A Novel Snf2 Protein Maintains *trans*-Generational Regulatory States Established by Paramutation in Maize

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Paramutations represent heritable epigenetic alterations that cause departures from Mendelian inheritance. While the mechanism responsible is largely unknown, recent results in both mouse and maize suggest paramutations are correlated with RNA molecules capable of affecting changes in gene expression patterns. In maize, multiple required to maintain repression (*rmm*) loci stabilize these paramutant states. Here we show *rmm1* encodes a novel Snf2 protein that affects both small RNA accumulation and cytosine methylation of a proximal transposon fragment at the *Pl1-Rhoades* allele. However, these cytosine methylation differences do not define the various epigenetic states associated with paramutations. Pedigree analyses also show *RMM1* does not mediate the allelic interactions that typically establish paramutations. Strikingly, our mutant analyses show that *Pl1-Rhoades* RNA transcript levels are altered independently of transcription rates, implicating a post-transcriptional level of *RMM1* action. These results suggest the RNA component of maize paramutation maintains small heterochromatic-like domains that can affect, via the activity of a Snf2 protein, the stability of nascent transcripts from adjacent genes by way of a cotranscriptional repression process. These findings highlight a mechanism by which alleles of endogenous loci can acquire novel expression patterns that are meiotically transmissible.

Introduction

The term “paramutation” describes a genetic behavior in which the regulatory state of specific alleles is heritably altered through interactions with their homologous partners in *trans* [1,2]. This behavior presents an exception to the Mendelian principle that alleles segregate from a heterozygous state unchanged [3]. Paramutations have been best characterized at loci encoding transcriptional regulators of pigment biosynthesis in maize, but similar behaviors have been described in other plant and animal systems, most recently in mice [4,5]. While the broader roles of paramutation in genome-wide regulation and evolution remain to be seen, the *Pl1-Rhoades* allele of the maize *purple plant1* (*pl1*) locus presents a tractable system to study the paramutation process.

The *pl1* locus encodes a Myb-like protein that acts as a transcriptional activator of genes required for anthocyanin pigment production [6]. Inheritance patterns illustrate that the *Pl1-Rhoades* allele can exist in quantitatively distinct regulatory states, reflected by differences in plant color. When individuals with a highly expressed reference state of *Pl1-Rhoades*, termed *Pl-Rh*, are crossed with plants having a repressed state, referred to as *Pl'*, only progeny with weak pigmentation are produced [7,8]. *Pl-Rh* states invariably change to *Pl'* in *Pl-Rh*/*Pl'* heterozygotes [7]; this is a typical hallmark of paramutation. Relative to *Pl-Rh*, the *Pl'* state displays reductions in both *Pl1-Rhoades* RNA levels (~10-fold) and transcription rate (~3-fold) that are associated with a reduction in plant pigment [8]. This repressed *Pl'* state is meiotically stable when maintained in a *Pl1-Rhoades* homozygote, with no reversion to *Pl-Rh* seen to date. *Pl'* can, however, revert to *Pl-Rh* when heterozygous with some *pl1* alleles other than *Pl1-Rhoades*, when maintained in a hemizygous condition, or in the presence of specific recessive mutations [9–12].

Genetic screens for ethyl methanesulfonate (EMS)–induced recessive mutations identify at least ten loci, including required to maintain repression (*rmm1*), *rmm2*, *rmm6*, and mediator of paramutation1 (*map1*), whose normal functions maintain the repressed *Pl'* state ([10,11,13]; J. B. H., unpublished data). These *rmm* mutations specifically affect the expression of *Pl1-Rhoades* and not other *pl1* alleles [10,11], indicating that the *Pl1-Rhoades* allele is a direct and specific target of paramutation-based epigenetic changes. *Map1* was recently identified [14,15] as encoding the putative ortholog of the *Arabidopsis* protein RDR2, a presumed RNA-directed RNA polymerase involved in siRNA-based maintenance of de novo cytosine methylation [16]. Recessive mutations defining *rmm1*, *rmm2*, and *rmm6* destabilize the repressed *Pl'* state, resulting in darkly pigmented plant tissues, an increase in *pl1* RNA levels, and meiotic transmission of *Pl'-revertant* states [10,11]. To
Author Summary

Genetics is founded on the principle that heritable changes in genes are caused by mutations and that the regulatory state of gene pairs (alleles) is passed on to progeny unchanged. An exception to this rule, paramutations—which reflect the outcome of interactions between alleles—produce changes in gene control that are stably inherited without altering the DNA sequence. It is currently thought that these allelic interactions cause structural alterations to the chromatin surrounding the gene. Recent work in both maize and mice suggests that RNA molecules may be responsible for paramutations. Several genes are required to maintain the repressed paramutant state of a maize purple plant1 (pl1) allele, and here we report that one of these genes encodes a protein (RMR1) with similarity to a protein previously implicated in facilitating genomic DNA modifications via small RNA molecules. Genetic and molecular experiments support a similar role for RMR1 acting at a repeated sequence found adjacent to this pl1 gene. Although loss of these DNA modifications leads to heritable changes in gene regulation, the data indicate these changes do not represent the heritable feature responsible for paramutation. These findings highlight an unusual but dynamic role for repeated genomic features and small RNA molecules in affecting heritable genetic changes independent of the DNA template.

Results

rmr1 Defects Affect pl1 RNA Stability

The rmr1 locus is defined by four recessive mutations (Protocol S1) characterized by a darkly pigmented plant phenotype that results from loss of Pl1 repression. Previous RNase protection experiments showed a 26-fold increase in pl1 RNA in floret tissue between rmr1–1 mutant plants and heterozygous siblings [10]. However, these experiments did not address if changes in pl1 transcript abundance correlated with changes in actual transcription at the pl1 locus.

In vitro transcription assays using nuclei isolated from husk leaf tissue revealed there was no statistically significant change in relative transcription rates of the Pl1-Rhoades allele between rmr1–1 mutants and heterozygous siblings (Figure S1). However, transcription rates of anthocyaninless1 (a1), a direct target of the PL1 transcriptional activator [7,19], were ~4-fold greater in rmr1–1 mutants (Figure S1), reflecting significantly increased PL1 activity. Transcription rates from colored plant1 (b1)—a locus encoding a basic helix-loop-helix factor genetically required for a1 transcription—remained unchanged. These results were recapitulated in comparisons between nuclei isolated from rmr1–3 mutants and heterozygous siblings in which in vitro transcription assays revealed no significant change in transcription rate of Pl1-Rhoades (Figures 1A and S1; n = 4, two-tailed two-sample t-test, t = 0.8, p = 0.5) while RNase protection experiments showed a 5.7-fold increase in pl1 RNA for rmr1–3 mutants (Figure 1B and 1C; n = 2, two-tailed two-sample t-test, t = 10.8, p < 0.01) using RNA isolated from the same tissues of the same individuals. Similar comparisons from identical tissues but in a different genetic background again showed that transcription rates at pl1 remained unchanged while pl1 RNA levels increased 7.52-fold in rmr1–3 mutants compared to heterozygous siblings (n = 1; see Protocol S1).

These RNA expression results sharply contrast those of previous reports using identical in vitro transcription assays
that detected significant differences in *Pl1-Rhoades* transcription rates between *Pl* and *Pl-Rh* states and between rmr6 mutants and non-mutants [8,11]. This indicates our in vitro results represent an accurate assessment of transcription rates and not a limitation of the assay to detect rate differences at the *pl1* locus. Combined, these results imply an increase of *pl1* RNA abundance disproportionate to insignificant changes in transcription rate in *rmr1* mutants, the most direct interpretation being that RMR1 functions at a post-transcriptional level to stabilize *Pl1-Rhoades* RNA.

**rmr1 Encodes a Novel Protein with a Snf2 Domain**

To better understand *Rmr1* function and the paramutation mechanism, we used a map-based approach to identify the *rmr1* gene. Using a polymorphic F2 population we looked for genetic linkage between the mutant phenotype and previously mapped chromosome markers [29]. The dark-color phenotype of *rmr1–1* homozygotes showed invariant cosegregation with the mutant parent polymorphism of SSLP markers *bnlg1174a* (680 chromosomes tested; <0.15 cM) and *npi252* (60 chromosomes tested; <1.7 cM), indicating *rmr1* was tightly linked to those markers in bin 6.05 on Chromosome 6. We used the high degree of synteny between this region and rice Chromosome 5 to identify candidate *rmr1* orthologs (Figure 2A and 2B).

Within the syntenic rice region we identified a gene model, *Os05g32610* (http://rice.tigr.org/), predicted to encode a Snf2 protein. The Snf2 protein family is composed of members similar to *Saccharomyces cerevisiae* Snf2p with a bipartite helicase domain containing Pfam SNF2_N and Helicase_C profiles, and includes many proteins involved in ATP-dependent chromatin remodeling [21,22]. While there was no public maize expressed sequence tag for this candidate, we used BLAST searches to identify genomic survey sequence similar to *Os05g32610*. Oligonucleotide primers were designed from these sequences and used to generate PCR amplicons spanning the maize *Os05g32610* ortholog, which were sequenced from individuals homozygous for *Rmr1* progenitor alleles and mutant derivatives (see Materials and Methods and Dataset S1). The maize sequence generated from each of the homozygous mutants revealed unique single transition-type base pair changes consistent with EMS mutagenesis relative to the progenitor (Figure 2C). The amino acid change associated with the *rmr1–1* allele is predicted to prevent proper folding of the helicase domain [23], while the non-conservative amino acid substitutions associated with the *rmr1–2* and *rmr1–4* alleles occur at highly conserved residues in the SNF2_N profile (Figure 2D). The *rmr1–3* allele is associated with a nonsense mutation predicted to truncate the peptide before the conserved helicase domain. CAPS markers were designed to the potential *rmr1–1* and *rmr1–3* lesions and used to show that the base pair polymorphisms at each of the probable lesions invariably cosegregate with the mutant phenotype (see Materials and Methods). These results support these polymorphisms as bona fide molecular lesions in the *rmr1* gene. Based upon molecular genetic mapping data, DNA sequencing results, and the relevance of the fact that Snf2 proteins affect chromatin environments, we conclude the *rmr1* locus encodes a protein containing a Snf2 helicase domain.

*Os05g32610* gene models and our cDNA sequencing analysis (see Materials and Methods) indicate *rmr1* encodes a 1,435-amino-acid protein. In addition to having the conserved Snf2 helicase domain, the protein has a large N-terminal region with no significant identity to any known or predicted proteins. Phylogenetic comparison with other known Snf2 proteins in maize, rice, *Arabidopsis*, and budding yeast shows RMR1 is a member of a Rad54-like subfamily defined by DRD1 (Figure 3). *Arabidopsis* DRD1 is a putative chromatin remodeling factor affecting RNA-directed DNA methylation (RdDM) patterns [24–26]. In the emerging RdDM pathway model, DNA sequences are targeted for de novo cytosine methylation by complementary siRNA molecules generated from “aberrant” RNA transcripts. The putative MOP1 ortholog in *Arabidopsis*, RDR2, is required in this pathway to presumably generate double-stranded RNA from these transcripts and provide a substrate for siRNA biogenesis through activity of a Dicer-like enzyme [27]. DRD1 is thought to be a downstream effector protein that facilitates de novo methylation of targeted DNA sequences, possibly by modulating chromatin architecture to provide access to de novo methyltransferases [24–26,28]. The DRD1 subfamily also includes the recently identified CLSY1 protein implicated in the systemic spreading of siRNA-mediated silencing in *Arabidopsis* [29].
DRD1 subfamily can be divided into three distinct monophyletic groups, with RMR1, DRD1, and CLSY1 defining different groups (Figure 3). The presumed maize ortholog of DRD1 is likely one of two proteins in the DRD1 subgroup. Chromatin remodeling complex subunit R 127 (CHR127) (http://chromdb.org), a partial protein predicted from maize expressed sequence tag sequences, or CHR156, a full-length protein predicted from maize genomic sequence (see Materials and Methods). RMR1 is more similar to Arabidopsis proteins predicted from At1g05490 and At3g24340. RNA interference knockdowns of these putative Arabidopsis orthologs are known to have little to no effect in response to DNA damage [30].

Taking into account the phylogenetic analysis of the predicted coding sequence, it is possible RMR1 function may be similar to, but distinct from, that of DRD1 and CLSY1. The three proteins may fulfill a similar role in RdDM, but perhaps function under different conditions or in distinct genomic contexts. Alternatively, they could perform different roles within an RdDM pathway, or function in separate epigenetic mechanisms altogether. Given the results of our pl1 RNA expression analyses, it is possible that RMR1 represents a Snf2 protein that links chromatin organization to RNA transcript stability.

RMR1 Maintains Cytosine Methylation and Small RNA Accumulation at Pl1-Rhoades

In the described Arabidopsis RdDM pathway, DRD1 maintains cytosine methylation at nonsymmetrical CNN sequences represented by siRNAs [24–26]. Many endogenous genomic targets of DRD1 appear to be repetitive elements [31]. At Pl1-Rhoades there is a 402-bp terminal fragment of a CACTA-like type II DNA transposon, similar to doppia, 129 bp upstream of the translational start site [8,32,33]. Assuming analogous functional roles of RMR1 and DRD1 we compared DNA methylation patterns at this upstream repetitive element in rmr1 mutants and non-mutant siblings.

Previous restriction-enzyme-based comparisons of DNA methylation status between Pl-Rh and Pl' states found no differences, although few 5' proximal sites were evaluated [8]. Using Southern blot hybridization analysis following digestion of genomic DNA with methylation-sensitive restriction enzymes, we found that the doppia fragment is hypomethylated at specific sites in plants homozygous for the rmr1–1 mutation compared to heterozygous wild-type siblings (Figures 4A, 4B, and S3). Consistent with findings in Arabidopsis RdDM mutants [16,34–36], the sites hypomethylated in rmr1 mutants were of the CNN context. A relative hypomethylation pattern in 5' sequences is also present in plants homozygous for mutations at either rmr6 or mop1 (Figures S4 and S5). In rmr6 mutants the extent of hypomethylation was greater than that of either rmr1 or mop1 mutants and encompassed CG methylation sites as well as non-CG targets, suggesting Rmr6 has a broader effect in cytosine methylation maintenance. The presence of these methylation differences in multiple mutant backgrounds indicates that this hypomethylation pattern reflects the chromatin status at doppia in plants where maintenance of repressed paramutant states is compromised.

Consistent with the Arabidopsis RdDM model, small RNAs (~26 nt) with sequence similarity to the doppia element are detected in wild-type Pl' plants in both sense and antisense orientations (Figures 4D and S6). These small RNAs are undetectable in rmr1 mutants, unlike in wild-type siblings. This result contrasts those in Arabidopsis showing that DRD1 deficiencies do not affect the abundance of endogenous siRNAs representing repetitive elements [31]. However, it has been reported that the abundance of endogenous siRNA and trans-acting siRNA populations are highly reduced in CLSY1 mutants [29].

To test if the doppia fragment hypomethylation was indicative of genome-wide changes we assayed the cytosine methylation status at centromeres and 45S repeat sequences. Cytosine methylation patterns were unaffected in either of these regions in rmr1 mutants as compared to non-mutant siblings (Figure S7). Additionally, we examined the methylation status of doppia-like loci genome-wide (Figure 4E) and found no obvious differences between rmr1 mutants and non-mutant siblings. These results indicate that while RMR1 acts on the doppia sequence upstream of Pl1-Rhoades, doppia elements appear unaffected throughout the genome. This specificity of RMR1 function may be due to its intimate and exclusive involvement with alleles that undergo paramutation, or may be indicative of differential regulation of repetitive elements depending on their genomic and epigenetic context.
If RMR1 is involved in maintaining cytosine methylation patterns characteristic of repressed paramutant states then a prediction would be that the methylation differences seen between mutants and non-mutants would reflect the prediction would be that the methylation differences seen patterns characteristic of repressed paramutant states then a upstream doppia states (Figures 4C and S8). These results suggest that while the upstream doppia element of Pl1-Rhoades is a target of multiple factors involved in maintaining the epigenetic repression associated with paramutation, the actual process of paramutation does not result in similar changes of DNA methylation at this element.

RMR1 Is Not Required for Establishment of Paramutant States

Based on a reverse transcriptase PCR (RT-PCR) expression profile (Figure S9) rmr1 appears to be expressed in all rapidly dividing somatic tissues, consistent with a role in maintaining paramutant states throughout development. However, since the methylation patterns maintained by RMR1 appear unrelated to the paramutant state of Pl1-Rhoades, we questioned whether RMR1 is directly required for paramutation to occur. This process results in the invariable establishment of the P′ state in Pl/Pl-Rh plants, as evidenced by the observation that only Pl′/Pl′ progeny are found when Pl′/Pl-Rh plants are crossed to Pl-Rh/Pl-Rh testers [7,8]. If RMR1 were directly involved in this process we would expect that an rmr1 deficiency might interfere with the P′ establishment event. To test this, we tracked the behavior of individual Pl1-Rhoades alleles in test crosses to assess the ability of the P′ state to facilitate paramutations in Pl′/Pl-Rh; rmr1–1/rmr1–2 plants. The Pl1-Rhoades allele in a Pl-Rh state was genetically linked (~1.5 cM) to a T6–9 translocation breakpoint (T6–9). The T6–9 interchange can act as a dominant semi-sterility marker, allowing us to trace specific Pl1-Rhoades alleles through genetic crosses [11]. rmr1 mutants heterozygous for the T6–9 interchange (T6–9 Pl-Rh/Pl′) were crossed to a Pl-Rh/ Pl-Rh tester (Figure 5; Table S1). If establishment of the P′ state was prevented in rmr1 mutants, we would expect all progeny receiving the interchange to display a Pl′/Pl′-like phenotype (light anther pigmentation), indicating that paramutation was established in the rmr1 mutant parent. It should also be noted that Pl-Rh/Pl′ plants, and those of an intermediate phenotype of partial pigmentation [7], were present in both progeny inheriting the interchange and those inheriting a normal chromosome. These results are consistent with previous work showing P′ can revert to a Pl-Rh state in rmr1 mutants [10].

Corresponding analysis of the establishment of paramutant states at the bl locus generated similar results (Table S2). The repressed B′ state of the B1-Intense allele [37] was established in B′/B-Intense rmr1 mutants greater than 95% of the time. While it is possible that rmr1 defects affect establishment efficiency, it will be difficult to differentiate any such effects from its clear role in maintenance [11]. These results point to an interesting duality in RMR1 function in which the wild-type protein is necessary for meiotic heritability of repressed epigenetic states, but is not required to establish these states. This duality is marked by different results generated in the analysis of DRD1, which was shown to be necessary for the maintenance, establishment, and removal of repressive epigenetic marks [24,25].

Discussion

RMR1 is the first protein identified whose function acts to maintain trans-generationally repressed states associated with paramutation, a genetic behavior that affects meiotically heritable epigenetic variation through allelic interactions at endogenous loci. The identification of RMR1 as a Snf2 protein highlights an emerging role of these proteins in establishing and maintaining epigenetic marks. In Arabidopsis the Snf2 proteins DRD1 and DDM1 [38,39] are known to maintain cytosine methylation patterns. Lsh1, the mammalian protein most closely related to DDM1, is also required for normal DNA methylation patterns [40–42]. There are some 42 Snf2 proteins in Arabidopsis and at least as many in maize.

Figure 4. Cytosine Methylation Patterns and Small RNA Accumulation Are Altered at Pl1-Rhoades in rmr1 Mutants

(A) Schematic of Pl1-Rhoades locus with exons highlighted in black and the upstream doppia element represented by the gray arrow. The methylation context of sites cut by methylation-sensitive enzymes are shown in parentheses. Open circles denote sites hypomethylated in rmr1–1 mutants while filled circles are sites methylated in both wild-type and rmr1 mutants. BsrI restriction sites and the regions used to generate probes for blot hybridization analysis, denoted A and B, are also shown. (B and C) Representative Southern blots hybridized with probe A showing methylation status at a Bstul site in rmr1 mutants and heterozygous siblings (B), as well as Pl′ and Pl-Rh plants (C) with a larger 2.9-kb band (upper arrow) representative of a fully methylated BsrI fragment, and a 2.1-kb band (lower arrow) indicative of a hypomethylated Bstul site. Additional primary blots shown in Figures S3 and S8. (D) Small RNA northern blot probing with doppia sequence from probe B showing changes in amount of small RNAs between rmr1–1 plants and wild-type (WT) siblings. (E) Southern blot of genomic DNA digested with BstNI (“B” lanes) and methylation-sensitive PspGI (“P” lanes) hybridized with probe B, showing no bulk changes in doppia methylation genome-wide.

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Plants with a T6–9 translocation chromosome carrying \( \text{Pl} \) allele were crossed to state (dark anther pigmentation) and heterozygous for the interchange chromosome in the \( \text{S9} \) closely matches that of \( \text{mop1} \) phenotype, indicating that the proposed pathway, the \( \text{MOP1} \) as an RDR2 ortholog [14,15]. Consistent with this placement of \( \text{RMR1} \) in an RdDM pathway based on its helicase functional specialization amongst these proteins. We have (http://chromdb.org/). This diversity likely represents great functional specialization amongst these proteins. We have placed \( \text{RMR1} \) in an RdDM pathway based on its helicase domain similarity to \( \text{DRD1} \) and the recent identification of \( \text{MOP1} \) as an RDR2 ortholog [14,15]. Consistent with this proposed pathway, the \( \text{mrm1} \) mRNA expression profile (Figure S9) closely matches that of \( \text{mop1} \) [15]. Additionally, both \( \text{RMR1} \) and \( \text{MOP1} \) are necessary to maintain cytosine methylation patterns at silenced transgenes [43], the \( \text{Pl1-Rho} \) doppia sequences, and certain \( \text{Mutator} \) transposable elements ([15,44]; J. B. H. and D. Lisch, unpublished data).

\( \text{DRD1} \) is also known to target repetitive elements found in euchromatic contexts through an RdDM pathway [31]. However, the role \( \text{RMR1} \) plays to maintain the repressed paramutant states at \( \text{Pl1-Rho} \) appears different than the function of \( \text{DRD1} \) in the \( \text{Arabidopsis} \) RdDM pathway, as \( \text{RMR1} \) has, in addition to its requirement for CNN methylation at \( \text{doppia} \), a role in the normal accumulation of small RNAs with similarity to that element.

It is unclear how \( \text{RMR1} \) mediates the post-transcriptional regulation of \( \text{pl1} \) transcripts as suggested by the in vitro transcription and RNase protection assays reported here. It is possible that \( \text{pl1} \) transcripts resulting from \( \text{Pl1-Rho} \) state are less stable than those produced from the \( \text{Pl-Rh} \) state because of differences in the chromatin environment of \( \text{Pl1-Rho} \). However, there do not appear to be any \( \text{Pl} \)-specific small RNAs produced from the \( \text{pl1} \) coding region [12]. In \( S. \text{pombe} \) it has been shown that the chromatin environment of a locus can affect RNA transcript levels without altering RNA polymerase II occupancy of that locus, leading to the proposal of a cotranscriptional gene silencing mechanism whereby nascent transcripts initiating in a heterochromatic environment are degraded by complexes targeted via heterochromatic small RNAs [17,18]. Chromatin differences in the upstream region of \( \text{Pl1-Rho} \) may favor recruitment of alternative RNA-processing factors or RNA polymerases, which in turn influence the stability of \( \text{pl1} \) transcripts. In plants, localization of the large subunit 1a of RNA polymerase IV to loci targeted for RdDM appears necessary for the biogenesis of siRNAs from these loci [28]. When \( \text{Pl} \) repression is disrupted in \( \text{rmr1} \) mutants, this alternate generation or processing of the \( \text{pl1} \) transcript may also be lost. Alternatively, our results may highlight a novel role for \( \text{RMR1} \)-like Snf2 proteins in directly interacting with nascent RNA transcripts via a helicase domain, or in recruiting factors that directly destabilize these transcripts.

Importantly, our analysis of \( \text{rmr1} \) mutants calls into question the relationship between \( \text{RMR1} \) function and the mechanism of paramutation at \( \text{Pl1-Rho} \). The mutational screens identifying \( \text{rmr1}, \text{rmr6}, \) and \( \text{mop1} \) were designed to discover genetic components necessary to maintain the repressed state of \( \text{Pl} \), not necessarily factors needed to establish this repressed state [10,13]. Therefore, it is possible that loci thus far identified may be indirectly related to the paramutation mechanism. Our results are consistent with a model wherein \( \text{RMR1} \) functions in an RdDM pathway, along with an RDR2-like enzyme, \( \text{MOP1} \), to maintain a persistent heterochromatic-like chromatin structure at the repetitive element found directly upstream of the \( \text{pl1} \) coding region. While it is not clear where \( \text{RMR1} \) acts in this pathway it presumably acts coordinately with the maize orthologs of known RdDM components identified in \( \text{Arabidopsis} \), namely \( \text{DCL3} \) [16,45], the \( \text{DRM} \) methyltransferases [36], \( \text{AGO4} \) [46,47], the RNA polymerase IV subunits, and the maize \( \text{DRD1} \) ortholog (Figure 6A). In this model, \( \text{doppia} \) transcripts, perhaps because of the repetitive nature of the \( \text{doppia} \) genomic elements and/or the numerous internal subterminal repeats that are present in these elements [32,48], are the source of aberrant RNA that is processed via \( \text{MOP1} \) and a \( \text{DCL3} \) enzyme into siRNAs. This small RNA production is carried out in a manner that is dependent on \( \text{RMR1} \) activity, possibly via direct interaction with a small RNA processing complex or by making the DNA accessible to factors necessary for siRNA precursor generation such as polymerase IVa. These siRNAs, through the activity of \( \text{AGO4} \), \( \text{DRM} \) enzymes, and polymerase IVb, then establish a heterochromatic state at the \( \text{Pl1-Rho} \) doppia-like element that is present in both \( \text{Pl-Rh} \) and \( \text{Pl} \) states. The methylation effects seen in \( \text{rmr1} \) mutants might indicate that this heterochromatization machinery depends on the activity of \( \text{RMR1} \) to feedback on the \( \text{doppia} \) element, or loss of \( \text{RMR1} \) may short circuit this pathway and thus affect methylation activity indirectly. An \( \text{RMR1} \) defect then affects stability of paramutant states at \( \text{pl1} \) because of the chromatin context of the \( \text{Pl1-Rho} \) allele, and not through direct disruption of components required.
for paramutations to occur. This is in line with a report that MOP1-dependent small RNAs produced at the b1 locus are insufficient to mediate paramutation [49].

The relationship between RMR1 action, the chromatin organization of Pl1-Rhoades, and the repressed Pl9 state is not clearly understood at this time. It is possible that derepression of the upstream repetitive element makes the region more accessible to general transcription factors whose actions could destabilize repressive Pl9 chromatin states that are independent of those maintained at doppia (Figure 6A). Indeed, RNA polymerase processivity can lead to changes in the chromatin environment through histone modifications or histone replacement [50,51]. Alternatively, Pl9 chromatin states may represent a spreading of the heterochromatic domain into a euchromatic region defined by the Pl1-Rhoades gene space (Figure 6B). In fission yeast, heterochromatic domains nucleated by small RNAs have the ability to spread in cis through successive H3 K9 methylation [52]. In this situation, loss of RMR1 function would alleviate Pl9 repression by disrupting maintenance of this expanded heterochromatic domain. In either of these situations RMR1 affects Pl1-Rhoades paramutations by virtue of its role in maintaining heterochromatic states at a proximal repetitive element.

McClintock was the first to describe derivative alleles in which transposons acted to control the expression patterns of attendant genes [53]. It is now clear that epigenetic modulations of the transposons themselves—what McClintock referred to as “changes in state”—can alter the regulatory properties of individual genes both somatically [54] and trans-generationally [55,56]. Our results indicate that even transient changes in state of the Pl1-Rhoades doppia fragment can have trans-generational effects on pl1 gene expression patterns. These experimental examples, in the context of McClintock’s thesis [53], point to a dynamic source of regulatory, and potentially adaptive, variation adjunct to the DNA itself. Precisely how this epi-variation relates to existing genome structure and function, as well as its evolutionary potential, remains a largely unexplored area of investigation.

Currently, well-characterized examples of paramutation are limited to loci where expression states have a clear phenotypic read-out, such as pigment synthesis. cis-Elements required to facilitate paramutation have been functionally...
identified at specific alleles of b1 and colored1 (rl) [57–59]. To date, there is no evidence that the chromatin status of these cis-elements is affected by mutations at trans-acting loci required for maintenance of repressed paramutant states. It appears that paramutations represent a type of emergent system wherein genomic context and maintenance of chromatin states interact to facilitate meiotically heritable epigenetic variation. In this view, it is possible that cis- and trans-elements necessary for maintenance of such variation might not interact in a direct and predictable manner. What remains to be seen is the extent to which this type of system acts throughout the genome. Genome-wide screens for paramutation-like behavior, in which expression states are affected by allele history, remain technologically and conceptually challenging. Recent work by Kasschau et al. [60] suggests that in Arabidopsis, few endogenous genes are regulated by proximal presumed RdDM targets. However, it is tempting to speculate that examples of paramutation represent an exception to this trend, representing a mechanism by which populations can quickly, and heritably, change their transcriptome profile and regulation.

Materials and Methods

Scoring of the PlI-Rhoades allele expression state and rmr mutants. Plants were scored as carrying Pl-Irh or Pl’ states through visual inspection of anther pigmentation and assignment of an anther color score as previously described [7]. Pl’Pl’ (anther color score 1 to 4) anthers show little to no pigmentation while Pl-Rh/Pi-Rh anthers show red to purple. Mutants were scored in the same manner, with rmp and mop mutants showing a Pl-Rh/Pi-Rh-like phenotype, except in the case of the F2 rmr mapping populations, in which mutants were chosen on the basis of a dark seedling leaf phenotype [10].

Genetic stocks. Elite inbred lines (B73, A619, and A632) were provided by the North Central Regional Plant Introduction Station (http://www.ars.usda.gov/main/site_main.htm?modecode=36-25-12-00). Color-converted versions of A619 and A632 inbred lines were created by introgressing the Pl-I-Rhodease allele into each [11]. The lmr-1, lmr-2, mop-1, and rmr-6-1 alleles have been previously described [8,10,13]. The lmr-1-7 allele was derived from identical materials used to isolate lmr-1 and lmr-2; lmr-1-4 was derived from EMS-treated pollen from an A619 color-converted line applied to a color-converted A632 line [11] (see Protocol S1 and Table S3 for complementation tests). The T6-9 translocation line carrying the Pl-I-Rhodease alleles used in Pl’ establishment tests has been described previously [11].

plI expression analyses. In vitro transcription assays (rmr-1 and rmr-1-3; Figures 1 and S1) and RNase protection assays (rmr-1 only; Figure 3) were carried out as described [8] with husk nuclei and RNA isolated from single ears of the same genetic stocks used to measure plI RNA differences in rmr-1 alleles [10]. The b1 and plI genotypes of these plants are as follows: B1-Intense (B-I)/B-I; Pl-I-Rhodease (Pl’)/Rmr1Pl’Pl’ rmr-1 and B-I/B-I; Pl’/Pl’ rmr-1-1Pl’/Pl’ rmr-1, or B-I/B-I; Pl’/Pl’ rmr-1Pl’/Pl’ rmr-1-3 and B-I/B-I; Pl’/Pl’ rmr-1-3Pl’/Pl’ rmr-1-3. Identical procedures were applied to single ears from plants homozygous for Pl’ and either homozygous or heterozygous for rmr-1-3 following a single backcross into the KYS inbred line [12]. Additional details regarding stock synthesis are available upon request.

Genome range of rmr1. A F2 mapping population was created from inbred (SO) rmr1-1/rmr1-1, Pl’Pl’, and color-converted A632 inbred (Pl’Pl’Pl’ > 93% A632) parents. DNA was isolated using the DNeasy 96 kit (Qiagen, http://www.qiagen.com) from F2 mutant seedlings, mapping parents, and F1 hybrid leaf tissue. These DNA samples were screened with SSLP markers developed from the Maize Mapping Project (http://www.maizegdb.org; US National Science Foundation award number 9827655; primer sequences and protocol available at http://maizegdb.org). Initial marker choice was restricted to Chromosomes 6 and 9 because of linkage of rmr1 to a T6-9 breakpoint. In addition to the rmr1-1 mapping population, a second mapping population created with inbred (SO) rmr1-1/rmr1-1, Pl’Pl’Pl’Pl’/Pl’Pl’Pl’Pl’; Pl’Pl’Pl’Pl’ and color-converted A632 parents showed similar cosegregation with marker bngl1174a (78 chromosomes tested; <0.56 Cm). CAPS [61] markers were designed to test cosegregation of the rmr1-1 and rmr1-3-associated lesions with the rmr1 mutant phenotype (see Protocol S1 for details). No recombinant chromosomes (876 chromosomes tested for rmr1-1, 268 chromosomes tested for rmr1-3) were found using either marker.

Candidate gene selection and sequencing. A BLAST search using the rice Os05g32610 ORF as a query identified maize GSS and sorghum expressed sequence tag sequences that were used to generate a contig representing the putative maize gene (see Protocol S1 for sequence identifiers). Oligonucleotide primers (Sigma-Genosys, http://www.sigmachrlik.com/Brands/Sigma_Genosys.html) were designed from these sequences and used in PCR amplification of genomic DNA from three separate individuals homozygous for each rmr1 mutant allele as well as functional reference alleles (rmr1-B73, Rmr1-A632, and Rmr1-A619). PCR amplicons were purified using QiAquick gel extraction kit (Qiagen) and dideoxy sequenced (UC Berkeley DNA Sequencing Facility, http://mcb.berkeley.edu/barker/). To verify the intron/exon structure of rmr1, DNA was generated from rmr1-1 mutants as well as non-mutant B73 plants as described [15], and rmr1 was amplified via RT-PCR. The resulting products, which were the predicted size for spliced rmr1 transcript, were sequenced to validate the intron/exon structure shown in Figure 2C. See Protocol S1 and Table S4 for all oligonucleotide primer sequences used.

Phylogenetic analysis. Sequencing reads from genomic and cDNA were aligned and edited with Sequencer (Gene Codes, http://www.genecodes.com) to create a contig representing rmr1. The N-terminal prediction is based on alignment of RMR1 with the protein model for Os05g32610. A search of the Pfam database (http://www.sanger.ac.uk/Software/Pfam) with the predicted RMR1 protein sequence was used to identify the conserved SNF2_N and Helicase_C protein profiles of the Snf2 helicase domain. MUSCLE [62] was used to generate an alignment between RMR1 and proteins from maize (CHR127 and CHR156), and budding yeast over the helicase domain (Figure S2). Sequences for CHR127 and CHR156 were retrieved from ChromDB (http://www.chromdb.org/). Additional sequence information for CHR156 was identified from BAC CHZ01-317 (GenBank accession AC194602), and gene model prediction was performed using FGENESH+ (Softberry, http://www.softberry.com) with RMR1 as similar protein support. A distance tree was created and bootstrap values were calculated using PAUP* 4.0 from the above alignment (Simionov Associate, Inc).

Small RNA northern blots. Small RNAs were prepared from 10-mm immature ear tissue and used to generate small RNA northern blots as previously described [63]. In Figure 4D the small RNAs were run with a 27-bp DNA oligonucleotide containing doppia sequence that hybridized with the riboprobe used to identify the small RNAs. The riboprobe was synthesized as described [63] from a plasmid containing the region denoted probe B in Figure 4A linearized at an Asel site so as to contain only doppia sequence.

Pl’ establishment tests. Establishment of the Pl’ state in rmr1 mutants was assayed essentially as described previously [11]. When the T6-9 interexchange pair is heterozygous with structurally normal chromosomes, the plants display <50% pollen sterility due to meiotic-segregation-induced aneuploidy in the resulting gametes. Pollen sterility was assayed in the field using a pocket microscope. rmr1 mutants were crossed to Pl-Rh/Pi-Rh A619 or A632 inbreds (Table S1), and the resultant progeny were scored with respect to Pl-I-Rhodease expression state.

Supporting Information

Dataset S1. Sequence Information for rmr1 Progenitor and Mutant Alleles

Found at doi:10.1371/journal.pone.0050275.s001 (42 KB PDF).

Figure S1. rmr1 Does Not Affect PlI-Rhodease Transcription Rates

In vitro assays with isolated husk nuclei show no differences in plI transcription rates between rmr1 mutants and non-mutant heterozygotes.

(A) In vitro radiolabeled RNAs corresponding to specific genes from
isolated husk nuclei of sibling plants detected with slot blot hybridizations (pBS, bacterial plasmid DNA; pl1, purple plant1; b1, colored plant1; a1, anthocyaninless1; uq, ubiquitin2).

(B) Quantification of relative mean transcription rates from five independent sets of rnr1–1 (open) and rnr1–3 (closed) siblings (+ standard error of the mean) showing no significant difference between Pfl transcription rates.

(C) In vitro radiolabeled RNAs from isolated husk nuclei of rnr1–3 mutants and heterozygous siblings used to generate quantification in Figure 1A.

Found at doi:10.1371/journal.pbio.0050275.sg001 (708 KB TIF).

Figure S2. RMR1 Is Structurally Related to Other Snf2 Proteins

Multiple species alignment of RMR1 helicase domain with other known and predicted Snf2 proteins.

Found at doi:10.1371/journal.pbio.0050275.sg002 (101 KB TIF).

Figure S3. rnr1 Affects DNA Methylation Patterns at Pl1-Rhoades

Additional Southern blots comparing the DNA methylation status of the Pl1-Rhoades upstream region in rnr1–1 mutants and heterozygous siblings.

(A) Genomic digests of an rnr1–1 mutant (−) and heterozygous sibling (+) using methylation-sensitive restriction enzymes in concert with BstI, hybridized with probe A (Figure 4A). These results were used to generate the methylation profile shown in Figure 4A.

(B) Blot hybridized with probe A comparing rnr1–1 mutants to heterozygous siblings with respect to methylation at a PspGI site.

Found at doi:10.1371/journal.pbio.0050275.sg003 (2.4 MB TIF).

Figure S4. rnr6 Affects DNA Methylation Patterns at Pl1-Rhoades

Southern blots comparing the DNA methylation status of the Pl1-Rhoades upstream region in rnr6–1 mutants and heterozygous siblings.

(A) Methylation profile similar to that shown in Figure 4A showing sites at the Pl1-Rhoades locus hypomethylated (open circle) in a rnr6–1 mutant as compared to heterozygous siblings.

(B) and (C) Blots shown in (B) and (C) were used to generate this profile and are analogous to the blot shown for rnr6 mutants in Figure S3A and S3B, respectively.

Found at doi:10.1371/journal.pbio.0050275.sg004 (2.2 MB TIF).

Figure S5. mop1 Affects DNA Methylation Patterns at Pl1-Rhoades

Southern blots comparing the DNA methylation status of the Pl1-Rhoades upstream region in mop1–1 mutants and heterozygous siblings.

(A) Methylation profile similar to that shown in Figure 4A showing sites at the Pl1-Rhoades locus hypomethylated (open circle) in a mop1–1 mutant as compared to heterozygous siblings.

(B) and (C) Blots shown in (B) and (C) were used to generate this profile and are analogous to the blots shown for mop1 mutants in Figure S3A and S3B, respectively.

Found at doi:10.1371/journal.pbio.0050275.sg005 (2.2 MB TIF).

Figure S6. rnr1 Affects Abundance of doppia Small RNAs

Additional small RNA northern blots showing that doppia small RNAs of both sense and antisense orientations are absent in rnr1 mutants. Small RNA northern blots were probed with probe B (Figure 4A) in both the sense (A) and antisense (B) orientations, showing small RNAs (~26 nt) with doppia sequence similarity are present in sense and antisense orientation in rnr1–1 heterozygotes (+) and are lost in rnr1–1 mutants. DNA oligonucleotides (22 and 21 nt) used as sizing standards are also shown.

Found at doi:10.1371/journal.pbio.0050275.sg006 (1.3 MB TIF).

Figure S7. Mutations at rnr1 Do Not Affect Genome-Wide Methylation Levels

Southern blots show that rnr1 mutations do not affect methylation levels at centromeric sequences and 45S ribosomal DNA repeats. Genomic DNA from four rnr1–1 mutants and non-mutant siblings digested with BstNI (“B” lanes) and a CNG methylation-sensitive enzyme PspGI (“C” lanes), which has the same recognition site, probed with (A) radiolabeled centromere sequence and (B) 45S repeat sequence. The comparison between the PspGI digests in mutant and non-mutant individuals reveals no gross methylation differences.

Found at doi:10.1371/journal.pbio.0050275.sg007 (6.2 MB TIF).

Figure S8. Pl-Rh and Pl’ States Have Identical DNA Methylation Patterns

Additional Southern blots show no changes in Pl-Rhoades methylation status between the Pl-Rh and Pl’ states. (A) The methylation status of upstream PspGI sites is compared for Pl’/Pl’ and Pl-Rh/Pl-Rh plants with the Pl1-Rhoades allele introgressed (>98%) into distinct A619 and A632 backgrounds via hybridization with probe A. The blot reveals no methylation differences at this site between the two Pl-Rhoades regulatory states. The “C” lanes indicate control lanes where the digest was carried out with BstNI, a methylation-insensitive restriction enzyme.

Found at doi:10.1371/journal.pbio.0050275.sg008 (1.9 MB TIF).

Figure S9. rnr1 Is Expressed in Rapidly Dividing Tissues

RT-PCR expression profile shows rnr1 is expressed primarily in tissues with high mitotic index. Tissue samples represented in the analysis include seedling leaf, adult leaf, shoot apical meristem, immature tassel, and immature ear. RT-PCR was carried out using primers that span the first and second introns of rnr1.

Found at doi:10.1371/journal.pbio.0050275.sg009 (358 KB TIF).

Protocol S1. Additional Methods Used to Generate Supporting Pieces of Data

Found at doi:10.1371/journal.pbio.0050275.sd002 (47 KB DOC).

Table S1. rnr1 Is Not Required to Establish pl1 Paramutation

Test cross results measuring acquisition of paramutation by Pl-Rh in T Pfl-Rh rnr1–2/2 pl1 rnr1–1 plants. Genetic assay used for this experiment is detailed in Results and Figure 5. Table details individual anther phenotypes using a 1–7 graded anther color score for specific test cross progeny. Progeny structural genotypes refer to the presence or absence of the reference T6–9 interchange chromosome.

Found at doi:10.1371/journal.pbio.0050275.st001 (127 KB DOC).

Table S2. rnr1 Is Not Required to Establish b1 Paramutation

Table details individual progeny plant phenotypes from crosses of rnr1 Lmr1; B-1B’ plants to Rml1 b1 testers. Two different mutant rnr1 alleles are assayed. The B-I and B’-plant phenotypes represent darkly pigmented and light or variegated pigment, respectively. With four exceptions, 206 test cross progeny had a B’ phenotype.

Found at doi:10.1371/journal.pbio.0050275.st002 (58 KB DOC).

Table S3. Two New rnr Mutations Are Alleles of rnr1

Table details individual progeny anther phenotypes graded using a 1–7 anther color score from crosses designed to test genetic complementation of various rnr mutations.

Found at doi:10.1371/journal.pbio.0050275.st003 (70 KB DOC).

Table S4. Oligonucleotides Used in This Work

Found at doi:10.1371/journal.pbio.0050275.st004 (45 KB DOC).

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