Maize RNA Polymerase IV Defines \textit{trans}-Generational Epigenetic Variation$^{\ddagger}$

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The maize (\textit{Zea mays}) RNA Polymerase IV (Pol IV) largest subunit, RNA Polymerase D1 (RPD1 or NRPD1), is required for facilitating paramutations, restricting expression patterns of genes required for normal development, and generating small interfering RNA (siRNAs). Despite this expanded role for maize Pol IV relative to \textit{Arabidopsis thaliana}, neither the general characteristics of Pol IV–regulated haplotypes, nor their prevalence, are known. Here, we show that specific haplotypes of the \textit{purple plant} \textit{Pl1} locus, encoding an anthocyanin pigment regulator, acquire and retain an expanded expression domain following transmission from siRNA biogenesis mutants. This conditioned expression pattern is progressively enhanced over generations in Pol IV mutants and then remains heritable after restoration of Pol IV function. This unusual genetic behavior is associated with promoter-proximal transposon fragments but is independent of sequences required for paramutation. These results indicate that \textit{trans}-generational Pol IV action defines the expression patterns of haplotypes using co-opted transposon-derived sequences as regulatory elements. Our results provide a molecular framework for the concept that induced changes to the heterochromatic component of the genome are coincident with heritable changes in gene regulation. Alterations of this Pol IV–based regulatory system can generate potentially desirable and adaptive traits for selection to act upon.

INTRODUCTION

Epialleles, epigenetic variants of isogenic alleles, are characterized in diverse eukaryotes as non-DNA-based sources of regulatory variation (Jablonska and Raz, 2009). One potential link between many mitotically, and sometimes meiotically, heritable epiallele states is their association with nearby repetitive DNA sequences, such as transposable elements (TEs) (Slotkin and Martienssen, 2007). TE sequences provide host genomes with a rapidly evolving source of gene regulatory capacity, as shown recently in maize (\textit{Zea mays}) where a \textit{Hopscotch} family retrotransposon insertion functions as an enhancer in specific haplotypes of a domestication locus (Studer et al., 2011). Though the prevalence of heritable epialleles in natural populations remains unknown (Richards, 2011), epigenetic regulation of endogenous haplotypes associated with TEs could provide a significant source of adaptive phenotypes that are \textit{trans}-generationally stable, yet reversible under certain conditions.

Specific maize haplotypes that undergo paramutation can be considered \textit{trans}-generationally stable epialleles that are unique in their ability to alter meiotically heritable regulatory states via trans-homolog interactions (Erhard and Hollick, 2011). Several maize haplotypes known to participate in paramutation interactions contain cis-regulatory elements that are repetitive in nature, and these examples provide experimental models for understanding the mechanism of regulation for repeat-associated epialleles (Erhard and Hollick, 2011). In a species such as maize, where the genome is composed of $\geq 85\%$ TEs (Schnable et al., 2009) and haplotype diversity between inbred lines is driven predominantly by TE mobilization (Wang and Dooner, 2006), a large number of TE-associated epialleles is possible.

Paramutation is typified by the distinct regulatory states and inheritance patterns exhibited by \textit{Pl1-Rhoades} haplotypes of the \textit{purple plant} \textit{Pl1} locus. When combined in a heterozygote, a \textit{Pl1-Rhoades} haplotype in the strongly expressed \textit{Pl-Rh} state becomes repressed in trans by a homologous haplotype existing in a weakly expressed \textit{Pl'} state and acquires the meiotically heritable \textit{Pl'} state and expresses a common paramutation allele. The mechanism of regulation remains unknown, though several proteins required for producing 24-nucleotide RNAs, usually termed small interfering RNAs (siRNAs), are required for maintaining repressed \textit{Pl'} states, as well as repressed states of other maize haplotypes subject to paramutation (Erhard and Hollick, 2011). siRNAs present in both maize (Nobuta et al., 2008; Barbour et al., 2012) and \textit{Arabidopsis thaliana} (Kasschau et al., 2007) are highly enriched for sequences of TEs and other repeats.
observations indicate a link between paramutation-based repression and cellular mechanisms presumably evolved to suppress potential deleterious effects of TE activity.

Plants use specialized RNA polymerases IV and V (Pols IV and V) to facilitate RNA-directed DNA methylation (RdDM) of repetitive genomic sequences via the production and targeting of 24-nucleotide siRNAs. Mutational analyses of genes encoding Pol IV– and V–specific subunits indicate that Pol IV acts upstream in this pathway, generating siRNA precursors, while Pol V acts downstream, generating nascent scaffold transcripts that mediate targeting of siRNAs to homologous loci (Haag and Pikaard, 2011). This siRNA-targeting process guides the deposition of chromatin marks, such as asymmetric (non-CG) cytosine methylation (Haag and Pikaard, 2011), presumably via recruitment of enzymes, such as de novo cytosine methyltransferases.

The biological importance of Pol IV/V-dependent chromatin modifications in Arabidopsis has remained unclear, as Pol IV/V subunits are largely dispensable for development, though Pol IV mutant plants are slightly delayed in flowering (Haag and Pikaard, 2011). In maize, the Pol IV largest subunit, RNA Polymerase D1 (RPD1 or NRPD1), is required for facilitating paramutations (Erhard et al., 2009) and for restricting the expression patterns of certain genes required for normal development (Parkinson et al., 2007). These results indicate an expanded role for Pol IV in maize, which is likely related to the higher TE load (Schnable et al., 2009) and more frequent juxtaposition of TEs and genes in the maize genome compared with Arabidopsis (Hale et al., 2009). P11-Rhoades and its derivatives are useful models for studying Pol IV function because their expression states and inheritance patterns can be visually tracked by pigmentation that is most apparent in the anthers of male flowers (Hollick et al., 2000). Here, we show that Pol IV defines the tissue-specific regulation of certain pl1 alleles through its trans-generational action on attendant DNA transposon fragments.

**Figure 1.** RPD1 Function Represses P11-Rhoades Expression in the Aleurone.

(A) Aleurone pigment phenotypes in representative wild-type (WT) ears on the left and in ears segregating 1:1 for +/rp1 heterozygotes and rp1/rp1 homozygotes on the right. Table below shows correlations of anther pigmentation phenotypes (scored at the time of flowering) among ear progeny described above after selection of kernels with the lightest and darkest pigmentation phenotypes, respectively. P1′ (+/rp1 genotypes) signifies weak anther pigmentation and P1-Rh (rp1/rp1 genotypes) strong anther pigmentation.

<table>
<thead>
<tr>
<th>Kernel phenotype</th>
<th>n=1241</th>
<th>n=1222</th>
</tr>
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<tbody>
<tr>
<td>Mature plant phenotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1′</td>
<td>1097 (88%)</td>
<td>1097 (87%)</td>
</tr>
<tr>
<td>P1-Rh</td>
<td>144 (12%)</td>
<td>144 (12%)</td>
</tr>
</tbody>
</table>

(B) RT-PCR analysis of RNAs isolated from individual aleurones of lightly pigmented (n = 4) and darkly pigmented developing kernels (n = 4) 25 days after pollination (DAP). Below kernel phenotypes are results of molecular genotyping for each individual (see Methods). Primers specific to the P11-Rhoades (P11-Rh) allele and a control gene Ala aminotransferase (Aat) (see Methods) were used to amplify identical quantities of oligo(dT)-primed cDNA templates; amplifications using control samples prepared with no reverse transcriptase (-RT) are shown below for each primer pair.
RESULTS

Absence of RNA Pol IV Reverts a Null Pigment Phenotype

Anthocyanin pigmentation in maize requires function from either the pi1 locus or its subfunctionalized paralog colored1 (c1) (Cone et al., 1993a): c1 alleles condition pigmentation of the aleurone, the outermost layer of the kernel endosperm, whereas most pi1 alleles condition pigmentation of only the vegetative and floral tissues of the plant (Cone et al., 1993a).

Unexpectedly, we observed various degrees of aleurone pigmentation (Figure 1) in progeny segregating 1:1 for rpd1 homozygous mutants and +/rpd1 heterozygotes in a background genetically null (c1/c1; Pl1-Rhoades/rpd1-Rhoades) for aleurone pigmentation (Figure 1A, left panel). Sequencing of the c1 allele resident in our rpd1 mutant stocks (see Methods) identified a 363-bp miniature inverted-repeat transposable element of the Heartbreaker (Hbr) family (Zhang et al., 2000) (see Supplemental Figure 1 online) in exon 2, which introduces a premature nonsense codon that likely renders this c1-Hbr allele nonfunctional. We also observed aleurone pigmentation in individuals homozygous for c1-1 (see Supplemental Table 1 online), a null c1 allele disrupted by several indels in its coding region (Scheffler et al., 1994). It was therefore unlikely that the aleurone pigmentation seen in the 1:1 segregating rpd1 lines described above is due to C1. These results indicate that Pol IV is responsible for the colorless kernel phenotype seen in many of our Pl1-Rhoades stocks.

Pol IV Restricts Tissue-Specific Expression of Pl1-Rhoades

Aleurone and anther pigmentations phenotypes documented over nine generations of 1:1 segregating rpd1 progeny showed that 93% of the darkest kernel classes correlated with Pl-Rh-like types (both rpd1-1/rpd1-1 and rpd1-2/rpd1-2 genotypes) and 88% of the lightest-colored and colorless kernel classes correlated with Pl'-like types (both +/rpd1-1 and +/rpd1-2 genotypes) (Figure 1A). Because of this strong correlation, we hypothesized that expansion of Pl1-Rhoades expression to the aleurone caused the observed kernel pigmentation seen in the absence of Pol IV. RT-PCR analysis (Figure 1B) of RNAs isolated from four darkly pigmented and four colorless aleurones indicated that Pl1-Rhoades RNA levels qualitatively correlated with the amount of aleurone pigmentation for these eight samples. Molecular genotyping for the rpd1-1 lesion (see Methods) confirmed that the aleurone phenotype reflected the presence (colorless) or absence (darkly pigmented) of RPD1 function (Figure 1B). These results indicate the aleurone pigmentation seen in our stocks results from an expansion of Pl1-Rhoades expression, conditioned by passage through rpd1 mutants.

Trans-Generational Absence of Pol IV Conditions a Fully Colored Kernel Phenotype

The intensity of aleurone pigmentation increased over generations in which Pl1-Rhoades was maintained in an rpd1 mutant background. We generated progeny ears representing different filial generations of 1:1 segregating rpd1-1 families (Figure 2A) during the same field season to control for environmental conditions and observed a progressive enhancement of Pl1-Rhoades expression in the aleurone over an increasing number of generations without RPD1 function. Quantification of five roughly graded classes of aleurone pigmentation (see Supplemental Figure 2A online) in the sibling cross progeny described (Figure 2A) confirmed a trans-generational increase in overall aleurone pigmentation (Figure 2B). Notably, by the F5 generation, some kernels had a fully colored phenotype (see Supplemental Figures 2A and 2B online) indistinguishable from that conferred by dominant C1 alleles (see Supplemental Figure 2C online), and the ratio of this C1-like class among ear progeny increased across generations (Figure 2B). These data indicate that Pl1-Rhoades, which lacks aleurone expression in reference state nonmutant backgrounds, can be
inherited in a fully functional aleurone expression state after several generations of conditioning in the absence of RPD1 function. Phenotypes resulting from epigenetic changes are often not heritable across meiosis (Jablonka and Raz, 2009). However, Pl1-Rhoades aleurone expression, conditioned in the absence of RPD1 function, was heritable though not 100% penetrant in F1 progeny having inherited at least one copy of wild-type Rpd1 from either the ear or pollen parent (see Supplemental Table 2 online). This expanded expression domain was also heritable for at least two meiotic cycles in the presence of RPD1 function (see Supplemental Table 2 online).

**Conditioned pl1 Aleurone Expression Is Correlated with a Proximal CACTA-Like Transposon Fragment**

Conditioned Pl1-Rhoades aleurone expression resembles the expression pattern of Pl1-Blotched (Cocciolone and Cone, 1993), a haplotype structurally similar to Pl1-Rhoades (Hoekenga et al., 2000) that does not undergo paramutation (Hollick et al., 2000; Gross and Hollick, 2007). We tested the effect of RPD1 loss on the aleurone expression of Pl1-Blotched alleles and observed increases from a weak, variegated phenotype (Figure 3A) to fully colored C1-like phenotypes among F2S2 progeny of rpd1-1 mutants (Figure 3B). This result indicates that sequences targeted by Pol IV and regulating aleurone expression are shared between Pl1-Rhoades and Pl1-Blotched but are insufficient for paramutation. Both alleles contain a 402-bp terminal fragment of a CACTA-like DNA transposon related to the doppia subfamily (Cone et al., 1993b) located 97 bp 5' of the annotated transcription start sites (Figure 3C).

We also observed aleurone pigmentation (see Supplemental Figure 3 and Supplemental Table 3 online) in progeny segregating 1:3 for rpd1-2 (formerly rmr6-2) mutant alleles (Hollick et al., 2005) and a distinct pl1 variant from the CML52 inbred line, Pl1-CML52 (Figure 3C), which does not display aleurone expression in non-mutant backgrounds (see Methods). Unlike Pl1-Blotched, however, Pl1-CML52 undergoes paramutation (see Supplemental Table 4 online). Sequencing of the Pl1-CML52 5' region (see Methods; see Supplemental Figure 4 online; Figure 4, Tables 1 and 2).
Methods) identified a doppiia fragment in the same location with respect to the two p{l} haplotypes described above but containing a 32-bp insertion (Figure 3C). Thus, all three p{l} haplotypes restricted from aleurone expression via RPD1 action contain a similar proximal DNA transposon fragment.

**Sequences Required for Aleurone Expression Are Separable from Those Necessary for Paramutation**

Analyses of a p{l}-Rhoades derivative, p{l}-R30, supported the hypothesis that p{l} aleurone expression is driven by doppiia sequences found in p{l} promoter regions. The p{l}-R30 haplotype (Figure 3C) was identified in a screen for recombinant p{l}-Rhoades derivatives defective for paramutation (see Methods; see Supplemental Figures 4A and 4B and Supplemental Table 5 online).

We applied sequential selections to identify potential recombinants between p{l}-Rhoades and p{l}-B73, a p{l} haplotype that does not facilitate paramutation (Gross and Hollick, 2007). Using phenotypic and molecular markers diagnostic of a specific T6-9 chromosome (T) containing p{l}-Rhoades in a paramutant p{l} state (T p{l}’), we selected for plants having potentially recombinant chromatids that contained the p{l}-Rhoades coding region but lacked ability to facilitate paramutation of p{l}-Rh in trans (see Supplemental Figure 4B and Supplemental Table 5 online). One individual (06-932-30) had an anther color score (ACS) of 7, the fully colored phenotype expected if paramutation had not occurred (Hollick et al., 1995) (see Supplemental Figure 4C online).

Molecular analyses of genomic DNA from individual 06-932-30 confirmed the presence of a recombinant chromatid having a derivative p{l} haplotype hereafter referred to as p{l}-R30. DNA gel blot results (see Supplemental Figure 5 online) showed that p{l}-R30 maintains a p{l}-Rhoades–like structure for at least 6 kb 3′ of the coding region, yet PCR results identified p{l}-B73 haplotype polymorphisms at more distal 3′ simple sequence length polymorphism (SSLP) markers bnlg2249 (~27 Mb away) and umc2006 (~5 Mb away) (see Supplemental Figures 4C and 4D online). Based on these results and the design of the recombination screen, p{l}-R30 represents a derivative p{l} haplotype consisting of centromere proximal 5′ sequences, coding region, and 3′ flanking sequences from p{l}-Rhoades with more distal 3′ sequences from p{l}-B73. The exact crossover point remains unknown but is located between ~6 kb and 5 Mb 3′ of the coding region.

Additional genetic tests confirmed that p{l}-R30 was deficient for facilitating paramutation (Table 1; see Supplemental Tables 6 and 7 online). The p{l}-R30 haplotype was combined with p{l}’ and resulting heterozygotes were reciprocally crossed to p{l}-Rh/p{l}-Rh testers. The progeny sets segregated both p{l}’-type and p{l}-Rh-type plants (Table 1), which was unexpected if p{l}-R30 was able to facilitate paramutation. Because p{l}-R30 was carried on the T6-9 chromosome, we were able to distinguish most of the p{l}-R30/p{l}-Rh progeny as these had semisterile pollen. All but four of the 29 semisterile plants had fully colored anthers, indicating that paramutation had not occurred (Table 1). The four other semisterile plants had high ACSs that are not typical of paramutant p{l}’. In total, the p{l}-R30 haplotype is deficient for functions required to acquire and/or facilitate paramutation.

Coincidentally, the p{l}-R30 recombinant also lost high levels of expression in seedling sheaths (Figure 4A; see Supplemental Table 8 online), mature leaves (Figure 4B), and anthers as measured by visual assessment of pigment phenotype (Figure 4C; see Supplemental Tables 6 and 8 online) and by RT-PCR (see Supplemental Figure 6 online). These results are consistent with the hypothesis that 3′ sequences required to facilitate paramutation also serve as an enhancer-like element.

We selected pigmented kernels from progeny segregating p{l}-R30, p{l}-Rhoades, and rpd1-1 alleles, and molecular genotyping (see Methods) confirmed that 6/33 individuals grown from these kernels were homozygous for the p{l}-R30 allele (Table 2). These results show that the 3′ sequences required for paramutation interactions between p{l}-Rhoades alleles are not required for p{l}-R30 to be expressed in the aleurone.

Combined, our allelic series comparisons indicate that sequences targeted by Pol IV and regulating aleurone expression are shared between p{l} haplotypes containing a proximal doppiia fragment (Figure 3C). Additionally, plants homozygous for p{l}-Blotted and p{l}-R30 alleles, which in wild-type backgrounds confer variegated and weak anther pigmentation phenotypes, respectively, also produce dark p{l}-Rh-like anther phenotypes in the absence of RPD1 function (see Supplemental Table 9 online). This observation indicates that loss of Pol IV function conditions an overall increase in the expression of doppiia-associated p{l} haplotypes, in addition to an apparent expansion of tissue-specific expression in the absence of sequences necessary for paramutation.

Because the p{l}-Rhoades promoter-proximal doppiia is a presumed target of Pol IV–facilitated RdDM (Hale et al., 2007), we asked whether loss of doppiia cytosine methylation correlated

### Table 1. Anther Phenotypes of Progeny Resulting from Reciprocal Crosses of p{l}/T p{l}-R30 and p{l}-Rh/p{l}-Rh

<table>
<thead>
<tr>
<th>Female Parent</th>
<th>Male Parent</th>
<th>Progeny Structural Genotype</th>
<th>No. of Progeny Sets</th>
<th>No. of Progeny Individuals with Indicated ACS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>p{l}-Rh/p{l}-Rh</td>
<td>p{l}/T p{l}-R30</td>
<td>++</td>
<td>2</td>
<td>0 0 7 7 0 0 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+T</td>
<td></td>
<td>0 0 0 0 1 8</td>
</tr>
<tr>
<td>p{l}/T p{l}-R30</td>
<td>p{l}-Rh/p{l}-Rh</td>
<td>++</td>
<td>3</td>
<td>1 3 14 2 2 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+T</td>
<td></td>
<td>0 0 0 2 17</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>++</td>
<td>5</td>
<td>1 3 21 2 2 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+T</td>
<td></td>
<td>0 0 0 2 2 25</td>
</tr>
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</table>

Pl-Rh/Pl-Rh (A619) was used in all test crosses.
with the increased aleurone pigmentation observed in advanced filial generations of 1:1 segregating rpd1-1 families (Figure 2). We determined the 5'-methylcytosine (5meC) profiles at the 5' border of the Pl1-Rhoades doppia fragment (see Supplemental Figure 7A online) by sequencing PCR amplicons from bisulfite-treated genomic DNA isolated from individual aleurones. We compared 5meC profiles of DNA isolated from darkly pigmented (molecularly genotyped as rpd1-1/rpd1-1) and lightly pigmented (molecularly genotyped as +/rpd1-1) kernels representing the F7 and F9 generations and in unconditioned control (+/+ ) kernels (see Supplemental Figure 7B online). These profiles indicate that cytosines both 5' of and within the doppia fragment are relatively hypomethylated (see Methods) in both F7 (10/37 hypomethylated cytosines) and F9 (14/37 hypomethylated cytosines) darkly pigmented kernels compared with their respective lightly pigmented siblings profiles (see Supplemental Figures 7C and 7D online). Specific cytosines in all contexts (CHG, CHH, and CG) were affected by the loss of RPD1 function, although predominant differences were biased for CHG and CHH context. These data confirm earlier results obtained with methylation-sensitive restriction enzymes (Hale et al., 2007) showing RPD1-dependent maintenance of 5meC patterns. In the presence of RPD1, 5meC patterns remained relatively unaffected in this region, even among the F7 and F9 genotypes (see Supplemental Figures 7C and 7D online). While aleurone pigmentation is correlated with the loss of RPD1 and 5meC across this region, the increased pigmentation seen in the presence of RPD1 following trans-generational conditioning is not. This result indicates that either 5meC patterns are differentially conditioned at regions not evaluated in our analysis or that a distinct epigenetic feature at this region is modified by such breeding designs.

Kernel Pigmentation Occurs in Other siRNA Biogenesis Mutants

Three other siRNA biogenesis pathway components are required for maintaining repressed Pl' states, including REQUIRED TO MAINTAIN REPRESSION1 (RMR1), a SUCROSE NONFERMENTING2-like ATPase (Hale et al., 2007); MEDIATOR OF PARAMUTATION1 (MOP1), a putative RNA-dependent RNA polymerase (Woodhouse et al., 2006; Alleman et al., 2006); and one of three presumed second largest subunits of Pol IV isoforms, RMR7/NRPD2a (Sidorenko et al., 2009; Stonaker et al., 2009). We found aleurone pigmentation in ear progenies segregating 1:1 for homozygous rmr1, mop1, and rpd2a mutants and heterozygous siblings. Cosegregation analyses, performed as described above for rpd1, indicated that RMR1, MOP1, and RPD2a also contribute to repression of Pl1-Rhoades in the aleurone layer (Figures 5A and 5B). However, distinctive from the loss of rpd1, we did not see evidence for enhancement of pigmentation in subsequent generations.

DISCUSSION

The characterization of meiotically heritable epialleles broadens our understanding of how selectable traits can be manipulated and transmitted across generations. Our results indicate that trans-generational Pol IV function defines specific expression domains of endogenous maize alleles. We find that epigenetic
conditioning of Pl1-Rhoades across multiple generations in rpd1 mutants converts a null allele for aleurone expression into a heritable, fully functional, dominant form. Our genetic analyses using Pl1-Rhoades derivatives and closely related alleles provide a correlation between nearby repetitive sequences and endogenous allele structures subject to siRNA-directed regulation. A similar relationship is supported by comparative genomics and siRNA signatures in divergent Arabidopsis accessions (Hollister et al., 2011).

Our results contrast with those of other known epialleles subject to siRNA-based control in that heritable changes in Pl1-Rhoades regulation are conditioned in the absence of Pol IV, which is required for RdDM (Haag and Pikaard, 2011). Both Arabidopsis silent epialleles FWA and SDC contain repetitive features targeted by RdDM, yet FWA is not derepressed in RdDM mutants (Cao and Jacobsen, 2002; Chan et al., 2006a) and the derepressed SDC found in similar mutants is not meiotically heritable (Chan et al., 2006b; Henderson and Jacobsen, 2008). These contrasts may reflect the general differences in haplotype structures (Hale et al., 2009) and/or Pol IV functions (Stonaker et al., 2009) seen in grasses relative to eudicots.

It remains unknown whether restriction of Pl1-Rhoades expression in the presence of siRNAs is mechanistically related to paramutation interactions occurring between Pl1-Rhoades states. The 3’ sequences required for paramutation interactions are not necessary for aleurone expression, but the 5’ doppia fragment may be necessary for paramutations. This hypothesis predicts that a genetic difference, defined by 3’ regulatory sequences, distinguishes the Pl1-Rhoades and Pl1-Blotched haplotypes, which is consistent with linkage data indicating that the Blotched factor is located ~1 centimorgan (cM) distal of Pl1-Rhoades (Rhoades, 1948). The recovery of pl1-R30 from a screen for recombinant alleles between pl1-B73 and Pl1-Rhoades demonstrates that the paramutagenic elements of Pl1-Rhoades are tightly linked but separable, from the Pl1-Rhoades coding sequence. The screen recovered one recombinant haplotype among ~13,655 chromatids screened, placing the paramutagenic element(s) ~0.0073 cM distal of the Pl1-Rhoades coding sequence. However, this estimate assumes that all such recombinants were recognized in our phenotypic screen.

Molecular, genetic, and phenotypic analyses of pl1-R30 indicate it is different from its progenitor alleles. pl1-R30 is weakly expressed in seedlings, unlike both Pl1-Rhoades and pl1-B73, and weakly expressed in anthers like pl1-B73 but unlike Pl1-Rhoades. The pl1-B73 enhancer for seedling expression may be 5’ of the coding sequence, and the pl1-B73 enhancer for anther expression may be 3’ of the coding sequence. However, in Pl1-Rhoades, both tissue-specific enhancers, or a single enhancer functioning in both seedlings sheaths and anthers, could be 3’ of the coding sequence. The paramutagenic element(s) of Pl1-Rhoades is located 3’ of the coding sequence, which could be identical to or closely linked to an enhancer(s) required for high expression. This scenario is similar to the model of the maize B1-Intense (B1-I) haplotype, in which an enhancer-type element ~100 kb 5’ of the B1-I coding region regulates both high expression and paramutagenic activity (Stam et al., 2002a, 2002b). Although the cis-acting features

<table>
<thead>
<tr>
<th>Genotype of Selfed F1 Parent</th>
<th>No. of Progeny Ears</th>
<th>Pl1-Rhoades/Pl1-Rhoades</th>
<th>Pl1-Rhoades/pl1-R30</th>
<th>pl1-R30/pl1-R30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pl1-Rhoades/pl1-R30; +/rpd1-1</td>
<td>2</td>
<td>15</td>
<td>12</td>
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Table 2. Cosegregation of Aleurone Pigmentation with pl1-R30 Homozygotes

Figure 5. Aleurone Pigmentation in siRNA Biogenesis Mutants. (A) Histogram showing the percentage of mature plant phenotypes (Pl’ and PI-Rh as described in Figure 1) among progeny segregating 1:1 for heterozygotes and homozygous mutants for each of the siRNA biogenesis factors listed, after selection of kernels with the lightest pigmentation of the progeny. (B) Same analyses as in (A) after selection of kernels with the darkest pigmentation.
required for Pl1-Rhoades paramutation interactions have not been identified, genetic and molecular evidence suggests that regulatory regions of pl1 and/or RNA transcripts produced by the pl1 coding sequence could be important for these trans-homolog interactions (Gross and Hollick, 2007). Thus, the trans-sensing interactions between pl1 haplotypes may occur between distal cis-regulatory sequences.

It will be important to determine the requirement of the 5’ doppia fragment for both paramutation interactions between Pl1-Rhoades alleles and its aleurone expression capacity. Cytosine methylation patterns at this feature appear identical in both Pi’ and Pi-Rh states (Hale et al., 2007). Most of the RdDM-like factors identified to date in forward genetic screens for paramutation defects may primarily affect the transcriptional status of Pl1-Rhoades via their action on the attendant doppia fragment, and these effects may be unrelated to the paramutation process responsible for trans-homolog communication. siRNAs alone appear to be insufficient for certain aspects of paramutation interactions between Pl1-Rhoades alleles (Erhard and Hollick, 2011; Barbour et al., 2012). For example, the 64% of siRNAs dependent on RMR1 function (Hale et al., 2009) are not required for the acquisition of Pi’ states (Hale et al., 2007), and the 85% of siRNAs dependent on RMR7/NRPD2a are not required to maintain the meiotically heritable Pi’ state (Stonaker et al., 2009). Therefore, the presumed RdDM-like mechanism suppressing Pl1-Rhoades expression in the aleurone may be functionally distinct from that which facilitates the trans-homolog interactions between Pl1-Rhoades alleles.

Our findings implicate the 5’ doppia fragment as a component of the regulatory module that conditions strong aleurone expression after transmission from siRNA mutants. A similar 226-bp doppia fragment acts as an aleurone-specific promoter in the R-r haplotype of the red1 (r1) locus (Walker et al., 1995), which encodes an anthocyanin regulator unrelated to pl1, suggesting that the doppia fragment in the promoter regions of the four pl1 alleles we examined (Figure 3C) may be responsible for their aleurone expression. It remains possible that heritable loss of Pol IV-directed cytosine methylation patterns within and surrounding the doppia sequence 5’ of Pl1-Rhoades influences or facilitates the trans-generational increase in pigmentation observed after multiple generations of conditioning in an rpd1 mutant background. It is also possible that other epigenetic regulatory features are progressively altered by such treatments.

In contrast with the progressive and meiotically heritable enhancement of aleurone pigmentation reported here, other developmental defects typically seen in rpd1 mutant plants such as problems with leaf polarity are not heritable (Parkinson et al., 2007). The difference in heritability of rpd1 mutant phenotypes in the mature plant and the Pl1-Rhoades aleurone expression domain may reflect a fundamental difference in the function of RdDM in embryo- and endosperm-derived cells. This difference may also reflect functional diversity in the allele or haplotype structures of RPD1 targets, as seen between Pl1-Rhoades and its derivatives. It also remains unclear why doppia-associated pl1 alleles conditioned in the absence of other siRNA biogenesis components are not progressively enhanced in pigmenting potential. This observation implicates a role of Pol IV in defining meiotically heritable regulatory patterns unrelated to a presumed RdDM pathway.

Pol IV function represents a molecular link between developmental gene control in maize and TE-associated epiallele regulation. Alleles, or haplotypes, that undergo paramutation are seemingly exceptional, but TE-associated haplotypes affected by Pol IV action may be far more numerous. We speculate that Pol IV has been co-opted during maize evolution and germplasm improvements to define the expression pattern of specific alleles that have incorporated repeat-like features. Changes to the heterochromatic component, as defined by maize Pol IV, thus leads to the appearance of phenotypic variation as previously surmised (McClymont, 1951). Our results indicate that Pol IV, together with repetitive genomic features, constitute a nuclear system serving as a capacitor of cryptic, heritable epigenetic variation available for breeding and evolution.

METHODS

Nomenclature

Alleles, phenotypes, and genotypes of maize (Zea mays) are designated as described (Hollick et al., 2005). The “c” delineates cis-elements in recombinant haplotypes, where descriptors on the left of the “c” indicate centromere proximal elements, and descriptors on the right of the “c” describe centromere distal features. For SSLP markers, polymorphisms derived from the T Pl1-Rhoades/T Pl1-Rhoades parent (see below) are referred to as “Rhoades” types, and polymorphisms derived from the B73 inbred line parent are referred to as “B73” types. Female-derived factors are placed before male-derived factors when stating diploid genotypes. Anther pigmentation is visually assessed using a 1 to 7 graded ACS as previously described (Hollick et al., 1995).

Genetic Stocks

The rpd1-1 and rpd1-2 stocks used to generate families segregating 1:1 for rpd1-1 and rpd1-2 homoygotes and heterozygous siblings (Figures 1 and 2, respectively) were previously described (Hollick et al., 2005; Parkinson et al., 2007) and contain the plant color genotypes Pl1-Rhoades (Pi’); b1; c1; R-r.

For the Pl1-Rhoades recombinant screen, the B73 inbred line and a Pl1-Rhoades line homozygous for the T6-9 (043-1) interchange (T), T Pl1-Rhoades/T Pl1-Rhoades (T/Pi’/Pi’), are as described (Hollick et al., 2005; Gross and Hollick, 2007). The 9S portion of the T Pl1-Rhoades chromosome carries a recessive waxy1 (wx1) allele and, thus, selection of opaque waxy kernels among starchary kernels aids selection of T Pl1-Rhoades homozygotes. The translocation breakpoint 1.5 cM from Pl1-Rhoades (≈1.1 cM) represents a dominant semisterility locus (Hollick et al., 2005). Translocation heterozygotes produce ≈50% sterile pollen, yet translocation homozygotes produce ≈100% fertile pollen (Patterson, 1994). T Pi-Rh/T Pi-Rh stocks were obtained by capturing a revertant Pi-Rh state from an ACS 7, rpd1-1/rpd1-1; T Pi’/T Pi’ individual as described (Hollick et al., 2005). Additional Pi-Rh/Pi-Rh testers are as described (Hollick et al., 2005; Gross and Hollick, 2007). A null pl1 stock, (pl1-987/ pl1-987; Pl-Rh/Pl-Rh) was provided by Karen Cone, University of Missouri, Columbia, MO. To generate screening material for recombinants between pl1-B73 and Pl1-Rhoades, pl1-B73/pl1-B73 females were crossed by T Pi’/T Pi’ males. F1 (pl1-B73/T Pi’/Pi’) individuals were crossed by T Pi-Rh/T Pi-Rh (ACS 7) males, and kernels resulting from this cross were screened as described below.

Inbred line CML52 used for the paramutation test (see Supplemental Table 4 online) was obtained from the North Central Regional Plant Introduction Stations, Ames, IA. Complementation tests indicate that the CML52 inbred line contains both a null c1 allele and a null seed
component of the r1 haplotype (r-r). Female and male parents, ear progenies examined, and kernel phenotypes are as follows: CML52, A632 (c1; R-r), 2, all colorless aleurones; CML52, K55 (C1; r-r), 2, all colorless aleurones; K55 (C1; r-r), 2, all colorless aleurones.

Color-converted A632 [Pl1-Rhoades (P1’); b1; c1; R-r] stocks used for outcrosses are described by Hollick et al. (2005). Color-converted Mo17 stocks (>93% Mo17; Pl1-Rhoades (P1’); B1-Mo17; c1; R-r) used for outcrosses were established through recurrent backcrosses to the inbred line in the same manner as described by Hollick et al. (2005). The A619 [Pl1-Rhoades (Pl-Rh); b1; c1; R-r] stock used as a control for bisulfite conversion (see below) was derived by introgressing T Pl-Rh containing a null c1 allele into the color-converted A619 [Pl1-Rhoades (Pl-Rh); b1; c1; R-r] stock described by Hollick et al. (2005).

Plant 06-932-30 (T pl1-R30/T Pl-Rh) was selfed, and resulting progeny were genotyped for the umc2006 marker to identify pl1-R30 homozygotes. Plant 06-932-30 was also crossed to both were genotyped for the Pl-Rh (Gross and Hollick, 2007): 5’-GAACAGTGTACATAGCAGTGACGA-3’ and 5’-CCAGCGTGCTGTCCAGTATT-3’. Plants with pl1-B73/Pl1-Rhoades phi031 polymorphisms were discarded (354 of 360 were pl1-B73/Pl1-Rhoades, respectively: 5’-TGTTTTGTCTTGACTGAGGGAGAAG-3’. The six remaining Pl1-Rhoades/Pl1-Rhoades individuals were scored for their ACS and pollen phenotypes as described (Hollick et al., 2005). Of the remaining 6 Pl1-Rhoades/Pl1-Rhoades plants, all were fully fertile (7T7), but five had PI1-like phenotypes and were discarded. Only one Pl1-Rhoades/Pl1-Rhoades individual (06-932-30) had fully fertile pollen and ACS 7 anthers. Select PI1-Rhoades/PI1-Rhoades individuals from family 06-932 were genotyped for the bnlq2249 SSLP polymorphism (Lee et al., 2002) to confirm the presence of a recombinant event distal to the pl1 locus using the primer pairs 5’-AGATGCCCTTAGCAAAGGA-3’ and 5’-C CCCCTAGTTGTCGATAA-3’.

Mapping of Recombinant pl1-R30

All SSLP mapping was performed using DNA from the individual heterozygous for the recombinant pl1 allele, 06-932-30 (T pl1-R30/T Pl1-Rhoades). The maize FPC map (http://www.grameye.org and http://www.maizesequence.org) was used to find sequenced or partially sequenced BACs anchored to maize FPC contig 280, on which pl1-B73 had been placed. Genetic distances between pl1, umc2006 (Lee et al., 2002; 5’-AGTCCATACCATCCATCTGGCC-3’ and 5’-CGACACTATGCAGTTAACCTTGTGCAAT-3’), and bnlq2249 markers were calculated using the maize IBM2 2004 Neighbors 6 map available at MaizeGDB (http://www.maizegdb.org); Lee et al., 2002). To coordinate the genetic distance between this high-resolution genetic map and F2 data, genetic distances of the IBM2 2004 Neighbors map were divided by a factor of 3.8 (Yao et al., 2002). This method places pl1 ~4.71 cM centromere proximal of umc2006 and 17.5 cM proximal of bnlq2249.

Molecular Analyses for Pl1-Rhoades

For PI1-Rhoades expression analysis (Figure 1B), aleurone layers were hand-dissected from individual developing kernels 25 d after pollination of an ear from a PI1-like (+/rdp1-1) plant by pollen from a PI-Rh-like (rdp1-1/rdp1-1) plant, and RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. cDNA was generated as previously described (Hale et al., 2007) using 1 µg of total RNA as template. Separate reactions were performed with RNA from four independent biological replicates for each of the two pigment/genotypes using no reverse transcriptase to control for genomic DNA contamination. Resulting cDNA samples were PCR amplified for 27 cycles using PI1-Rhoades—specific primers (Gross and Hollick, 2007) and primers specific to Ala amino-transferase as described (Hale et al., 2007), yielding a 171-bp product and a 290-bp product, respectively. RT-PCR products were sized on a 2% agarose gel and stained with ethidium bromide for visualization and qualitative comparison between samples.

For sequencing of c1 and pl1 alleles, genomic DNA was isolated from seedling or mature plant leaves as described (Hale et al., 2007). DNA from PI1-type plants in a 1:1 rdp1-1-segregating family from stocks described above was amplified via PCR using c1-specific primers homologous to sequences in the 5’ untranslated region and intron 2, respectively: 5’-CCTATTGACCC-CATGCTA-3’ and 5’-GAACAGTGTAGATGGACGA-3’. DNA from CML52 inbred plants was PCR amplified using PI1-specific primers homologous to sequences 5’ of the doppia fragment and the intron 1-exon 2 boundary, respectively: 5’-TGTTTCTGGTCCAGTGGAGGAAAG-3’ and 5’-AAAGCTG-CACACACCATC-3’. PCR amplicons were purified as described (Hale et al., 2007) and sequenced at the UC Berkeley DNA Sequencing Facility (http://mcb.berkeley.edu/barker/dnaseq/index.html).
Derived cleaved amplified polymorphic sequence primers (Neff et al., 1998) were designed to molecularly identify the rpd1-1 lesion (Erhardt et al., 2009): 5’-TAGAATGTTCTATGGGCAAGCTACAGG-3’ and 5’-GGGTGTTCAAAAGCTCCATA-3’. During the PCR, the forward primer introduces a Bgfi restriction site only when amplifying genomic DNA or cDNA containing the wild-type sequence over the region containing the rpd1-1 lesion. PCR amplicons (139 bp) were digested for 1 h at 37°C with Bgfi (New England Biolabs) and analyzed by agarose gel electrophoresis to determine the restriction polymorphism (22 bp) of each sample.

All materials for pl1-R30 transcript analyses were grown in soil and vermiculite with fertilizer under standard greenhouse conditions. Shoot tissue below the 1st leaf was collected from 2-week-old seedlings. Developing tassel florets were collected just prior to opening, identified by their proximity to florets that had previously opened and extruded anthers. All tissues for transcript analysis were collected near mid-day. RNA was extracted and RT-PCR was performed as described (Gross and Hollick, 2007) starting with 10 μg total RNA.

A probe for the ~1300 region of Pl1-Rhoades used in DNA gel blot analyses (see Supplemental Figure 5 online) was generated as follows. Available Pl1-Rhoades sequence was compared with additional maize sequences deposited in GenBank using BLASTn (Altschul et al., 1997). A region extending from ~1318 through ~2316 was found to be unique to pl1. This sequence was amplified by PCR using primers Bh_Eco_L (5’-TGGCCC TTTCAATCCAGTGGTA-3’) and Bh_Eco_R (5’-CCCCGACCTT-TGTGACCTTTG-3’) from the Pl1-Blotched 2.4-kb EcoRI plasmid provided by Karen Cone, University of Missouri, Columbia, MO. PCR conditions were as follows: 95°C for 35 s, 60°C for 35 s, 72°C for 1 min, for 35 cycles. PCR amplicons for the ~1300 probe were gel purified using the Freeze’nSqueeze gel extraction kit (Bio-Rad) prior to radiolabeling. DNA for DNA gel blot analysis was extracted as described (Voelker et al., 1997). Four micrograms of genomic DNA was cut with indicated enzyme according to the manufacturer’s instructions (New England Biolabs), separated on 1% agarose gels, and blotted to Hybond-N membranes (GE Healthcare Life Sciences). The ~1300 probe was labeled as described (Feinberg and Vogelstein, 1983). Hybridized blots were exposed to phosphor screens for 3 d and imaged as described (Gross and Hollick, 2007).

Bisulfite Sequencing

Darkly blotched and lightly blotched aleurones were hand-dissected from kernels of F7 and F9 progeny ears and from kernels of Pl1-Rhoades (A619) 2 d postimbibition, and genomic DNA was isolated as previously described (Voelker et al., 1997) with the following modifications: Aleurones were frozen in liquid nitrogen and vigorously ground using a mortar and pestle with lysis buffer (7 M urea, 0.25 M NaCl, 50 mM Tris-HCl, pH 8.0, 1% Na-laurylsarcosine, 20 mM EDTA, and 0.25% β-mercaptoethanol) until thawed. After a 5-min incubation at 37°C, DNA samples were extracted from pulverized aleurone tissue with phenol/chloroform/isooamyl alcohol, precipitated in isopropanol, and resuspended in TE. Five hundred nanograms of genomic DNA was bisulfite converted following manufacturer’s specifications using the EZ DNA Methylation Kit (Zymo Research). Degenerate primers (Doppia-bis-F, 5’-GTGATAGTGTAAAGTGGGAGG-3’, and Doppia-bis-R: 5’-TACCRACRAAATACCATTTG-3’) were designed using the Kismeth web suite (Gruntman et al., 2008) to amplify the 5’ portion of the doppia fragment (~660 to ~190 nucleotides from the Pl1-Rhoades transcription start site) from both untreated and bisulfite-treated templates. Gel-purified (Freeze’n Squeeze gel extraction kit) 470-bp PCR amplicons were cloned using the pGEM-T Easy vector system (Promega) following vendor instructions, and the resulting plasmids were transformed into TOP10 cells (Invitrogen). Validated clones were sequenced by the Plant-Microbe Genomics Facility (The Ohio State University, Columbus, OH) using the Doppia-bis-F primer. Geneious software (Biomatters) was used to trim sequences and perform quality control before bisulfite analysis with Kismeth. Data included in bisulfite sequence analysis using the Kismeth web suite (parameters: 0.8 min fraction of positive matches; 0.25 min fraction of length) comprise sequenced clones from one A619 aleurone, two F7 light blotchy aleurones, three F7 dark blotchy aleurones, three F9 light blotchy aleurones, and three F9 dark blotchy aleurones. Standard error and analysis of variance tests were performed with JMP software (SAS). We called a cytosine relatively hypomethylated if the percentage of methylation averaged from the biological replicates of one genotype was lower by at least one standard error than the average of the comparable cytosine from another genotype.

Accession Numbers

DNA sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: c1-Hbr (JX556412) and Pl1-CML52 (JX556413)

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. c1-Hbr Allele Structure.
Supplemental Figure 2. Representative Aleurone Pigmentation Phenotypes.
Supplemental Figure 3. Aleurone Pigmentation in Stocks Containing Pl1-CML52.
Supplemental Figure 4. Selection of pl1-R30.
Supplemental Figure 5. DNA Gel Blot Analysis of pl1-R30.
Supplemental Figure 6. RT-PCR Analysis of pl1-R30.
Supplemental Figure 7. Cytosine Methylation at the Pl1-Rhoades doppia Element.
Supplemental Table 1. c1-n Cosegregation with Aleurone Pigment Phenotype.
Supplemental Table 2. Aleurone Pigmentation Phenotypes of Ear Progeny Resulting from Outcrossing Individuals Grown from Pigmented Kernels.
Supplemental Table 3. Pl1-CML52 Cosegregation with Aleurone Pigment Phenotype.
Supplemental Table 4. Progeny Phenotypes Resulting from Cross of Pl1-CML52/T Pl’ × T Pl-Rh/T Pl-Rh.
Supplemental Table 5. Frequencies and Descriptions of Expected Genotypes and Phenotypes of wx1/wx1 Seedlings and Plants Resulting from pl1-R73/T F’ × T PI-Rh/T Pl-Rh.
Supplemental Table 6. Anther Phenotypes of Genotyped Progeny Resulting from Outcrosses of 06-932-30 (T pl1-R30/T PI-Rh) to Indicated Testers.
Supplemental Table 7. Anther Phenotypes of Progeny Resulting from Outcrosses of 06-932-30 (T pl1-R30/T PI-Rh) to Indicated Testers.
Supplemental Table 8. Anther and Seedling Phenotypes and umc2006 Genotypes for F2 Progeny of 06-932-30 (T pl-R30/T Pl-Rh).
Supplemental Table 9. pl1-R30 and Pl1-Blotched Anther Phenotypes in rpd1 Mutants.

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AUTHOR CONTRIBUTIONS

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REFERENCES


