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Paramutation: a process for acquiring *trans*-generational regulatory states

Karl F Erhard Jr and Jay B Hollick

Basic tenets of Mendelian inheritance are violated by paramutations in which *trans*-homolog interactions lead to heritable changes in gene regulation and phenotype. First described in plants, similar behaviors have now been noted in diverse eukaryotes. Genetic and molecular studies of paramutations occurring in maize indicate that components of a small interfering RNA (siRNA) biogenesis pathway are required for the maintenance of meiotically heritable regulatory states. Although these findings lead to a hypothesis that siRNAs themselves mediate paramutation interactions, an assessment of existing data supports the opinion that siRNAs alone are insufficient. Recent evidence implies that transcription of paramutation-associated repeats and siRNA-facilitated chromatin changes at affected loci are involved in directing and maintaining the heritable changes in gene regulation that typify paramutations.

Address
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, United States

Corresponding author: Hollick, Jay B (hollick@berkeley.edu)

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Introduction

Paramutations have been best characterized in *Zea mays* at specific alleles of the *red1 (r1)*, *booster1 (b1)*, *purple plant1 (pl1)* and *pericarp color1 (p1)* loci, all of which encode pigment regulators [1]. In all examples described to date [2], the expression of an allele inherited in a paramutable state (Box 1) is repressed when combined in a heterozygote with a partner allele inherited in a paramutagenic state (Box 1). The altered regulatory state of a newly repressed allele is meiotically heritable, and is transmitted in a paramutagenic state (Figure 1a). The mechanism responsible for acquiring (Figure 1a) and maintaining (Figure 1c) these *trans*-generationally stable regulatory states is not fully understood. Studies in both maize [3] and mice [4] implicate an RNA-based mechanism for transferring regulatory information between alleles, leading to the speculation that some aspects of paramutation are conserved across the eukarya [5]. Mutational analyses in maize indicate that paramutations are affected by components of a small interfering RNA (siRNA) biogenesis pathway (Figure 2). These findings raise the possibility that paramutation represents an 'extreme manifestation' of an RNA interference (RNAi)-type pathway [6].

A myriad of small RNA-based regulatory systems have now been described across the kingdoms of life [7]. Small RNAs can program the epigenome of gametes in both Drosophila ovaries [8••] and *Arabidopsis* pollen [9••], implicating a role for some siRNAs in transmitting epigenetic information across generations. In plants, the majority of non-symmetrical cytosine methylation patterns are maintained through the action of 24 nucleotide (nt) siRNAs generated from repetitive sequences by alternative RNA polymerase (RNAP) complexes (Figure 2) [3]. Recent genetic and molecular studies in maize indicate that both the largest [10] and second largest [11•,12] subunits (RPD1 and RPD2a, respectively) of RNA Polymerase IV (Pol IV) (Figure 2) affect paramutation-based repression [13], siRNA biogenesis [10,11•,12], cytosine methylation patterns [14] and transposon regulation [15••].

Many mechanistic features of paramutation remain unresolved, such as its developmental timing, the epigenetic feature(s) that defines heritable paramutagenic states (Box 1), and the molecular roles that *trans*-acting factors play in affecting either the acquisition (Figure 1a) and/or maintenance (Figure 1c) of paramutagenic states. This review highlights recent studies of the paramutation mechanism and argues the opinion that, while siRNAs influence paramutation behaviors, these same siRNAs are insufficient to account for paramutation interactions occurring at defined maize loci.

Mechanistic link between paramutation and siRNAs

Mutational analyses indicate that molecules responsible for producing or stabilizing 24nt siRNAs (Figure 2) are required to either facilitate and/or maintain paramutations [10,11•,12,14,16]. These findings lead to hypotheses in which siRNAs mediate *trans*-homolog interactions as diffusible molecules with the potential to transfer regulatory information between alleles [17]. However, the exact role siRNAs play in paramutation is still unclear. Searches for potential siRNA signatures of paramutation have focused on the functionally important *cis*-linked

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Box 1 Paramutation Glossary

Paramutable: A state of gene regulation that can be heritably changed either spontaneously or through *trans*-homolog interactions

Paramutagenic: Possessing the ability to facilitate heritable changes of gene regulation *in trans*

Spontaneous Paramutation: Paramutable states in maize are unstable, and can change spontaneously to paramutagenic states; these changes can occur either somatically or germinally [36] and are transmitted through meiosis

Facilitated or Induced Paramutation: When combined with a paramutagenic partner, paramutable states are invariably transmitted from such heterozygotes in a meiotically heritable paramutagenic state

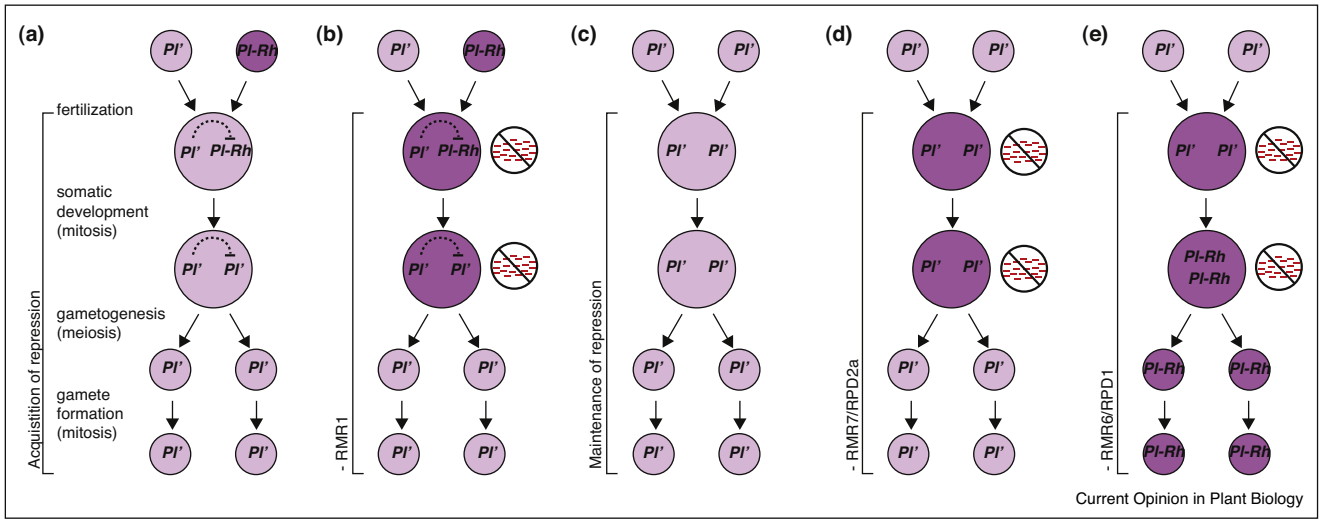
Reversion: A form of paramutation in which a paramutagenic state reacquires a non-paramutagenic form. For example, the repressed *Pl′* state of the *Pl1-Rh* allele can revert to a highly expressed, meiotically heritable *Pl-Rh* state after transmission through *rmr* homozygous mutants or if it is transmitted from either a hemizygous condition or heterozygous condition with certain other *pl1* alleles

***Trans*-repression**: The repression of gene expression from a paramutable state in sporophytes dictated *in trans* by a paramutagenic partner. Loss of *trans*-repression can occur in *rmr* and *mop* homozygous mutants.

repeat sequences located approximately 100 kb 5′ of the *B1-I* allele (upstream repeats) (Table 1). Recently, Arteaga-Vasquez *et al.* found no difference in siRNA profiles from *B1-I* alleles in either the *B′* (paramutagenic) or the *B-I* (paramutable) regulatory states using both small RNA deep sequencing and Northern blots [18•]. However, overexpression of a transgenic hairpin construct designed to produce upstream repeat-like siRNAs does appear to facilitate paramutation (Box 1) of a naïve *B-I* allele [18•]. These two results indicate that if siRNAs themselves do facilitate *b1* paramutation, then either tissue-specificity and/or a threshold level of siRNA production from the upstream repeats are probably important to their function. Tissue-specific profiles of both siRNAs and the molecular changes they facilitate at affected loci may be needed to implicate specific siRNA functionality in paramutation. As the heritable regulatory changes associated with paramutation are functionally tied to meiosis, or a process tightly linked to meiosis (see discussion of this point below), tissues enriched for inflorescence meristems, gametogenic cells and haploid gametes will be relevant to assay.

Mutant analyses described to date do not appear to support the hypothesis that siRNAs are required for all paramutation behaviors. *Pl′* states are always transmitted from *Pl′*/*Pl′* plants that are deficient for RPD2a [11•] (Figure 1d) yet mostly *Pl-Rh* states are transmitted from *Pl′*/*Pl′* plants lacking RPD1 [10,13] (Figure 1e). This contrasting behavior is especially curious, as both RNAP molecules are required for the majority of 24nt siRNA accumulation

Figure 1



Acquisition and maintenance, or lack thereof, of repressed *Pl′* states in wild-type and *required to maintain repression (rmr)* loss-of-function genetic backgrounds. **(a)** Acquisition of repression of a *Pl1-Rhoades* (*Pl1-Rh*) allele in a *Pl-Rh* state. Dark purple color represents strong expression conditioned by the *Pl-Rh* state, light purple color represents weak expression normally conditioned by the *Pl′* state in a wild-type background. Small top and bottom circles represent haploid nuclei, larger circles in between represent diploid nuclei. Dotted line from *Pl′* to *Pl-Rh* represents somatic *trans*-repression of *Pl-Rh* by *Pl′*. The exact timing of the transition from *Pl-Rh* to *Pl′*, here represented in a somatic diploid nucleus for convenience, is not known. **(b)** Alleles inherited in a *Pl-Rh* state can acquire the repressed *Pl′* state [as in **(a)**] in an *rmr1* mutant background, though maintenance of *Pl′* repression is lost in the sporophyte. Red dashes outside of nucleus represent small interfering RNAs (siRNAs), the majority of which are lost in *rmr1* homozygous mutants. **(c)** Maintenance of *Pl′* repression is necessary across both mitotic and meiotic cell divisions. **(d)** *Pl′* alleles are sexually transmitted in a *Pl′* state after exposure to *rmr7*/*rpd2a* homozygous mutant backgrounds for one generation, though maintenance of *Pl′* repression is lost in the sporophyte. **(e)** *Pl′* alleles are most often sexually transmitted in a *Pl-Rh* state after exposure to *rmr6*/*rpd1* homozygous mutant backgrounds for one generation. The exact timing of this reversion event, here represented in a somatic diploid nucleus for convenience, is not known.

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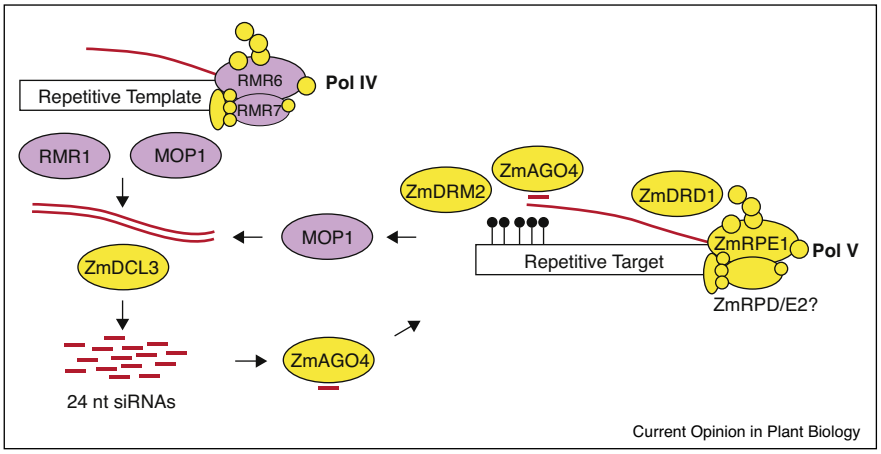
Figure 2



Model for an RNA-dependent DNA methylation pathway in maize. Purple symbols represent bona fide maize components and yellow symbols represent orthologs of *Arabidopsis* components known to exist in the maize genome (unpublished JBH, KFE). Presumed transcription of repetitive templates by RNA polymerase IV (Pol IV), possibly facilitated by the ATPase function of RMR1, generates single-stranded RNAs that are recognized by the putative RNA dependent RNA polymerase MOP1. MOP1 probably synthesizes double-stranded RNAs (dsRNAs), which are cleaved into 24 nucleotide (nt) small interfering RNAs (siRNAs) by a Dicer-like ribonuclease (ZmDCL3). siRNAs are loaded onto an Argonaute4 (ZmAGO4) protein, and guided to homologous loci in the genome by non-coding scaffold RNAs produced by Pol V, facilitated by the Snf2-like protein DRD1. The Domains Rearranged Methyltransferase2 (ZmDRM2) is recruited by an unknown mechanism to RdDM targets, presumably via interaction with AGO4. MOP1 could potentially use siRNAs or Argonaute4-sliced RNAs as a primer for multiple rounds of dsRNA production, generating a Pol IV-independent positive feedback loop to maintain a threshold level of siRNAs.

[10,11•,12]. Additionally, paramutation can be facilitated (Box 1) at naïve paramutable *Pl-Rh* alleles in the absence of RMR1-dependent siRNAs [14] (Figure 1b). Plants with *B′*/*B′*; +/*mop2-1* genotypes have weakly pigmented *B′* phenotypes even though RPD2a-dependent siRNAs are depleted in these individuals [12]. However, neither *pl1* or *b1* paramutation can be acquired in *rmr6*/*rpd1* [13] or *mop1*/*rdr2* mutants [19]. In total, these studies indicate that siRNA action alone is insufficient for certain aspects of paramutation, such as acquisition of paramutagenic *Pl′*

Table 1

Functional features associated with paramutation

Locus	Allele	*cis* Regulatory Sequence	Function in paramutation	Molecular function	Refs
red1 (r1)	*R-r:standard (R-r:std)*	Promoter region (σ) that drives expression from two *r1* genes in tail-to-tail orientation	Deletion of σ attenuates paramutability of *R-r:std* haplotype derivatives	Hyper-methylation near transcription start sites associated with paramutation	[27]
r1	*R-stippled (R-st)*	Repeated *r1* genes within the *R-st* haplotype	Number of *r1* gene repeats within the *R-st* haplotype correlates with paramutagenic strength	Hyper-methylation near transcription start sites associated with paramutation	[26,35]
r1	*R-marbled (R-mb)*	Repeated *r1* genes within the *R-mb* haplotype	Number of *r1* gene repeats within the *R-mb* haplotype correlates with paramutagenic strength	Hyper-methylation near transcription start sites associated with paramutation	[28,35]
booster1 (b1)	*B1-Intense (B1-I)* (B-I *and B′* states)	Seven direct tandem repeats of 853 bp ~100 kb 5′ of the *b1* coding region	Serves as a long-range enhancer element and is required for *b1* paramutation. Ability to facilitate paramutation correlated with the number of repeats	- Physically interacts with *b1* transcription start site in a tissue-specific manner - Repressive chromatin marks at repeats are associated with repressed *B′* states	[29,34•,43]
pericarp color1 (p1)	*P1-rr* (*B-I* and *B′* states)	Direct tandem repeat sequences 5′ of the *p1* coding region	Serves as an enhancer element and is required for *p1* paramutation	Hyper-methylation of endogenous repeat sequences are associated with a paramutant *P1-rr′* state	[30]

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states (Box 1) and maintenance of repressed *B′* and *Pl′* states in the sporophyte. It is possible that RMR1-dependent siRNAs are immaterial for paramutation or that RMR6/RPD1 and MOP1/RDR2 function, separate from their roles in siRNA biogenesis, are required to facilitate the acquisition of a meiotically heritable paramutagenic state. It is also possible that only specific components affect transcription, rather than siRNA biogenesis, of paramutation loci, leading to heritable de-repression.

Small RNAs can also act in a non-cell autonomous manner and there are now many examples in which small RNAs generated from companion cells or nuclei are able to target sequences in adjacent cell types or nuclei [8••,9••,20,21•,22•,23••]. If paramutagenicity were dependent on siRNAs per se, gametophytes harboring a particular siRNA-producing locus would produce respective sperm and eggs containing these siRNAs. However, non-equivalent sperm cells can be produced in a single pollen grain as a result of chromosome non-disjunction events and, in those cases that have been examined, only the sperm cell receiving the paramutagenic locus can generate plants in which paramutation events continue [24]. This result indicates that any germline transmitted siRNAs are insufficient to facilitate paramutation in the next generation.

Paramutation and transcriptional control of repetitive sequences

The largest subunit of Pol IV, RPD1, is required for maintenance of transcriptionally repressed paramutant states of the *Pl1-Rh* and *B1-I* alleles [10,13], yet the mechanism by which this repression occurs is still unclear. Expression analyses of Long Terminal Repeat (LTR) retrotransposons in *rmr1*, *mop1*/*rdr2* and *rmr6*/*rpd1* mutants indicate that RPD1 represses LTR-type sequences by competing with RPB1 (Pol II largest subunit) for template recruitment and/or Pol II holoenzyme assembly at these sites, and can do so in the absence of RMR1 and MOP1 function [15••]. The RNAP competition model proposed by Hale *et al.* [15••] might account for the different developmental phenotypes observed between *rmr6*/*rpd1* and *rmr7*/*mop2*/*rpd2a* mutants [10,11•,15••,25]. In addition, RPD2a, one of three second-largest polymerase IV-type subunits encoded by the maize genome [11•,12], is required for siRNA accumulation, but is not required for potential RPD1 interference with Pol II [11•]. These findings indicate that either RPD1 plays a role in repressing repetitive sequences separate from its role in siRNA biogenesis as a subunit of Pol IV, or that there are diverse Pol IV complexes in maize that use different RPD2-like subunits, perhaps in a tissue-specific manner. Differential RNA expression patterns of the three *rpd2*-encoding genes comport with such a model [12].

Repetitive sequences are genomic targets of an RNA-dependent DNA methylation (RdDM)-type machinery (Figure 2) that maintains transcriptional repression of paramutant states [3]. Perhaps not surprisingly, the *cis*-acting sequences functionally required for paramutation at the *r1*, *b1* and *p1* loci [26–30] are repeated sequences (Table 1). In all three examples, the *cis*-acting sequences act as transcriptional regulatory elements, indicating that transcription of paramutagenic states is important to the mechanism responsible for paramutation interactions. In *Arabidopsis*, transcription of intergenic regions by Pol V [31•] and Pol II [32] produce scaffold RNAs that can guide Pol IV-dependent AGO4-bound siRNAs to targeted loci (Figure 2). Scaffold RNA-producing transcription guiding RdDM to repetitive loci presents an attractive model that accounts for the relationship between Pol IV-dependent siRNA function and transcription of paramutation-associated repeats. The *b1* upstream repeats are transcribed in both directions [16], primarily by Pol II [18•], though no significant differences in transcription rates between *B′* and *B-I* have been noted [16]. Determining the relationship between transcription of repetitive sequences by diverse RNAPs, siRNA signatures and the epigenetic changes mediated by siRNAs will be important for resolving the function of siRNAs in paramutation.

Recent findings by Brzeska *et al.* [33•] indicate that a CXC-domain DNA binding protein – CBBP – may be sufficient to induce paramutation of a naïve *B-I* allele. Identified in a yeast one-hybrid screen for proteins interacting with a portion of the upstream repeats, CBBP was found to form multimers that bind preferentially near a repeat junction [33•]. Because repression of the *B-I* state resulted from overexpression of *cbbp* from a constitutively expressed transgene construct, it remains unknown whether the amount of CBBP binding or the timing of its binding are significant parameters of its function. Repression of *B-I* facilitated by *cbbp* overexpression is also less heritable than that facilitated (Box 1) by an endogenous *B′* allele [33•], indicating that additional changes to the upstream repeats, besides accumulation of the CBBP protein, are necessary for the stable change seen in *B′* paramutation. Whether CBBP is associated with all repeats required for paramutation or just with the *b1* upstream repeats is unknown, nor how CBBP binding may influence RNAP assembly at, and transcription of, the repeats. Interestingly, CBBP produced from expression of the identical transgenic construct was not detectable by Western blot at the upstream repeats in *B-I*/*B-I* individuals [33•], suggesting its binding may depend on specific chromatin marks that are not present at the repeats in *B-I* states (see discussion below).

Transmitting chromatin-based paramutant states through meiosis

Genetic studies of paramutations occurring at the *pl1* locus [11•,13,14] show that somatic repression *in trans* of a susceptible *pl1* allele (Box 1) is distinct from a

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meiotically heritable change. As discussed above, the maintenance, but not the acquisition, of paramutation is affected by *rmr1* mutations (Figure 1b) [14]. A recent study of the *b1* upstream repeats (Table 1) in different developmental stages of the sporophyte was designed to distinguish between potentially heritable chromatin marks associated with paramutation and chromatin marks associated with somatic, tissue-specific regulation of *B′* and *B-I* [34•]. Measuring cytosine methylation, nucleosome occupancy and histone modifications associated with transcriptionally active [Histone 3 Lysine 9 acetylation (H3K9ac) and H3K4 methylation (H3K4me2)] and repressed [H3K27me2, H3K27me3 and H3K9me2] chromatin, Haring *et al.* [34•] found changes consistent with the idea that cytosine methylation plays a role in the progression from paramutable to paramutagenic states (i.e. *B-I* to *B′* transition) [34•]. The upstream repeats of *B′* states are hypermethylated compared to those of *B-I* states in two week old seedlings [34•]. This result comports with similar analyses of the *r1* locus [Table 1] showing that paramutagenic haplotypes are hypermethylated near their transcription start site relative to non-paramutagenic states [27,35]. Histone modification differences seen at the *b1* upstream repeats are most consistent with tissue-specific regulation of *B-I* and *B′* rather than any meiotic-specific chromatin status of either regulatory state. Interestingly, in a *B′*/*B-I* heterozygote, cytosine methylation at the internal tandem repeat junctions of the *B-I* allele progressively accumulates during sporophyte development to resemble a more *B′*-like signature [34•].

The findings of Haring *et al.* [34•] appear at odds with results of genetic mosaic analyses using *B′*/*B-I* heterozygotes [36], which indicate that acquisition of a mitotically stable paramutation (Figure 1a) at the *b1* locus occurs late in development. Irradiation of *B′*/*B-I* materials at different timepoints of zygotic and early seedling development induce somatic sectors derived from cells lacking the chromosome arm carrying the *B′* allele. Such sectors allow direct observation of plant color phenotypes conditioned by a *B-I* allele that has been exposed to a *B′* allele for different numbers of mitoses during somatic development. Sectors found relatively late in development (10 leaf stage) still had a *B-I*-like pigment level [36] indicating that 1) *B-I* retains its capacity for high expression even after exposure to *B′* during somatic development and 2) that *B-I* is initially repressed *in trans* by *B′* before commitment to a mitotically and/or meiotically heritable *B′* state. One interpretation of these results in relation to the cytosine methylation profiles of *B1-I* alleles described by Haring *et al.* [34•], is that cytosine methylation accumulated at a *B-I* allele up until the 10 leaf stage cannot be sufficient for its heritable repression. However, accumulated cytosine methylation at the *b1* upstream repeats may predispose a *trans*-repressed *B-I* allele in somatic tissue for a meiosis-dependent transition to a heritable *B′* state. This hypothesis is consistent with the fact that in plants, *trans*-generational inheritance of epigenetically defined regulatory states relies on their propagation through many rounds of mitosis as well as meiosis [37] and gametophyte development. Whether or not cytosine methylation defines the mitotic and/or meiotic heritability of paramutagenic regulatory states can now be tested using mutants in which acquisition (Figure 1e) and maintenance (Figure 1b, d) of paramutation are differentially affected [11•,13,14] and in which cytosine methylation at repetitive sequences is lost [14,15••].

Conclusions

The mechanism involved in facilitating paramutation interactions requires the function of RdDM components that presumably evolved to control potentially pathogenic nucleic acids such as transposons [38]. Though the extent to which paramutations occur in maize and other eukaryotes is unknown, paramutation-like inheritance patterns may be a common mechanism of gene regulation in repeat-rich genomes. For example, the ~2.3 Gb maize genome consists of >75% LTR retrotransposon sequences [39] that present a large number of potential targets for RdDM regulation even in gene-rich regions [15,39]. Because of the non-essential nature of plant pigments, paramutations described in maize present excellent model systems to study a potentially common and unappreciated mode of inheritance and gene regulation.

A study of the effect on temperature and light on the extent of paramutation occurring at the *r1* locus during early development [40•] established a link between paramutation and external environment sensing. Mikula's findings are intriguing given that potentially heritable cytosine methylation marks are associated with the allelic interactions that facilitate paramutation [27,28,30,34]. One can infer that the *trans*-acting components required for paramutation are also potentially involved in mediating heritable changes to gene regulation in response to environmental stimuli. Supporting this idea is the fact that siRNA biogenesis components, including RPD1, are required for the generation of biotic and abiotic stress-induced small RNAs [41,42], though the expression changes associated with these responses are not heritable. As factors responsible for paramutation in maize have also been linked to developmental canalization [25], it will be of interest to determine the extent to which diverse paramutation-like interactions [2,5], and their underlying mechanisms, are evolutionarily conserved.

The potential for spontaneous, heritable changes to gene regulation similar to paramutations is intriguing in terms of a mechanism for maintaining cryptic, phenotypic variation within a species, or an inbred line of plants. The fact that different allelic combinations can create heritable diversity has exciting implications for how an epigenetic

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regulatory system like paramutation can influence a phenomenon like hybrid vigor, which may be partially dependent on the interaction of different alleles [24]. Further research on the mechanism underlying paramutation promises further insights into the relationship between heritability of phenotypes and epigenetic regulation of repeat-rich genomes, as well as the characteristics of allelic interactions that lead to heritable changes in expression. Such information may facilitate novel strategies for future plant improvement efforts.

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