regulator, establishing and maintaining paramutant states of *pl1*. However, although *pl1-Rhoades(ems9711)* and *pl1-Rhoades(ems9718)* are not predicted to make functional DNA-binding proteins, they still transmit paramutagenicity through meiosis and stabilize *Pl′* in *trans*. Additionally, sequenced *pl1* alleles (notably *Pl1-Blotched*, *pl1-W22*, and *pl1-B73*) are predicted to produce proteins identical or similar to *Pl1-Rhoades* (data not shown), yet they do not acquire paramutagenicity. Collectively, our results strongly argue against PL1 polypeptides acting as a meiotically heritable agent of paramutagenicity or affecting the *trans*-stabilization of *Pl′*.

Prior to this study, there were indications that some *pl1* alleles were less efficient at stabilizing *Pl′* than others (HOLLICK and CHANDLER 1998). Our results suggest that two classes of neutral *pl1* alleles exist: a stabilizing class that effectively maintains paramutagenic *Pl′* states on the homologous chromosome in *trans* and an amorphic class (BRINK 1964) that acts similarly to a deficiency in facilitating overdominance by allowing *Pl′* to revert to nonparamutagenic states. Our results with various *pl1* alleles highlight this apparent functional diversity even though many inbred lines used in this study derive from the same racial complex of U. S. Corn Belt Dents (GOODMAN and BROWN 1988; HALLAUER *et al.* 1988). While it is still formally possible that specific background modifiers affect *Pl′* stability, the fact that we isolated an amorphic *pl1-Rhoades* derivative demonstrates that such *cis*-linked diversity can exist. Moreover, we found no evidence of dominant modifiers affecting *Pl′* reversion as no *pl1/T Pl′* heterozygotes had ACS 7 anthers. This is a difference from previous cosegregation studies (HOLLICK *et al.* 1995; HOLLICK and CHANDLER 1998) in that certain heterozygous combinations can lead to a meiotically heritable loss of paramutagenicity in the absence of *Pl1-Rhoades* expression increases in those heterozygotes. This suggests that either the *Pl′* state, in the context of the *T6-9* interchange, is exceedingly stable or reversion to *Pl-Rh* occurs very late in somatic development and is therefore not manifest by pigment increases in the F_1 heterozygote. In light of our genetic analysis of neutral *pl1* alleles, we propose that *Pl1-Rhoades* has distinct *cis*-elements for stabilizing *Pl′* states in *trans* and for facilitating heritable paramutational change in *trans*. If our bipartite hypothesis is correct, the coincident impairment of paramutagenicity and *Pl′ trans*-stabilizing functions in *pl1-Rhoades(mum9515)* implies that the lesion disrupts either two, presumably close, distinct elements or a single element with epistatic properties. Because the RFLP patterns of *pl1-Rhoades*

(mum9515) appear identical to those of *Pl1-Rhoades*, the lesion either is relatively small or exists in more distant 5′ or 3′ flanking regions for which there are no cloned reagents available for more detailed analysis. *Pl1-Blotched*, which is nearly identical to *Pl1-Rhoades* and is predicted to produce an identical RNA transcript (Cocciolone and Cone 1993), fails to acquire paramutagenicity yet acts to stabilize *Pl′* (this study and Hollick *et al.* 2000). In terms of our bipartite model, *Pl1-Blotched* has *cis*-elements for *Pl′* stabilization, but not for paramutagenicity. Given the strong sequence identity between *Pl1-Rhoades* and *Pl1-Blotched*, it seems likely that the element(s) responsible for paramutagenicity is far removed from the *pl1*-coding sequence. This is not without precedence as tandem repeats affecting both expression and paramutagenicity of the *B1-Intense* (*B1-I*) allele are nearly 100 kb 5′ of the *b1*-coding region (Stam *et al.* 2002a,b).

The finding that *mop1* encodes a putative RDR2 ortholog (Alleman *et al.* 2006; Woodhouse *et al.* 2006b) implicates RNAi in the maintenance of *Pl′* states. The *pl1* RNA could provide the target specificity necessary for a *trans*-acting substance, and the apparent *trans*-suppression of *pl1-W22* by paramutagenic *pl1* alleles is consistent with this proposal. Our Northern analysis shows that small sense-oriented *pl1* RNA species are produced from *Pl-Rh*, *Pl′*, stabilizing alleles (*pl1-B73* and *pl1-W22*), and an amorphic allele (*pl1-CO159*). As these small RNAs representing the *pl1*-coding sequence are not specific to either paramutagenic or stabilizing alleles, it appears unlikely that they act as mediators of *pl1* allelic interactions. Interestingly, recent studies of Drosophila have discovered Piwi-interacting RNAs (piRNAs) that are typically more abundant in one strand and are associated with silencing of repetitive elements and proper germline cell development (reviewed in Parker and Barford 2006). Whether the sense-oriented *pl1* small RNAs are analogous to piRNAs remains to be discovered.

Our inability to detect small RNAs associated with *Pl′* states does not preclude RNA-mediated *trans* interactions in *pl1* paramutation. One possibility is that small RNAs are generated from *Pl1-Rhoades* regulatory regions rather than from the coding region. Provided that *pl1* alleles share regulatory sequence homology with *Pl1-Rhoades*, they could be sensitive to small RNAs generated by paramutagenic states of *Pl1-Rhoades*. Alignments between *Pl1-Rhoades* and *pl1-B73* genomic sequences show that much of the 5′ proximal sequences in *pl1-B73* are present but rearranged in *Pl1-Rhoades* (not shown). Thus there appear to be significant stretches of sequence similarity between the two alleles upon which homology-dependent mechanisms such as RNAi may operate. The observation that *pl1* paramutagenic states suppress *pl1-W22* in *trans* also supports this hypothesis. Alternatively, temporal small RNAs or longer *pl1* transcripts, not detected by our analysis of husk and seedling tissues, may be operationally important. The regulatory mutation of *pl1-Rhoades(mum9515)* either may directly affect a region generating *trans*-acting RNAs or simply prohibit transcription of the locus, resulting in a failure to generate appropriate *trans*-acting RNAs from the coding sequence. Functional identification of the lesion disrupting *pl1-Rhoades(mum9515)* expression should help to distinguish between these possibilities.

Although our observation that paramutagenic states repress *pl1-W22* is consistent with an RNAi-type *trans*-sensing interaction, it is also possible that *pl1-W22* cannot compete effectively against *Pl′* for a limited pool of transcriptional activators. The apparent dosage sensitivity of *pl1-W22* when heterozygous with *pl1-CO159* is consistent with such competition models. Whether the apparent *trans*-interaction between paramutant states and *pl1-W22* is mediated by RNAi-type repression or due to interallelic competition between binding sites, the processes are not mutually exclusive. One way to discriminate between these processes will be to examine the *trans*-repressive interaction between *Pl′* and *pl1-W22* in *mop1* and *rmr* mutant plants. A requirement for functional *Mop1* and *Rmr* alleles to maintain repression of *pl1-W22* by *Pl′* would suggest that the *trans*-repression is related to paramutagenic activity and not a result of promoter binding site competition.

Given that *trans*-sensing behaviors can lead to overdominant-type variation, it will be important to understand the molecular nature of these interactions and the genomic features responsible. Reversion of repressed epigenetic states is not limited to *Pl′*. Like *Pl′*, paramutant *R-r′* states become unstable when heterozygous with neutral *r1* haplotypes or the *r-x1* deficiency (Styles and Brink 1969). Some silenced transgenes can also be reactivated in hemizygous states (de Carvalho *et al.* 1992; Nap *et al.* 1997; Sutherland *et al.* 2000; De Wilde *et al.* 2001), suggesting that certain forms of repressive epigenetic information are generally maintained by *trans*-stabilizing interactions. However, not all alleles displaying paramutation require *trans*-stabilization; paramutant states of *B1-I* (*B′*) are exceedingly stable and have not been observed to revert when heterozygous with neutral *b1* alleles (Chandler *et al.* 2000). Insight into these interactions at *pl1* will guide an understanding of similar behaviors within the maize genome, which may contribute to general heterosis. We are currently using recombination-based strategies to genetically map these functionally relevant regions at *Pl1-Rhoades*, and we can now use mutations affecting paramutation (Hollick and Chandler 1998; Dorweiler *et al.* 2000; Hollick *et al.* 2005) to identify other genomic targets through differential transcript profiling. Ultimately, a better understanding of these interallelic relationships and the features responsible for them should prove useful to mine epigenetic diversity for plant improvement through standard marker-assisted selection. Our work with *pl1* shows that specific allelic combinations can have *trans*-generational consequences through modifying heritable sources of epigenetic variation.

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