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Paramutation: a *trans*-homolog interaction affecting heritable gene regulation

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Paramutation describes both the process and results of *trans*-sensing between chromosomes that causes specific heritable changes in gene regulation. RNA molecules are implicated in mediating similar events in maize, mouse, and *Drosophila*. Changes in both small RNA profiles and cytosine methylation patterns in *Arabidopsis* hybrids represent a potential molecular equivalent to the interactions responsible for paramutations. Despite a seemingly unifying feature of RNA-directed changes, both recent and historical works show that paramutations in maize require plant-specific proteins and lack expected hallmarks of a *trans*-effect mediated solely by RNAs. Recent examples of nearby transposons affecting RNA polymerase II functions lead to an opinion that paramutations represent an emergent property of the transcriptional dynamics ongoing in plant genomes between repetitious features and nearby genes.

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Introduction

Paramutation was first used to describe the directed heritable changes occurring among specific haplotypes of the maize *red1* (*r1*) locus [1]. Kernel pigmentation conditioned by *R-r* is heritably reduced following transmission from *R-r*/*R-stippled* heterozygotes [2]. Co-segregation tests showed that this behavior is strictly dependent on *R-stippled* [3] implicating a type of *trans*-homolog interaction (THI) rather than cytoplasmic inheritance.

Haplotypes subject to paramutation exhibit dynamic behaviors. Repressed *R-r* (denoted *R-r′*) transmitted from *R-r*/*R-stippled* plants coincidently acquires the ability to facilitate a similar THI in subsequent *R-r′*/*R-r* heterozygotes [4]. Additionally, *R-r′* reverts to *R-r* when maintained in hemizygous condition [5]. These behaviors are most consistent with heritable epigenetic changes. Thus 'paramutation' describes a unique type of meiotically heritable epigenetic change in gene regulation that is facilitated by an unknown THI.

These apparent exceptions to Mendelian principles have profound implications for our concepts of genetics and evolutionary biology [6]. Specific examples of paramutation in maize mirror the breeding behaviors of inbreeding depression and hybrid vigor [7,8]. As such, there are well-motivated interests in understanding the genomic features making a haplotype susceptible to paramutation and the cellular mechanisms acting on these.

Though it remains unclear how conserved the mechanism(s) might be, RNA molecules continue to be implicated in various paramutation examples. This is a provocative connection in light of recent work showing RNAs themselves as meiotically heritable sources of epigenomic information [9••,10••,11]. As plant small RNAs (sRNAs) can mediate long distance silencing [12,13] it is also possible that paramutations serve a regulative role in plant development. While paramutations may have both phylogenetic and ontogenic functions, simple RNA-mediated events do not seem to account for both historical and recent experimental results in maize. A relationship emerging between the transcription of genes and nearby transposons by different RNA polymerases provides a different mechanistic perspective.

Maize paramutation

Well-characterized paramutation examples occur among specific haplotypes of the *r1*, *booster1* (*b1*), *pericarp color1* (*p1*), and *purple plant1* (*pl1*) loci [2,14–16]. Unusual inheritance behaviors of these haplotypes are visually traceable as each locus encodes a transcriptional activator of flavonoid pigment biosynthesis [17]. The extensive genetic diversity in maize [18,19] makes it probable that similar haplotypes can be identified at many different loci. Indeed, by following the inheritance patterns of kernel phytate levels, a *low phytic acid1* (*lpa1*) haplotype exhibiting paramutation was discovered [20]. Each paramutation example has slightly unique inheritance behaviors [15,21]. Although each example is governed by distinct genomic features [16,22–26], these are often repetitive and all play a role in affecting transcription [16,22,26,27].

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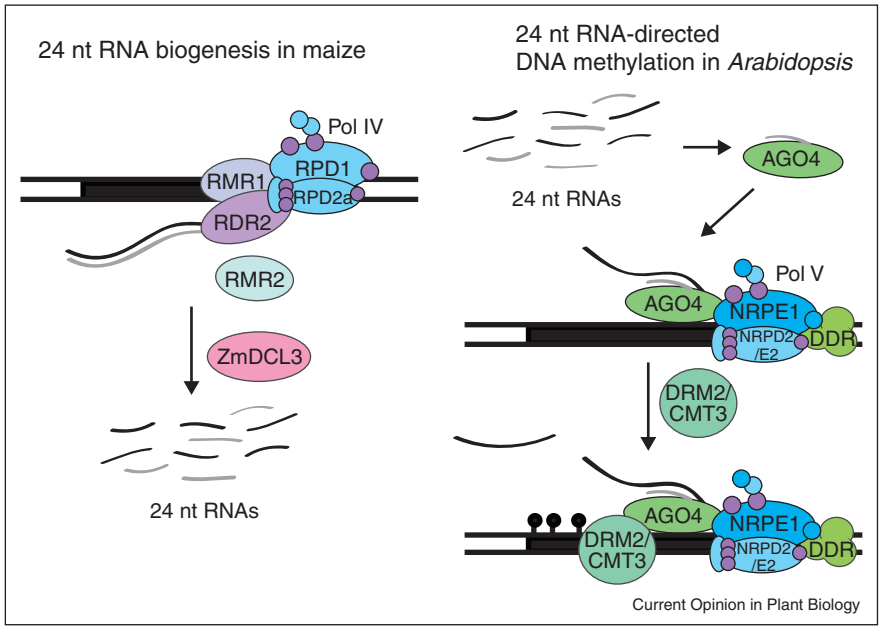
Figure 1



24 nt RNA biogenesis in maize and RdDM pathway in *Arabidopsis*. On the basis of mutant analyses, sRNA profiles, and presumed orthologies with *Arabidopsis* proteins, a complex containing maize Pol IV (RPD1 and RPD2a represent one of potentially multiple Pol IV holoenzyme isoforms in maize [31,32]), RMR1, and RDR2 produces a double stranded RNA (dsRNA) from a repetitive genomic feature (solid black line). The dsRNA is presumably cleaved by a maize Dicer-like3 (ZmDCL3) into 24 nt RNAs. RMR2 is also required for accumulation of 24 nt RNAs, but its location in the pathway remains unknown [34•]. In *Arabidopsis*, 24 nt RNAs associate with AGO4. These sRNA/AGO4 complexes dock with nascent Pol V transcripts and aid recruitment of *de novo* cytosine methyltransferases DRM2 and CMT3. DDR is composed of DRD1, DMS3/IDN1 and RDM1 which together associate with Pol V [see Matzke, Pikaard, and Wierzbicki reviews this issue].

Mutational approaches looking for factors required to maintain repressed paramutant states identify proteins responsible for 24 nt RNA accumulation. Several have *Arabidopsis* orthologs placed in the sRNA biogenesis portion of an RNA-directed DNA methylation (RdDM) pathway (see Matzke, Pikaard, Wierzbicki, reviews this issue) (Figure 1). Loci identified by mutations in screens using repressed states (*B′* and *Pl′*) of the *B1-Intense* and *Pl1-Rhoades* haplotypes are designated *mediator of paramutation* (*mop*) and *required to maintain repression* (*rmr*), respectively. *mop1*/*rdr2* encodes a likely RNA-dependent RNA polymerase (RDR) related to *Arabidopsis* RDR2 [28,29]. *rmr6*/*rpd1* encodes the largest subunit of RNA polymerase IV (Pol IV) related to *Arabidopsis* NRPD1 (NUCLEAR RNA POLYMERASE D1) [30]. *rmr7*/*mop2*/*rpd2a* encodes one of three second largest subunits available for assembly in either Pol IV or Pol V similar to *Arabidopsis* NRPD2/E2 (NUCLEAR RNA POLYMERASE D2/E2) [31,32]. *rmr1* encodes a Rad54-like ATPase required for co-transcriptional regulation of *Pl′* RNAs that founds a distinct, yet related, clade to *Arabidopsis* CLASSY1 (CLSY1) and DEFECTIVE IN RNA-DIRECTED-DNA-METHYLATION 1 (DRD1) proteins [33].

Very recently, *rmr2* was shown to encode a novel protein having no obvious catalytic function but containing a conserved C-terminal domain that defines a new family of plant-specific proteins [34•]. RMR2 represents a grass-specific clade of these proteins with no obvious *Arabidopsis* ortholog. RMR2 is required for maintaining transcriptional repression of *Pl′* and for defining specific cytosine methylation (5meC) patterns in CHG sites found 3′ of the *Pl1-Rhoades* coding region but is not required for facilitating paramutation at *r1* (Table 1). However, paramutation can be impaired at *pl1* in the absence of RMR2, highlighting a molecular distinction in the mechanisms responsible for THIs at different loci (Table 1).

All of these maize proteins are required for accumulation of >60% of 24 nt RNAs found in unfertilized ears [30,31,34•,35,36••] although the extent to which these RNA profiles overlap remains unknown. All 24 nt signatures appear reduced in the maize *rdr2* mutant [35] as well as in the *rmr6*/*rpd1* mutant, which has only ~15% of non-mutant levels [30]. Levels of sRNAs representing both repetitious and unique regions are affected with signatures of transposable elements (TEs) being predominant [35,36••]. From these associations, the mechanism required to maintain repressed states of paramutant haplotypes appears related to a grass-specialized version of RdDM targeting TEs.

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Table 1

***Trans*-acting factors required to facilitate paramutations.**

Locus	Molecular identity	Affected examples				Refs
		r1	*b1*	*pl1*	*p1*	
mop1/rdr2	RDR2	Yes	Yes	Yes	Yes	[51,63]
rmr1	Rad54-like ATPase	Unreported	No	No	Untested	[33]
rmr2	Novel protein	No	Untested	Partially[a]	Untested	[34•]
rmr6/rpd1	RPD1	Yes	Yes	Yes	Untested	[58]
rmr7/mop2/rpd2a	RPD2a	Yes	Yes[b]	Yes	Yes	[31,32]
cbbp	CBBP	Untested	Partially[c]	Untested	Untested	[47]

[a] Of progeny from five separate test-crosses: 17 (52%) had phenotypes indicating that paramutation had occurred in the absence of RMR2 while 8 (24%) had full-color anthers and another 7 (21%) had chimeric tassels (displaying several distinct pigment levels) both indicating an impairment of paramutation in the absence of RMR2 [34•].
[b] The *B-I* state was maintained when exposed to *B′* from an *rmr7-1* male, but not from the reciprocal cross with *B′* originating from an *rmr7-1* female [31].
[c] *FLAG-cbbp* transgene driven by a maize ubiquitin promoter induces silencing of *B-I* state to a *B′*-like state [47]. However, in the absence of the transgene, this repressed state is meiotically stable in only half of the progeny and any ability to facilitate paramutation remains unclear [47].

Trans-homolog interactions via RdDM

An RdDM-type model for paramutation has been proposed in which 24 nt RNAs produced from a paramutant haplotype on one chromosome, such as *B′*, recruit de novo DNA methyltransferases to nascent RNA scaffold transcripts produced from DNA sequences on the homologous chromosome (see Wierzbicki, review this issue). This hypothetical THI in maize would effectively transfer a 5meC profile across homologs with two required consequences: the newly acquired 5meC pattern results in reduced transcription of the gene in question and the acquired state now produces similar 24 nt RNAs.

Trans-chromosomal methylation (TCM) — possibly analogous to the above model — was recently reported in *Arabidopsis* hybrids of Landsberg *erecta* and C24 accessions [37•]. Some differentially methylated regions (DMRs) between parents become homogenized or altered in hybrids in ways interpreted as either representing TCM or *trans*-chromosomal demethylation events. While the mechanism remains unknown, similar observations regarding regions of differential 24 nt RNA levels may implicate an RdDM-like mechanism [38•,39]. Surprisingly, regions having the greatest parental difference in sRNA levels show the greatest reductions of sRNAs in hybrids [38•]. The extent to which these genome-wide dynamic changes in sRNA and 5meC patterns might represent paramutation-type events affecting heritable regulation of genic sequences remains to be seen. It is conceptually transformative to envision that both spontaneous and concerted heritable changes recently documented in plant methylomes may represent epigenetic sources of heritable phenotypic variation for selection to act upon [40,41,42••,43].

The RdDM-type model for paramutation has been evaluated at a group of seven tandem repeats (TRs) of an 853 bp non-coding sequence found ~100 kb 5′ of the *b1* coding region in the *B1-Intense* haplotype. The TRs collectively operate as both a long-distance transcriptional enhancer [27] and as a necessary feature for paramutation [25]. The TRs are transcribed by Pol II in both directions [28,44•], are the source for RDR2 and RPD2a-dependent 24 nt RNAs [32,44•], and have DMRs between paramutant (*B′*) and non-paramutant (*B-I*) states [45•]. Moreover, Haring *et al.* [45•] showed that 5meC of the *B-I* TRs increases during development of *B′/B-I* sporophytes. Chromatin conformation capture assays show that the hypermethylated *B′* TR has relatively weaker associations with the *b1* promoter [46]. While these data largely agree with a simple RdDM-type model, other expectations remain unmet.

One fundamental expectation is that specific sRNAs mediate the THI. Surprisingly, Arteaga-Vazquez *et al.* [44•] found that TR sRNAs are present — at approximately similar levels based on northern blots — in *B-I/B-I* and *B′/B′* plants, and even in *b1/b1* plants not exhibiting paramutation. Furthermore, run-ons showed the TRs are equivalently transcribed in *B-I/B-I* and *B′/B′* plants. This result indicates that even though the *B′* and *B-I* TRs represent DMRs, they do not produce unique sRNAs or obviously distinct sRNA levels. Curiously, expression of either a TR binding protein (CBBP), or an inverted-repeat hairpin TR transgene can induce changes of *B-I* to a heritably repressed state similar to *B′* but this induced state is not always able to facilitate paramutation on a naïve *B-I* [44•,47]. The reason for this difference remains unknown but clearly the sRNAs derived from the specific hairpin construct cannot fully recapitulate the requirement for a THI involving *B′*.

Similar exceptions to the model are seen in the absence of RMR1 and RMR2 — components required for accumulation of ~65% of 24 nt RNAs [33,34•,36••]. Both RMR1 and RMR2 maintain repressed *Pl1-Rhoades* states (*Pl′*) in the soma but the fully expressed *Pl-Rh* state can change to a meiotically heritable *Pl′* state in *Pl-Rh/Pl′* plants

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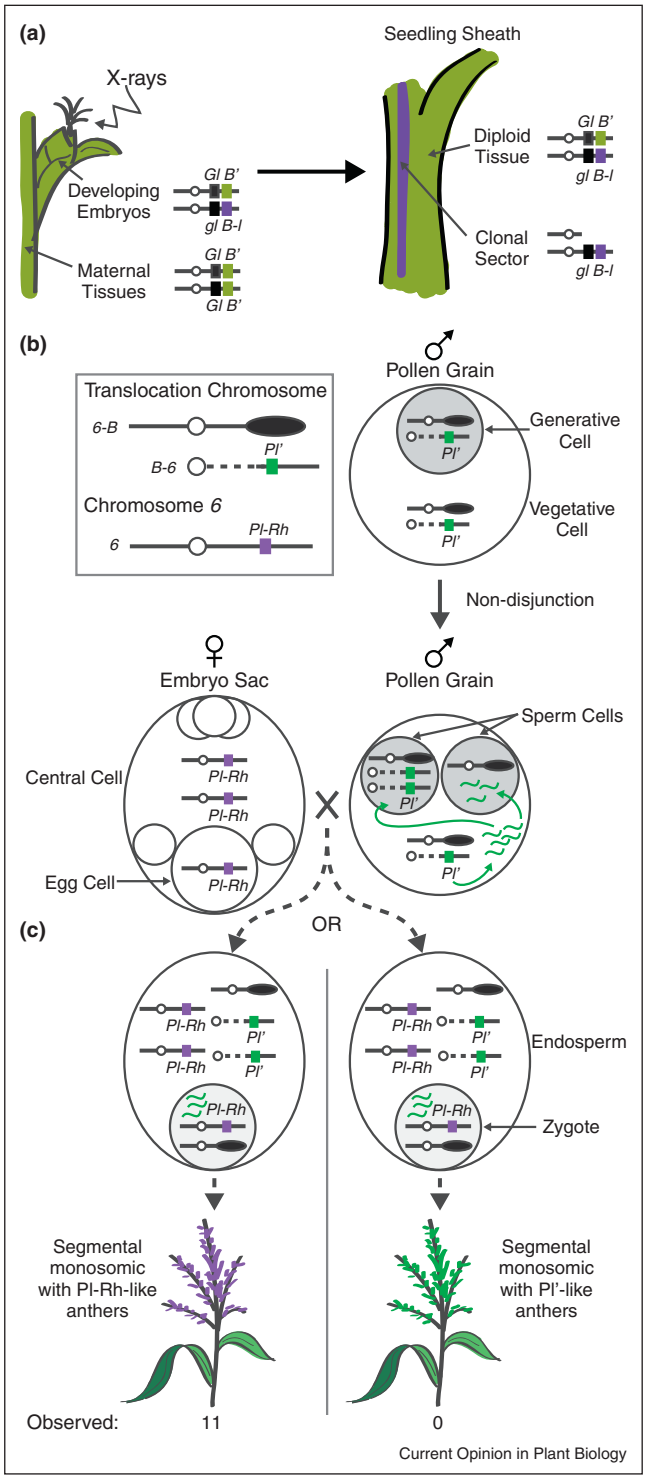
Figure 2



Cell-autonomous action of *trans*-repression. **(a)** *Trans*-repression of a *B-I* state requires cell-autonomous presence of a *B′* chromosome region [48]. Embryos (*Gl B′/gl B-I*) irradiated 1–2 days post-fertilization (dpf) produced fully colored (B-I-like) and glossy seedlings (8/2053 examined) and embryos treated 2–5 dpf produced seedlings with glossy, B-I-like, sectors (6/1100 examined). Irradiations of developing *Gl B′/gl B-I* sporophytes at both the 4-leaf and 10-leaf stages resulted in B-I-like sectors in older tissues including cobs [48]. **(b)** Heritable repression of *Pl-Rh* is chromosome-dependent and cell-autonomous. Pollen transmitting a translocation having a *Pl′* state linked to a *B* centromere can produce non-equivalent sperm cells via non-disjunction during mitosis of the generative cell. Both sperm cells are assumed to have identical cytoplasmic components including sRNAs (green squiggles) provided from the vegetative cell [10••] but one has two doses (left), and the other has none (right), of the chromosome region including *Pl′*. Fertilization of egg cells containing *Pl-Rh* by sperm cells deficient for *Pl′* could produce two different progenies, depending on whether or not *Pl′* itself is required for *trans*-repression. **(c)** If cytoplasmic components such as sRNAs are themselves sufficient for the THI, then segmentally monosomic progeny should have weakly colored Pl′-like anthers. However, if the chromosome region carrying *Pl′* is required then these progeny should have fully colored Pl-Rh-like anthers. All eleven segmentally monosomic progeny from such crosses had Pl-Rh-like anthers (J Hollick, unpublished results) indicating the THI is chromosome-dependent.

deficient for either RMR1 [33] or RMR2 [34•]. Similarly, *R-r* still changes to *R-r′* in *R-r/R-stippled* plants in the absence of RMR2 [34•]. These results indicate that neither the sRNA profiles dictated by RMR1 and RMR2, nor the 5meC patterns dependent on them, are responsible for mediating paramutation.

Additionally, not a single maize ortholog of downstream RdDM components has been found in the genetic screens completed to date (Figure 1). While this could reflect some degree of genetic redundancy or vital function, it is also possible that small RNAs and their effectors are immaterial to the THI. For instance, 5meC patterns at a fractured CACTA-like DNA transposon found immediately 5′ of *Pl1-Rhoades* are dependent on RPD1, RDR2, and RMR1 [33] but these patterns are no different between *Pl-Rh* and *Pl′* states [33].

Given that sRNAs can act as mobile silencing signals [12,13], it might also be predicted that paramutations act in a non-cell autonomous manner. This is not the case — at least in horizontal directions — as clonal sectors of paramutant identity can be found in both *B-I/B-I* and *Pl-Rh/Pl-Rh* plants [48] (J Hollick, unpublished observations). Moreover, sectors of either *B-I* / -genotype or *Pl-Rh* / -genotype have sharp clonal boundaries with adjacent *B-I* / *B′* and *Pl-Rh* / *Pl′* tissues [48] (Figure 2a) (J Hollick, unpublished results). Thus, although identification of maize sRNA accumulation factors leads to a simple RdDM-type model, many of the experimental results are at odds with this idea.

The role of transcription in maize paramutation

Some of the exceptions noted for a simple RdDM-like model may be related to the various requirements needed to ensure heritable transmission of a paramutation event (Box 1). Because the assay for whether or not a heritable change has occurred requires sexual transmission, it is difficult to identify the defining event and specific molecular changes responsible for paramutation.

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Box 1 Requirements for establishing paramutations.

There are at least three conceptual features to establishing paramutations that could be affected by different mechanisms [62].

First, a THI must occur early in development to repress the non-paramutant state in *trans* since reduction in plant pigments is seen in seedling tissues. Evidence for this is seen in segmental monosomic sectors such as *B-I*-induced by irradiation of *B-I*/*B′* embryos and developing sporophytes [48] (Figure 2a). Similarly, *Pl′* can repress a distinct *Pl1* haplotype (*Pl1-W22*) during somatic development but both haplotypes are transmitted unchanged [26].

Second, somatic maintenance of repressed states is required on both homologs. Evidence for this function can be seen in *rpd1*, *rdr2*, and *rmr1* mutants in which heritable reversions of *Pl′* to *Pl-Rh* occur in both *Pl*/*Pl′* and *Pl-Rh*/*Pl′* plants [33,54,58,63]. Additionally, some clonal sectors derived from losing the *Pl′* — containing chromosome arm in *Pl-Rh*/*Pl′* plants can display a Pl′-like phenotype indicating that a mitotically stable state has been attained (J Hollick, unpublished observations).

Third, the repressed state must be transmitted through meiosis and gametophyte development. So far, only Pol IV and RDR2 could potentially serve this function. It also remains possible that transmission of a repressed state is solely an outcome of being able to both facilitate the THI and maintain somatic repression. RPD1 and RPD2a constitute at least one isoform of maize Pol IV [31,32] and RDR2 is likely associated with maize Pol IV as it is in *Arabidopsis* [52]. Although all these proteins are required to facilitate paramutations at *r1*, *b1*, and *pl1* (Table 1), *Pl′* does not heritably revert to *Pl-Rh* in the absence of RPD2a [31]. This indicates that RPD2a defines a specific role in ensuring meiotic transmission of a new paramutant state conditioned in *Pl-Rh*/*Pl′* plants.

Failure to see a repressed phenotype, for example, in *Pl-Rh*/*Pl′* plants, does not indicate that paramutation is impaired; paramutation can still occur in the absence of RMR1 or RMR2 [33,34•]. Reciprocally, a repressed phenotype, for example, in *B-I*/*B′* plants, does not mean that an irreversible event has taken place (Figure 2a) [48]. This later point is potentially reflected in the developmentally progressive increase of TR 5meC in *B-I*/*B′* plants that lags behind early reductions in pigmentation [45•]. *B′* is also distinguished by attenuated associations of another 5′ enhancer located ~47 kb away with the *b1* promoter region and by higher H3K27me2 levels along the coding region [45•]. Thus, pigment reductions seen early in the development of *B-I*/*B′* plants may be associated with more immediate changes to the chromatin and loop domain architecture making any cause or effect relationship between specific epigenetic changes and either transcriptional or post-transcriptional events difficult to interpret.

Transcription rates are reduced in all cases of paramutation tested to date [49,50,51] and this agrees with epigenetic changes in enhancer-type sequences [16,25,27] that are functionally required for paramutation [16,25]. This relationship has focused attention to the enhancers as primary targets of Pol IV, sRNA biogenesis, and THI. Transcription of the coding regions may, however, be interrelated as promoter-deleted derivatives of *R-r* and mutant derivatives of *Pl1-Rhoades* blocking RNA production both fail to facilitate paramutation [23,26].

Additionally, transcription of *Pl′* appears abnormal as these transcripts are relatively unstable. RMR1 has no effect on *Pl′* transcription rates but it does affect co-transcriptional stability of the RNA [33]. Interestingly, both RDR2 and the potential *Arabidopsis* RMR1 orthologs are associated with Pol IV [52] suggesting that all the maize RMR and MOP proteins are associated in a specific Pol IV complex. Because RMR1 affects *Pl′* RNA stability, Pol IV may be competing with Pol II for *Pl1-Rhoades* transcription and thereby affecting the chromatin status of both the genic template and associated transcriptional enhancers [36••].

Pol IV and Pol II competitions likely occur at many repetitious features in the maize genome. Hale *et al.* [36••] found sense-specific polyA+ RNAs from *CRM2* and *Prem2* retrotransposons in the absence of RPD1 but not in the absence of RDR2 or RMR1. *rpd2a* mutants also had no effect [31] indicating that transcriptional repression of *CRM2* and *Prem2* elements are mediated by an RPD2a-independent Pol IV isoform. These results indicate, quite surprisingly, that transcriptional repression of certain retrotransposons is the result of specific RNA polymerase (RNAP) competitions rather than the expected repression through sRNAs and/or 5meC patterns. This hypothetical model could also account for the specific developmental defects observed in RPD1 mutants that are not seen in the other mutants affecting 24 nt RNA levels [30,31,34•,36••,53,54].

A role for transposable elements in paramutation?

Nearly all maize genes are flanked by TEs since over 85% of the genome is TEs [55,56]. In *Arabidopsis*, Hollister *et al.* [57•] found a compelling association between proximal TEs (<1 kb) targeted by 24 nt RNAs and reduced gene expression in comparisons of *Arabidopsis thaliana* and *A. lyrata* congeners. The proximate TE at the 5′ of *Pl1-Rhoades* is a constant target for RdDM in both *Pl′* and *Pl-Rh* states [33] yet somehow high levels of *Pl-Rh* transcription remain insulated.

The *Pl1-Rhoades* 5′ TE itself is insufficient for paramutation behaviors as the weakly expressed *Pl1-Blotched* haplotype has the identical TE and coding sequence as *Pl1-Rhoades* yet does not show paramutation behaviors [8,26]. However, loss of RPD1 leads to elevated transcription of *Pl′* [58] and to increased expression of *Pl1-Blotched* (K Erhard, J Hollick, unpublished observations) implicating a role for this TE in maintaining transcriptional repression. This idea is consistent with RNAP competition models and suggests integrative roles for TEs in specifying epigenomic landscapes of transcriptional control.

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Companion cells providing small RNAs to the gametes may be a general mechanism for ensuring transgenerational TE repression [9••,10••]. In plants, companion cells reside inside haploid gametophytes. Thus, sRNAs produced from pollen or egg companion cells could provide the locus-dependent trigger required for initiating repression in subsequent zygotes. If so, then even sperm cells that lose a chromosome containing a paramutant haplotype during gametophyte development should be competent to initiate paramutation in the next generation. Experimental results using BA translocations that often undergo non-disjunction at the second pollen mitosis show that segmental nullisomic sperm cells derived from *Pl′* pollen grains do not transmit a paramutation specific inducer (Figures 2b and c). This result indicates that either sRNAs are not the heritable feature responsible for *pl1* paramutation, or that transcription of the *Pl′* state is required to maintain the persistence of such sRNAs.

Conclusions

McClintock characterized many TEs as 'controlling elements' and inferred that changes to heterochromatin were responsible for 'changes in state' of these elements [59]. As 24 nt RNAs and RNA scaffolds appear to represent a molecular form of 'heterochromatin', it is likely that both features are involved in maintaining and changing particular epigenetic states. Genome and developmental dynamics affecting both RNAP distributions and sRNA profiles have the potential to effect heritable changes in state of specific TEs that then impact the regulation of attendant genes. Selection [60••], breeding designs [7] and environmental influences [61] may all impinge on this nuclear system to create and maintain epigenetic sources of heritable regulatory variation.

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- •• of outstanding interest

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