

Trans-Homolog Interactions Facilitating Paramutation in Maize

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Paramutations represent locus-specific trans-homolog interactions affecting the heritable silencing properties of endogenous alleles. Although examples of paramutation are well studied in maize (*Zea mays*), the responsible mechanisms remain unclear. Genetic analyses indicate roles for plant-specific DNA-dependent RNA polymerases that generate small RNAs, and current working models hypothesize that these small RNAs direct heritable changes at sequences often acting as transcriptional enhancers. Several studies have defined specific sequences that mediate paramutation behaviors, and recent results identify a diversity of DNA-dependent RNA polymerase complexes operating in maize. Other reports ascribe broader roles for some of these complexes in normal genome function. This review highlights recent research to understand the molecular mechanisms of paramutation and examines evidence relevant to small RNA-based modes of transgenerational epigenetic inheritance.

Transgenerational epigenetic changes affecting gene regulation have been implicated in agricultural performance, human disease, and evolution (Groszmann et al., 2013; Jablonka, 2013; Heard and Martienssen, 2014). Paramutation represents one well-studied mechanism for generating and establishing such changes (Hollick, 2010). Alexander Brink first applied the term paramutation to describe directed, yet reversible, changes in maize (*Zea mays*) kernel pigment patterns resulting from trans-homolog interactions (THI) of certain *red1* (*r1*) alleles (Brink, 1958). Mottled pigmentation conditioned by the *R-r:standard* (*R-r*) allele is invariably suppressed after exposure to *R-stippled* (*R-st*) in heterozygotes (Brink, 1956). The suppressed *R-r* state (denoted *R-r'*) is meiotically heritable, induces a similar change to naive *R-r* (secondary paramutation), and gradually reverts back to an *R-r* reference state when maintained in hemizygous conditions (Styles and Brink, 1969). These behaviors classify *R-r* as an epiallele having variable silencing properties influenced by THI. This type of non-Mendelian inheritance has remained a great unsolved mystery of genetics research and is a topic of considerable interest.

Paramutation has a genetic definition intended for invariable occurrences of heritable, although potentially reversible, changes of an allele when heterozygous with another specific allele of the same gene (Brink, 1958). Used in the same sense originally assigned to mutation, paramutation represents both the process and outcome of these THI without reference to specific molecular features. Alleles inducing paramutation, such as *R-st* and *R-r'*, are termed paramutagenic. Alleles susceptible to paramutation, like *R-r*, are paramutable, and alleles neither paramutagenic

nor paramutable are termed neutral. Some neutral alleles, similar to deficiencies, facilitate the reversion of an existing paramutation to the paramutable reference state, while others do not (Gross and Hollick, 2007). These behaviors can generate and maintain wide phenotypic variation from specific allelic combinations and/or transgenerational conditioning at a single locus.

The generality of paramutation in other eukaryotes remains unclear. Well-described examples occur among certain alleles of several other maize loci (Table I), including those, like *r1*, encoding transcription factors responsible for anthocyanin pigment biosynthesis: *booster1* (*b1*; Coe, 1959), *purple plant1* (*pl1*; Hollick et al., 1995), and *pericarp color1* (*p1*; Sidorenko and Peterson, 2001). Another example occurs at the maize *low phytic acid1* locus (Pilu et al., 2009). Paramutation-like behaviors have also been noted in other plants, fungi, and metazoans (for review, see Chandler and Stam, 2004), including more recent examples in *Mus musculus* (Rassoulzadegan et al., 2006; Wagner et al., 2008; Worch et al., 2008; Grandjean et al., 2009; Yuan et al., 2015), *Drosophila melanogaster* (Seong et al., 2011; de Vanssay et al., 2012), and *Caenorhabditis elegans* (Ashe et al., 2012; Shirayama et al., 2012; Seth et al., 2013; Wedeles et al., 2013; Sapetschnig et al., 2015). A common feature for many of these examples is the possible involvement of RNA-based mechanisms related to basal RNA interference (RNAi) machinery (for review, see Castel and Martienssen, 2013). Here, we present an RNAi-based working model for paramutation in maize and discuss experimental results addressing key aspects of the model.

A WORKING MODEL FOR PARAMUTATION IN MAIZE

Forward genetic approaches have begun to identify molecules relevant to the paramutation mechanism.

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Table 1. Characteristics defining examples of paramutation in maize

N/A, Not applicable.

Locus	Allele	Paramutation Properties					Proteins Required for ^a	
		Paramutable	Paramutagenic	Efficiency	Reversion	Secondary Paramutation	Somatic Repression	Induction of Paramutation
<i>red1 (r1)</i>	<i>R-r:standard</i> ^b	Yes ^b	Yes (<i>R-r'</i>) ^b	100% ^b	Yes ^{b,c}	Yes ^{d,e}	RPD1 ^f	RPD1 ^f RDR2 ^g RP(D/E)2a ^h [RMR1] ⁱ [RMR2] ^j
	<i>R-stippled</i> ^b	No ^b	Yes ^b	100% ^b	N/A	N/A	N/A	RPD1 ^f RDR2 ^g RP(D/E)2a ^h [RMR1] ⁱ [RMR2] ^j
<i>booster1 (b1)</i>	<i>B1-Intense</i> ^k	Yes ^{k,l}	Yes (<i>B'</i>) ^{k,l}	100% when induced by <i>B</i> ^{k,l} , less than 100% when induced by most transgenes ^{m,n}	Not when induced by <i>B</i> ^{k,l,o} , yes if initiated by transgenes ^{m,n}	Yes when induced by <i>B</i> ^{k,l} , variable if initiated by transgenes ^{m,n}	RDR2 ^g	RDR2 ^g RPD1 ^f RP(D/E)2a ^{h,p} [RMR1] ⁱ Parent-of-origin effect [RMR2] ^j RMR2 ⁱ
<i>purple plant1 (p1)</i>	<i>P1-Rhoades</i> ^q	Yes ^q	Yes (<i>P1'</i>) ^q	100% ^q	Yes ^{q,r,s}	Yes ^q	RDR2 ^g	RDR2 ^g RMR2 ⁱ
<i>pericarp color1 (p1)</i>	<i>P1-rr</i> ^{t,u}	Yes ^{t,u}	Yes (<i>P1-rr'</i>) ^{t,u}	0%–95% ^{t,u,v,w}	Yes ^t	Yes ^{t,u,v}	RMR1 ⁱ RPD1 ^f RP(D/E)2a ^{h,p} RMR2 ^j Putative <i>ufo1</i> protein ^w [RDR2] ^u	RDR2 ^g RPD1 ^f [RMR1] ⁱ [RP(D/E)2a] ^{h,p} RDR2 ^v
	<i>P1-pr</i> ^u	No ^u	Yes ^u	Variable ^u	Yes ^u	Yes ^u	Putative <i>ufo1</i> protein ^{u,w} [RDR2] ^x	Not tested
<i>low phytic acid1 (lpa1)</i>	<i>lpa1-241</i> ^x	No ^x	Yes ^x	<100% ^x	Yes ^x	No ^x	[RDR2] ^x	Not tested

^aProteins shown not to be required for the indicated paramutation behaviors are bracketed. ^bBrink (1956). ^cStyles and Brink (1969). ^dBrink et al. (1960). ^eBrown and Brink (1960). ^fHollick et al. (2005). ^gDorweiler et al. (2000). ^hSidorenko et al. (2009). ⁱHale et al. (2007). ^jBarbour et al. (2012). ^kCoe (1959). ^lCoe (1966). ^mArteaga-Vasquez et al. (2010). ⁿBelele et al. (2013). ^oChandler et al. (2000). ^pStonaker et al. (2009). ^qHollick et al. (1995). ^rHollick and Chandler (1998). ^sGross and Hollick (2007). ^tSidorenko and Peterson (2001). ^uGoettel and Messing (2013). ^vSidorenko and Chandler (2008). ^wSekhon et al. (2012). ^xPilu et al. (2009).

Recessive mutations of the *required to maintain repression* (*rmr*; Hollick and Chandler, 2001) and *mediator of paramutation* (*mop*; Dorweiler et al., 2000) loci identify functions required to maintain repressed pigmentation associated with paramutations (paramutant states) of *p1* and *b1* alleles, respectively. A dominant mutation identifying the *unstable factor for orange1* (*ufo1*) locus similarly affects *b1* and *p1* alleles (Sekhon et al., 2012). Among the estimated 14 *rmr* loci (J.B. Hollick, unpublished data), four encode orthologs of *Arabidopsis*

(*Arabidopsis thaliana*) proteins required to generate 24-nucleotide RNAs, including catalytic subunits of a plant-specific DNA-dependent RNA polymerase IV (Pol IV; Erhard et al., 2009; Sidorenko et al., 2009; Stonaker et al., 2009), an RNA-dependent RNA polymerase (RDR2; Alleman et al., 2006), and a Rad54-like ATPase (RMR1; Hale et al., 2007). In addition to RMR2, a small (366-amino acid) pioneer molecule defining a plant-specific clade of novel proteins (Barbour et al., 2012), all these maize proteins are required for complete 24-nucleotide

RNA biogenesis (Hale et al., 2007; Nobuta et al., 2008; Erhard et al., 2009; Stonaker et al., 2009; Barbour et al., 2012). In Arabidopsis, 24-nucleotide RNAs complexed with Argonaute-type proteins direct sequence-specific de novo cytosine methylation (5meC) through a process known as RNA-directed DNA methylation (RdDM; Matzke and Mosher, 2014; Matzke et al., 2015). Thus, current working models and hypotheses regarding the paramutation mechanisms in maize are largely based on a presumed RdDM framework (Fig. 1).

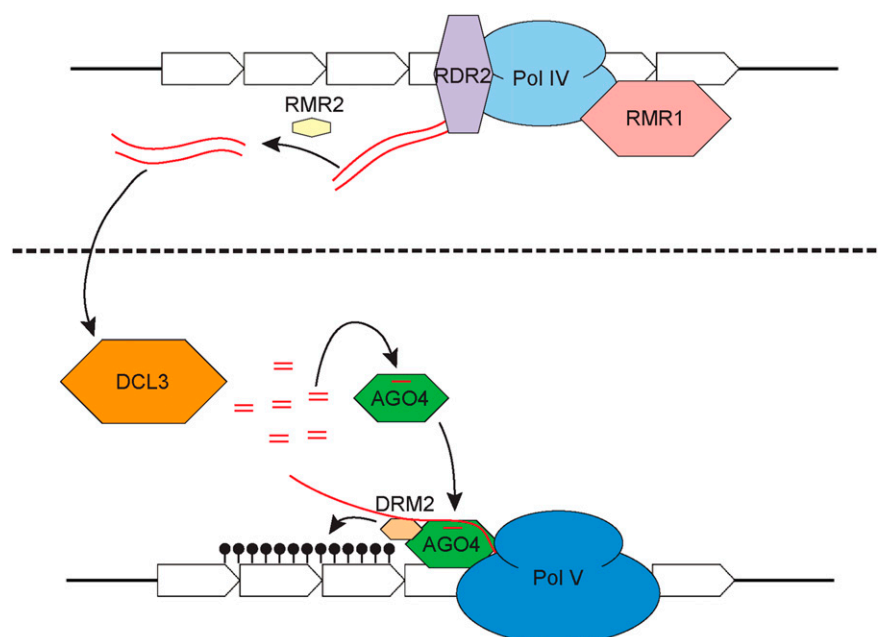
The general outline of an RdDM-type working model posits that important regulatory sequences of paramutagenic alleles are transcribed by a Pol IV: RDR2 complex (Haag et al., 2012; Li et al., 2015) to produce double-stranded RNAs. A dicer-like3 (DCL3) endoribonuclease cleaves these RNAs into 24-nucleotide fragments that are incorporated into an Argonaute protein and help recruit de novo DNA methyltransferases to identical or similar sequences in nascent transcripts produced from another DNA-dependent RNA polymerase (Pol V). In this model, 24-nucleotide RNAs represent a cache of epigenetic information that mediates THI with 24-nucleotide RNAs produced from a paramutagenic allele influencing 5meC patterns of a paramutable allele in trans. This model is supported by the requirement of the maize Pol IV largest subunit (*rna polymerase d1* [RPD1]) and RDR2 for facilitating paramutation at multiple loci (for review, see Hollick, 2012); however, it fails to explain why other RMR components are either only involved in locus-specific behaviors or are only required to maintain repressed expression states in somatic lineages (Table I). These relationships indicate a complexity not reflected by the current Arabidopsis RdDM paradigm. Other observations inconsistent with a simplistic RdDM model are discussed below.

DIVERSE ROLES FOR MULTIPLE DNA-DEPENDENT RNA POLYMERASES

An RdDM-based model for paramutations occurring in maize likely involves multiple Pol IV and Pol V subtypes. Arabidopsis has only one functional second largest subunit shared by both Pol IV and Pol V, but maize and other grasses have three (Haag et al., 2014; Huang et al., 2015). Consistent with genetic data showing diverse functions of more than one Pol IV holoenzyme (Stonaker et al., 2009), recent proteomic profiles support the presence of at least two Pol IV (Pol IVa and Pol IVb) and three Pol V (Pol Va, Pol Vb, and Pol Vc) subtypes in maize callus (Fig. 2; Haag et al., 2014). Second largest subunits *rna polymerase (d/e)2a* [RP(D/E)2a] and RP(D/E)2b define Pol IV and Pol V subtypes a and b, respectively, and RPE2c specifies subtype Pol Vc. Both Pol IV and Pol V associate with distinct accessory proteins, although the specificities of these proteins for each polymerase subtype remain unresolved (Fig. 2; Haag et al., 2014). Both polymerase subunits and accessory proteins may specify unique functions of these distinct complexes at different loci, cell types, and/or in certain developmental phases. Such functional diversity might account for the different RMR requirements observed at specific paramutable alleles (Table I).

Recent results show that maize Pol IV also has more general effects on gene regulation, possibly unrelated to RdDM (Erhard et al., 2015). Loss of RPD1 function results in strong developmental defects, possibly explained by the ectopic expression of specific genes (Parkinson et al., 2007; Erhard et al., 2013, 2015). Yet, similar defects are not seen in *rp(d/e)2a* or other *rnr* mutant plants (Hale et al., 2007; Stonaker et al., 2009; Barbour et al., 2012), thus implicating Pol IVb in gene regulation. RDR2-dependent 24-nucleotide RNAs are

Figure 1. An RdDM-type working model for paramutation. Maize components identified by mutations are presented above the dotted line, and presumed orthologs of select Arabidopsis RdDM proteins are presented below. In this schematic, Pol IV is transcribing a repetitive enhancer element (arrowed boxes), the nascent RNA is copied by RDR2, and the resulting double-stranded RNA (red lines) is cleaved by DCL3 to create 24-nucleotide RNAs. These small RNAs, in complex with argonaute4 (AGO4), facilitate the association with Pol V nascent RNAs and the recruitment of a de novo methyltransferase (domains-rearranged DNA methyltransferase2 [DRM2]) to accomplish site-directed cytosine methylation (black lollipops). All proteins are to relative scale based on amino acid content.



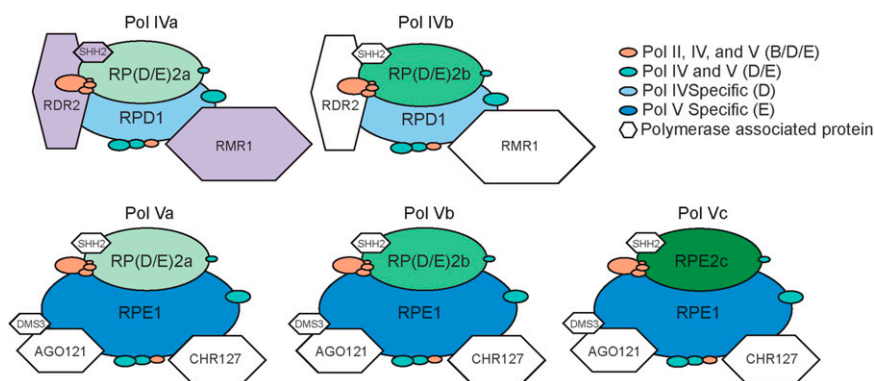


Figure 2. Maize DNA-dependent RNA polymerase diversity. Polymerase subunit and associated protein compositions of Pol IV and Pol V subtypes are based on coimmunoprecipitation profiles using epitope-tagged versions of RPD1, RPE1, and RP(D/E)2a in maize callus and epitope-tagged versions of RP(D/E)2a and RDR2 in developing ears with 3- to 5-cm cobs (Haag et al., 2014). All proteins are represented by sizes reflecting primary amino acid contents. Polymerase subtypes (ovals) are distinguished by combinations of largest and second largest subunits. Subunits are color coded according to the legend at top right and arranged relative to *Saccharomyces cerevisiae* Pol II structural information (Cramer et al., 2000) and presumed Arabidopsis compositions (Ream et al., 2009). Polymerase-associated proteins (hexagons) are coded white or purple to indicate ambiguous or unambiguous assignments, respectively. Peptides specific for RMR1 and another RMR1-like protein (CHR167) coimmunoprecipitated with RPD1, and the unambiguous placement of RMR1 with at least one of possibly multiple Pol IVa complexes is inferred from genetic results (Hale et al., 2009; Stonaker et al., 2009). Peptides identifying two distinct SHH2-like proteins coimmunoprecipitated with RPE1, RP(D/E)2a, and RDR2, placing these unambiguously with the Pol IVa subtype and ambiguously with all other subtypes.

found enriched at both genic transcription initiation sites and pretermination regions (Gent et al., 2013; Erhard et al., 2015), consistent with nascent transcript profiling showing that RPD1-containing complexes generally affect Pol II transcription at all gene boundaries (Erhard et al., 2015). These data support models in which Pol IV competes with Pol II for both genic and nongenic transcription (Hale et al., 2009; Stonaker et al., 2009; Erhard et al., 2015). Such models were proposed to account for the observations that polyadenylated RNAs (Pol II products) from certain transposable elements (TEs) accumulate in the absence of RPD1 but not in the absence of RdDM components [RMR1, RDR2, and RP(D/E)2a] required for 24-nucleotide RNA biogenesis (Hale et al., 2009; Stonaker et al., 2009). At least 183 alleles in the reference B73 genome (Schnable et al., 2009) are differentially transcribed in the absence of RPD1 (Erhard et al., 2015), and it is possible that some of these differences are responsible for the observed developmental defects.

Maize also has two Pol II subtypes defined by paralogous rna polymerase b2 subunits (Haag et al., 2014). In Arabidopsis, Pol II can provide scaffold transcripts for RdDM-type modifications (Zheng et al., 2009), so it is also possible that one or both of the maize Pol II subtypes play a role in the paramutation process. Run-on transcription results show that the sequences mediating paramutation at *b1* are transcribed by Pol II (Arteaga-Vazquez et al., 2010), but it is unknown which subtypes are responsible.

Methylome profiles of *rpm1*, *rp(d/e)2a*, and *rdr2* mutants show that maize Pol IV influences 5meC patterns (Li et al., 2014b), consistent with an RdDM mechanism. One or more of the maize Pol V complexes likely function

in an RdDM-type capacity, as some RPE1-associated proteins are orthologous to those interacting with Arabidopsis Pol V, including DEFECTIVE IN MERISTEM SILENCING3, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (CHR127), ARGONAUTE4 (AGO105 and/or AGO119), and AGO6-type (AGO121) proteins (Fig. 2; Haag et al., 2014). This evidence strongly indicates the existence and function of at least one maize RdDM pathway. The diversity of maize DNA-dependent RNA polymerases and AGO-type proteins (Singh et al., 2011) makes it likely that unique and/or expanded roles are assigned to various polymerase complexes. Paramutations may be dependent on one or more of these diversified functions.

GENOMIC FEATURES AFFECTING PARAMUTATION PROPERTIES

Paramutation only occurs among specific alleles having unique features responsible for their behaviors. In an RdDM-type working model (Fig. 1), these features would attract both Pol IV and Pol V complexes to mediate a 24-nucleotide RNA trans-acting signal. Previous studies functionally implicated transcriptional enhancers at both *P1-rr* and *B1-Intense* (*B1-I*; Sidorenko and Peterson, 2001; Stam et al., 2002), direct repeats at *R-st*, *P1-rr*, and *B1-I* (Kermicle et al., 1995; Sidorenko and Peterson, 2001; Stam et al., 2002), and promoter sequences at a subcomponent of the complex *R-r* haplotype (Fig. 3; Kermicle, 1996; Walker, 1998). All these features identified either genetically and/or by transgenesis have direct effects on the expression of

the genes being assayed (Eggleston et al., 1995; Kermicle, 1996; Walker, 1998; Sidorenko and Peterson, 2001; Stam et al., 2002).

TEs may also play a role in mediating paramutation behaviors (Walker, 1998; Erhard et al., 2013; Goettel and Messing, 2013), since TEs are a primary source of 24-nucleotide RNAs (Nobuta et al., 2008; Gent et al., 2014). Promoter sequences required for both kernel-specific expression and paramutation at *R-r* are mostly composed of one terminal end of a CACTA-type TE named *doppia* (Fig. 3B; Walker et al., 1995). At the paramutable *P11-Rhoades* (*P11-Rh*) allele, 5meC patterns at a similar promoter-proximate *doppia* fragment are dependent on RPD1, RDR2, and RMR1, indicating that Pol IV targets these features (Hale et al., 2007). In the

absence of these Pol IV components, or RP(D/E)2a, *P11-Rh* is ectopically expressed in the aleurone layer of the endosperm (Erhard et al., 2013) in a mottled pattern reminiscent of *P11-Blotched* (Cocciolone and Cone, 1993), a related *pl1* allele having an identical DNA sequence (including the *doppia* fragment) that does not participate in paramutation (Hollick et al., 2000; Gross and Hollick, 2007). Although these results indicate that the *doppia* sequences are insufficient for paramutation at *P11-Rh*, their potential necessity remains unknown. The *doppia* sequences, however, are very likely necessary for paramutation at *R-r* (Kermicle, 1996; Walker, 1998).

Because pigmentation from *P11-Blotched* is also enhanced in other *rmr* mutants, it is hypothesized that

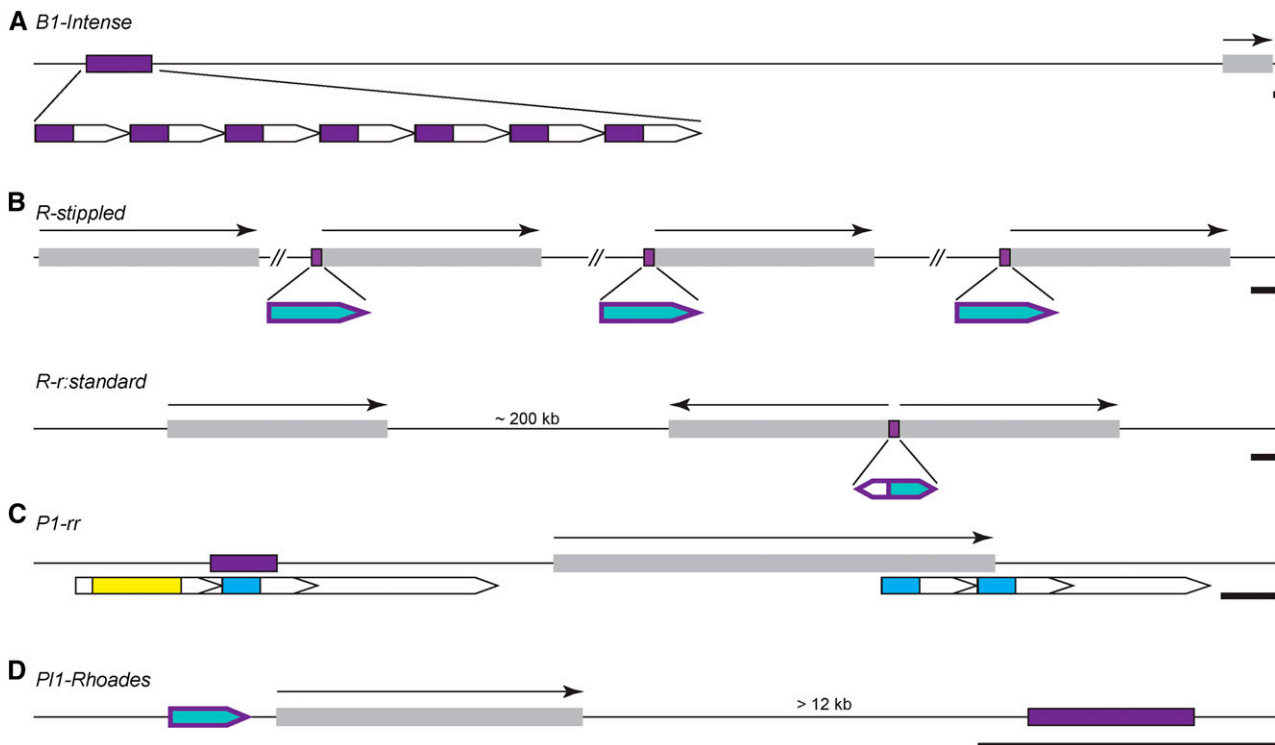


Figure 3. Regulatory features implicated in paramutation behaviors. Schematic representations of alleles showing paramutation behaviors are drawn to scale (black bars = 1 kb) unless indicated otherwise. Gray boxes with overhead black arrows delimit transcribed genic regions. Pentagons identify repeated sequences. Purple features represent regions implicated as having regulatory importance to paramutation behaviors. A, The *B1-I* distal enhancer (purple box) is composed of seven 853-bp tandem repeat (TR) units (pentagons enlarged below) with important 413-bp subregions (purple; Stam et al., 2002; Belete et al., 2013). B, *R-st* has four tandem *r* gene duplications, with the centromere-distal three having promoter-proximate *doppia* sequences (purple boxes on the top line and purple-rimmed teal pentagons enlarged below; Matzke et al., 1996). The sizes and directions of these *doppia* sequences are unknown (Matzke et al., 1996). Hashed lines indicate distances of unknown length. *R-r* has three *r* gene coding regions with a 226-bp *doppia* fragment (purple box on the top line and purple-rimmed teal pentagon enlarged below), a 161-bp region (purple-rimmed white pentagon), and a 15-nucleotide *doppia* terminus (purple tip) serving as a promoter for the inverted pair (Walker et al., 1995). C, The *P1-rr* coding region is flanked by a large duplication (large pentagons below the line) containing internal tandem duplications approximately 1.2 kb in length (smaller pentagons) each containing approximately 600 bp of unique sequence and a *Mutator*-like TE fragment (blue boxes; Goettel and Messing, 2013). The 5'-most repeat contains a *hAT* TE (yellow box). The area highlighted by the purple box is sufficient to induce paramutation (Sidorenko and Peterson, 2001; Sidorenko and Chandler, 2008). D, *P11-Rh* has a promoter-proximate *doppia* fragment (purple-rimmed teal pentagon) required for kernel-specific expression but insufficient to facilitate paramutation. A genetically defined downstream transcriptional enhancer of unknown size (purple box) associated with paramutagenic function (Erhard et al., 2013) is highlighted.

the *doppia* fragment serves as a Pol IV-regulated controlling element specifying aleurone expression (Erhard et al., 2013). By recurrent crossing of heterozygous (+/*rpdl*) and homozygous (*rpdl/rpdl*) mutant siblings, *P11-Rh*-specified aleurone expression continually intensifies to a solid-color phenotype (Erhard et al., 2013). This ectopic aleurone expression persists even in the presence of normal RPD1, indicating that heritable changes in gene regulation can be conditioned by this breeding scheme. Although the loss of RPD1 reduces the *doppia* fragment 5meC levels as expected, heritable increases in aleurone pigmentation seen in the presence of RPD1 are not associated with changes in 5meC patterns, leaving the nature of the meiotically heritable mark unknown. Because similar heritable conditioning is not seen in *rdr2*, *rnr1*, or *rp(d/e)2a* mutants (Erhard et al., 2013), it is inferred that some unique or complementary function of Pol IVb is required to define this transgenerational epigenetic variation. These results implicate the *doppia* region as a Pol IV-dependent regulatory element of *p11* potentially unrelated to the paramutation mechanism.

Similar to *B1-I*, *P11-Rh* also has a distal feature necessary for both high levels of expression and paramutation (Erhard et al., 2013). This 3'-localized distal (greater than 12 kb) element (Fig. 3D) was genetically identified by a recombinant derivative of *P11-Rh* that coincidentally lost its ability to undergo paramutation. The nature of these 3' paramutation sequences is currently unknown, but paramutation-defective derivatives isolated from active *Mutator* TE lines and γ -irradiation coincidentally lose high levels of *p11* expression (Gross, 2007; Gross and Hollick, 2007), indicating, as with *B1-I*, that enhancer function is related to paramutation behaviors. At *B1-I*, the 5' distal (approximately 100 kb) enhancer (Fig. 3A) and two other intervening regions physically loop to the *b1* gene promoter in frequencies correlated to the strength of *b1* transcription (Louwers et al., 2009). The distal enhancers in both *B1-I* and *P11-Rh* represent independent examples in which paramutation affects long-distance regulatory interactions.

The *B1-I* enhancer is currently the best characterized feature necessary for paramutation, and recent transgenesis results indicate its sufficiency (Belele et al., 2013). Both paramutagenic strength and enhancer-promoter associations depend on the enhancer's seven TRs of a unique 853-bp sequence (Fig. 3A; Stam et al., 2002; Louwers et al., 2009). Belele et al. (2013) found that either the entire unique sequence or just the 5' half of this repeat unit (413 bp) recapitulates paramutation behaviors in an RDR2-dependent manner, but only in TR organizations. The remaining half of the repeat unit is insufficient, even in tandem arrays, to affect changes at endogenous *B1-I*. Not all TR transgenes containing the 413-bp subfragment were initially able to induce paramutation, but some acquired this ability following exposure to endogenous paramutagenic *B'*. Thus, most of the TR transgenes could accomplish essentially the same behaviors as the endogenous 5' heptarepeat enhancer (*b1TR*) but from various positions in the genome. The

efficiency of these TR transgenes in inducing meiotically heritable *B'* states, however, was often (eight of 11) less than 100% (Table I), indicating that other unknown aspects of the THI were not fully replicated. In opposition to the RdDM model, not all TR transgenes that produced small RNAs (sRNAs) could induce paramutation (Belele et al., 2013). This finding agrees with previous results showing no correlations between *b1TR* sRNAs and either induction or maintenance of *B1-I* paramutations (Arteaga-Vazquez et al., 2010).

NATURE OF THE MEIOTICALLY HERITABLE EPIGENETIC MARK

In maize, paramutation is genetically defined by a locus-specific behavior without the apparent contribution of cytoplasmic factors (Brink et al., 1960; Coe, 1966; Hollick et al., 1995; Hollick, 2012). In both *D. melanogaster* and *C. elegans*, meiotically heritable paramutation-like behaviors occur in germline lineages and require the cytoplasmic transmission of sRNAs (Ashe et al., 2012; de Vanssay et al., 2012; Shirayama et al., 2012; Seth et al., 2013; Wedeles et al., 2013; Sapetschnig et al., 2015). Analogous to the canonical *Schizosaccharomyces pombe* RNAi mechanism, *C. elegans* germline sRNAs utilize Argonaute proteins and Pol II scaffold transcripts to index self (germline-expressed genes) and nonself (e.g. TEs and foreign transgenes) sequences (for review, see Youngman and Claycomb, 2014). Perhaps the inheritance of paramutations occurring in plants similarly requires the germline transmission of sRNAs in addition to alleles poised to recruit Pol V. This scenario would account for the locus specificity of paramutation seen in plants as well as a requirement for extrachromosomal sRNAs. So far, however, paramutation-specific sRNAs have not been identified (Gross and Hollick, 2007; Arteaga-Vazquez et al., 2010; Belele et al., 2013).

Cytosine methylation is commonly cited as both a mitotically and meiotically heritable epigenetic mark in many organisms (Richards, 2006), although many species (e.g. *S. pombe* and *D. melanogaster*) with clear examples of meiotically heritable epigenetic information lack this methylation machinery. In Arabidopsis, Pol IV can be recruited to chromatin having generally repressive histone H3 modifications (unmethylated or monomethylated Lys-4 and dimethylated Lys-9 [H3K9me2] residues) via a tandem-Tudor domain found in the SAWADEE HOMEODOMAIN HOMOLOG1 (SHH1) protein (Law et al., 2013). Haag et al. (2014) found that at least one or more maize Pol IV and Pol V complexes contain related proteins (SHH2a/SHH2b; Fig. 2) that might provide similar recruitment functions. Arabidopsis Pol V is recruited directly to DNA via two redundant noncatalytic histone methyltransferases that bind 5meC (Johnson et al., 2014), and the H3K9 methyltransferase KRYPTONITE is similarly recruited to 5meC residues (Du et al., 2014). In combination with H3K9me2 binding of the DNA methyltransferases CHROMOMETHYLASE3 (CMT3) and CMT2, a

self-reinforcing maintenance of non-CG-context 5mC and H3K9me2 is achieved. Thus, in Arabidopsis, non-CG 5mC patterns appear sufficient to initiate a feed-forward RdDM cycle. Maize apparently has no CMT2 ortholog, and recent methylome profiles show that Pol IV defines only a small fraction of the genome-wide 5mC patterns (Li et al., 2014b), unlike in Arabidopsis (Stroud et al., 2013).

Several studies have examined correlations between 5mC and paramutagenicity. The RdDM-based working model for paramutation predicts that 5mC patterns at important features should be altered in manners coincident with gene repression. The 1.2-kb enhancer of *P1-rr* is one such feature recently evaluated in detail (Fig. 3C; Sekhon et al., 2012; Goettel and Messing, 2013). From transgenesis results, these *P1-rr* sequences can induce paramutation at naive *P1-rr* (Sidorenko and Peterson, 2001; Sidorenko and Chandler, 2008); however, similar sequences found in the neutral *P1-ww* allele are insufficient to acquire paramutagenicity (Sidorenko and Chandler, 2008). The observation that higher 5mC levels at *P1-rr* enhancers are associated with paramutagenic reference states agrees with the RdDM model (Haring et al., 2010; Sekhon et al., 2012). These correlations were further delineated across the upstream 1.2-kb enhancer to short *p1*-specific repeated sequences (approximately 600 bp) flanked and/or interrupted by TE fragments (*hAT* [for *hobo Activator Tam3*] and *Mutator*-like elements) not present in the otherwise nearly identical *P1-ww* enhancer sequences (Goettel and Messing, 2013). Goettel and Messing (2013) hypothesize that transitive spreading of 5mC from these highly methylated TEs across the intervening sequence could be responsible for the paramutation behavior of these enhancer sequences at *P1-rr*.

Evidence that 5mC changes represent either paramutation signatures or triggers remains equivocal, particularly at *B1-I* (Haring et al., 2010; Bebele et al., 2013). Bebele et al. (2013) found that paramutagenic *b1TR*-based transgenes could have 5mC levels (measured with methylation-sensitive restriction digests) even lower than those seen at nonparamutagenic *B1-I* alleles, implying that extensive 5mC modifications are not an essential feature of paramutagenicity. The efficacy of transgene-induced paramutagenicity at endogenous *b1TRs*, however, was generally correlated with increased 5mC levels. Other correlations were noted during the induction of paramutation at *B1-I* (Haring et al., 2010) and more recently at the *p1* enhancer sequences (Goettel and Messing, 2013). During the induction of *B1-I* paramutation, significant reductions in pigmentation precede major 5mC changes (Haring et al., 2010) at the *b1TRs*, although minor changes detected immediately following germination may be responsible for inhibiting *b1TR* enhancer activities. Maximum *B'*-like 5mC levels are only acquired late in development (Haring et al., 2010), at a time consistent with genetic mosaic results showing that somatically heritable repression of *B1-I* is not established until after the 10-leaf stage (Coe, 1966). It appears as though some form of transrepression occurs during early

development and is followed by progressive increases in 5mC. For paramutation induction at *P1-rr*, Goettel and Messing (2013) found no correlation between the repression of cob pigmentation and 5mC levels using bisulfite sequencing of leaf-derived DNA, leading them to speculate that 5mC changes lag behind the establishment of a repressed epigenetic expression state. These observations at both *b1* and *p1* are consistent with the idea that heritable changes, possibly related to extensive 5mC modifications, do not occur until very late in development, perhaps during meiosis (Coe, 1966).

In both maize and Arabidopsis, recent methylome comparisons between parents and hybrids identify dominant 5mC patterns suggestive of paramutation-like events (Eichten et al., 2013; Regulski et al., 2013; Greaves et al., 2014). Importantly, however, neither the locus specificity (showing that methylation functions genetically map to a specific allele or locus), functional relevance to gene regulation, nor subsequent trans-silencing properties of these events is known. Several studies have now catalogued thousands of differentially methylated regions among inbreds or ecotypes, and many of these 5mC patterns show strong trans-generational stability (Becker et al., 2011; Schmitz et al., 2011; Li et al., 2014a). Changes to these differentially methylated region 5mC patterns have been highlighted as potential examples of paramutation (Eichten et al., 2013; Regulski et al., 2013; Greaves et al., 2014) without considering other available mechanisms or explanations that may account for these changes. Further pedigree-based methylome studies combined with allele-specific transcriptome profiling should begin to distinguish these possible mechanisms.

Another possible meiotically heritable mark accounting for transgenerational inheritance of paramutagenic states may be histone based. There is currently little information regarding specific histone modifications at the *b1* and *p1* enhancer sequences (Haring et al., 2010; Sekhon et al., 2012), and no histone-modifying components have been reported among *rmr* and *mop* loci. Nonetheless, given the apparent integral role of H3K9me2 in maintaining 5mC patterns in Arabidopsis (Du et al., 2014), it is predicted that such modifications would similarly correlate with paramutable and paramutagenic states. Indeed, both *b1* and *p1* enhancers have elevated H3K9me2 levels coincident with the reduced gene expression associated with paramutagenic states, but Haring et al. (2010) showed that this was a tissue-restricted correlation, which was not expected from a persistent somatically inherited mark of paramutation. The dominant *ufo1-1* mutation also coincidentally affects both 5mC and H3K9me2 levels in pericarp tissues where gene expression is monitored (Sekhon et al., 2012). These initial results indicate that H3K9 modifications are likely to be correlated with gene function, but their role as potential meiotically heritable changes remains unclear.

Other histone modifications may be important, including changes at H3 Lys-27 (H3K27) residues

(Haring et al., 2010). Recently, Crevillén et al. (2014) implicated reversible modifications of H3K27 in the transgenerational reprogramming necessary for vernalization in *Arabidopsis*. Intriguingly, Li et al. (2015) speculate that *Arabidopsis* Pol IV recruitment at specific regions may require H3K27 monomethylation marks, a feature potentially heritable by replication-dependent deposition of H3.1 variant histones (Jacob et al., 2014). These features remain to be evaluated for inducible, stable, and reversible paramutagenic states. Although the nature of the meiotically heritable epigenetic marks responsible for the transgenerational changes associated with paramutation remains unclear, there are now multiple paramutation-specific sequences and mutant stocks available for detailed molecular study.

CHALLENGES TO THE EMERGING RdDM-TYPE WORKING MODEL

Based on the design of genetic screens performed to date, all *rmr* and *mop* factors are required to maintain the somatic repression of pigmentation coincident with paramutagenic states. Only some of these factors, however, are necessary to maintain the meiotically heritable information specifying paramutagenic function. For instance, paramutagenic *P11-Rh* can revert back to a meiotically stable paramutable form in the absence of RMR1 (Hollick and Chandler, 2001; Hale et al., 2007; Barbour et al., 2012) but not in the absence of RMR2 (Barbour et al., 2012). Such reversion events occurring in specific mutant backgrounds can make it difficult to distinguish between functions required for establishing versus maintaining paramutant states (for review, see Hollick, 2012). In considering evidence relevant to an RdDM-based working model, it is important to distinguish between these various functions because they may reflect mechanistically distinct features. Accordingly, the following observations need to be accommodated by any paramutation working model.

Components Required Downstream of 24-Nucleotide Biogenesis Have Not Been Implicated in Examples of Paramutation

Despite nearly saturation-level screens for *rmr* factors (J.B. Hollick, unpublished data), all cloned loci encode proteins specific for the 24-nucleotide RNA biogenesis part of a presumed RdDM pathway. The maize genome encodes downstream RdDM orthologs, including de novo methyltransferases (Li et al., 2014b), yet none have been discovered that affect paramutation behaviors. It remains possible that one or more of the nine remaining uncharacterized *rmr* loci will identify such molecules. This part of the pathway may be essential for gametophyte function and/or proper embryonic development, and some components may have retained paralogs with functional redundancy. These possibilities are consistent with failed attempts to obtain maize double mutants for some of

the presumed CMT3-like orthologs (Li et al., 2014b). Given the large number of Pol V-associated proteins required for *Arabidopsis* RdDM (Matzke et al., 2015) and the identification of maize orthologs by sequence similarity, it is surprising that none of these maize orthologs have been identified in the *rmr* and *mop* screens. It remains plausible that paramutation is dependent on strictly Pol IV-based functions that may or may not require sRNAs. It is also possible that these screens, designed to identify functions required to maintain the somatic repression associated with paramutant states, do not always detect functions related to RdDM initiation.

Paramutation-Specific RNAs Have Not Been Identified

The *b1TR* enhancer sRNAs are also produced, in seemingly similar quantities, from neutral *b1* alleles (Arteaga-Vazquez et al., 2010) and even from non-paramutagenic *b1TR*-derived transgenes (Belele et al., 2013), indicating that the presence of these sRNA species alone is insufficient. However, the fact that inverted repeat transgenes producing Pol IV-independent 24-nucleotide *b1TR* RNAs can induce paramutation (Arteaga-Vazquez et al., 2010; Sloan et al., 2014) indicates direct actions of these 24-nucleotide species. Overexpression of a CXC domain-containing protein that binds to the insufficient portion of the *b1TR* (Belele et al., 2013) can also induce *B1-I* paramutation (Brzeska et al., 2010), indicating that non-sRNA triggers can suffice. Thus, (1) sRNAs are not causal; (2) the tissue(s), phase(s), or time point(s) in which a difference in sRNAs occurs has not been assayed; (3) important differences in sRNA levels have been too subtle to discern; or perhaps (4) there are other features, such as developmentally regulated or cell type-specific Pol V scaffold transcripts, required to interpret the sRNA signals.

Mutations of *rpm1* and Other *rmr* Genes May Not Specifically Affect Paramutation

Because pigmentation from *P11-Blotched* is intensified in the absence of RPD1 (Erhard et al., 2013), this allele could have been used to identify *rmr*-type factors. *P11-Blotched* does not show paramutation behaviors, despite having the same *doppia* element and coding sequences as *P11-Rh* (Gross and Hollick, 2007), so it is possible that the genetic screens are not specific for the paramutation mechanism per se. Mutations affecting the general behavior of TEs may have either direct or indirect regulatory consequences on the regulation of specific alleles. Given that the maize genome composition is greater than 85% TE (Baucom et al., 2009; Schnable et al., 2009) and that many TEs flanking genes are likely sources or targets of RdDM (Gent et al., 2014), there is great potential for such heritable regulatory effects (McClintock, 1951).

Paramutation Can Occur in the Absence of Some sRNA Biogenesis Components

The absolute genetic requirement for *rdr2* (Dorweiler et al., 2000) and *rpm1* (Hollick et al., 2005) functions for the induction of *r1*, *b1*, and *pl1* paramutation strongly indicates the role of 24-nucleotide RNAs, but paramutations can occur in other 24-nucleotide-deficient mutants. Paramutations at *r1*, *b1*, and *pl1* all occur in the absence of RMR1, an ATPase associated with one or both Pol IV complexes (Haag et al., 2014), despite a major depletion of 24-nucleotide RNAs (Hale et al., 2007). Another RMR1-like protein, CHR167, also copurifies with RPD1 (Haag et al., 2014), so it remains possible that a Pol IV complex defined by CHR167 provides essential sRNAs. Loss of RMR2 also depletes 24-nucleotide RNAs yet has no effect on the induction of paramutation at *r1* and is only partially required for induction at *pl1* (Barbour et al., 2012). Neither RMR2 nor its two paralogs was found complexed with RPD1, RPE1, RP(D/E)2a, or RDR2 (Haag et al., 2014), but these paralogous proteins could provide semiredundant functions.

Paramutation at *b1* Can Be Induced in the Absence of Pol IVa in an Apparent Parent-of-Origin Fashion

Paramutation could be induced in *rp(d/e)2a* mutant offspring when paramutagenic *B'* was transmitted from *rp(d/e)2a* mutant mothers but not *rp(d/e)2a* mutant fathers (Stonaker et al., 2009). With recent proteomic data confirming the existence of Pol IVb subtypes, this finding may indicate that maternal-specific information is required for paramutation induction. One speculative hypothesis is that maternally provided Pol IVb-derived sRNAs are amplified only in female gametophytes transmitting paramutagenic templates. Developing cobs at the stage of egg sac development are a highly abundant source of 24-nucleotide RNAs (Hale et al., 2007; Nobuta et al., 2008; Erhard et al., 2009; Stonaker et al., 2009; Barbour et al., 2012), so perhaps these represent sRNAs sufficient to induce paramutation in the absence of Pol IVa. In *Arabidopsis*, there is compelling evidence of pollen-transmitted sRNA information (Slotkin et al., 2009; Calarco et al., 2012). Maize sperm cells, however, do not appear to transmit paramutagenic sRNAs by themselves (J.B. Hollick, unpublished data; for discussion, see Hollick, 2012), so paramutagenic alleles transmitted from male gametophytes may require Pol IVa in the newly formed zygote to produce the types of sRNAs that induce paramutation. Because some paramutation-like behaviors in metazoans also appear to involve the maternal transmission of sRNAs, similar ideas deserve further experimental attention in plants.

These apparent inconsistencies mentioned here no doubt highlight interesting aspects of paramutation biology yet to be discovered. Many unanswered questions remain to be addressed, but an important role for a basal RNAi mechanism appears increasingly certain.

CONCLUSION

The potential diversity of maize RdDM-type pathways combined with the non-RdDM functions of Pol IV represent a different landscape of epigenetic control relative to *Arabidopsis* (Li et al., 2014b; Erhard et al., 2015). Whether examples of paramutation remain unique to this landscape remains to be seen. Nonetheless, the *Arabidopsis* RdDM pathway provides a useful paradigm with which to model paramutation behaviors and generate meaningful hypotheses. It is widely expected that continued research on examples of paramutation in maize will broaden our understanding of sRNA biology while exposing increasing evidence of rapid regulatory evolution in the grasses.

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