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Review

Paramutation in maize and related behaviors in metazoans

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ABSTRACT

Paramutation refers to both the process and results of *trans*-homolog interactions causing heritable changes in both gene regulation and silencing abilities. Originally described in plants, paramutation-like behaviors have now been reported in model metazoans. Here we detail our current understanding of the paramutation mechanism as defined in *Zea mays* and compare this paradigm to these metazoan examples. Experimental results implicate functional roles of small RNAs in all these model organisms that highlight a diversity of mechanisms by which these molecules specify meiotically heritable regulatory information in the eukarya.

Contents

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1. Introduction

Paramutation is a genetic term used to describe both the process and outcome of directed and meiotically heritable changes in both gene regulation and silencing abilities that are influenced by *trans*-homolog interactions (THI) [1]. Usage of the term is similar to that of classical "mutation" without regard to molecular hallmarks. Unlike mutations, however, paramutations occur in predictable, invariant, and sometimes reversible manners [2].

Deviations from expected Mendelian ratios of trait transmission – such as exclusive inheritance of a dominant trait – are one hallmark of paramutation events. However, pedigree analyses following independent genetic and/or cytogenetic markers distinguish examples of paramutation from other modes of transmission ratio distortion (TRD) [3] including cytoplasmic inheritance [4], preferential chromosome segregations [5], gametic competitions [6], and zygotic lethalities [7]. Dominant inheritance of abnormal leaf morphologies characteristic of the "rogue" phenotype in garden peas is commonly cited as the first published example of paramutation [8,9], without genetic evidence excluding other TRD models.

Several TRD examples occurring in metazoans have paramutation-like properties (see other contributions to this volume) fuelling the opinion that paramutation is also widespread in animals. This review defines the paramutation process as originally described in *Zea mays* (maize) and evaluates the similarities and differences among these metazoan examples. The involvement of small RNAs (sRNAs) in all these cases is specifically highlighted.

2. Definitions

Alleles typically conform to the Mendelian expectation of segregating unchanged from heterozygous condition (Fig. 1A). Deviations from this expectation can be due to various mechanisms including cytoplasmic inheritance (Fig. 1B). Inheritance patterns of seed pigment conferred by the *red1* (*r1*) locus in maize [10] established a definition of paramutation [1] as an invariant, locus-specific, yet parent-of-origin-independent, behavior (Fig. 1C). By following the inheritance of genetic markers from heterozygous individuals, a specific *r1* allele (*R:stippled*; *Rst*) was found to influence heritable properties of the alternate *r1* allele, *R-r:standard*; (*R-r*) [11,12].

2.1. Nomenclature

The terms "paramutable" and "paramutagenic" were applied to *r1* alleles either susceptible to, or capable of facilitating (or inducing) paramutations, respectively [1]. Certain *r1* alleles (*Rst* and *R:marbled*) are strictly paramutagenic [10,13] while others (*R-r*) are paramutable [10]. Another hallmark known as "secondary paramutation" occurs when paramutable alleles become paramutagenic (e.g. *R-r* is transmitted from *Rst*/*R-r* plants in a paramutagenic form denoted *R-r′*) [11]. This behavior distinguishes paramutations from other examples of heritable *trans*-dominant silencing [14]. Alleles neither paramutable nor paramutagenic are termed "neutral". In some cases, neutral alleles are genetically similar to "nulls" as reversions of *R-r′* to *R-r* occur in both *R-r′*/- hemizygotes and *R-r′*/*r-g* heterozyogtes [15]. These reversion behaviors indicate that THIs are needed for both inducing and stabilizing paramutations.

2.2. Genetic definition of paramutation

Paramutation, as defined by the inheritance behaviors of endogenous *r1* alleles, occurs at three other maize loci (Table 1) [16–19] and at one in *Lycopersicum esculentum* (tomato) [20]. In all cases, results of reciprocal crosses show that there are no parent-of-origin effects. Additionally, all examples show locus-specific behavior: only meiotic products transmitting a paramutagenic allele confer paramutation to offspring (Fig. 1C). This behavior demonstrates a particularly important genetic proof that distinguishes paramutation from other TRD mechanisms. Lastly, all examples show secondary paramutation. Thus, paramutation has a classic genetic definition based on specific inheritance properties of alleles conferring a phenotypic trait.

2.3. Molecular definition of paramutation

There is currently no genomic context of a paramutation in any sense similar to that of a mutation. Recent examples of *trans*-dominant cytosine methylation and demethylation behaviors occurring in both *Arabidopsis thaliana* and maize hybrids have been cited as potential paramutation examples [21–24] without compelling evidence that such changes in 5-methylcytosine (5meC) patterns are causal to gene regulation. Locus-specificity and secondary paramutation tests also remain to be evaluated. Given these uncertainties, and the potential for confusion between genetic and molecular definitions, it remains prudent to reserve the term paramutation for strictly genetic behaviors related to gene regulation.

3. Paramutation in maize

3.1. Examples

Specific alleles of the *booster1* (*b1*), *purple plant1* (*pl1*) and *pericarp color1* (*p1*) loci all exhibit paramutation (Table 1) similar to that seen at *r1* [16–19,25]. Each locus encodes a transcription factor required for flavonoid biosynthesis, and these paramutable alleles are highly expressed in their respective tissues [26] conferring strong pigment production. High transcription rates are facilitated by specific enhancer sequences: seven tandem repeats (TRs) ~100 kb 5′ of the *b1* coding region [27], a genetically defined region 3′ of the *pl1* coding region [28], and a promoter-proximal region of a direct repeat flanking the *p1* transcription unit [18]. These high expression states are inherently unstable and can spontaneously change to transcriptionally repressed forms coincident

Table 1
Common features of paramutation-like behaviors across species.

Locus	Affected allele	Inducing agents	Genetic properties			Small RNAs implicated	*Trans*-acting factors implicated	Molecular associations	References
			Parent-of-origin effect	Locus-specific	Secondary silencing				
Zea mays									
r1 (*red1*)	*R-r:standard*	*Rst*, *Rmb*, *R-r′*	No	Yes	Yes	24-nt	RPD1, RP(D/E)2a, RDR2	5meC	[1,10,11,13,34,35,37,39,40,46,47]
b1 (*booster1*)	*B1-Intense*	*B′*, *b1* TR-based transgenes	No	Yes	Yes	24-nt	RPD1, RP(D/E)2a, RDR2	5meC, H3K27me2	[16,25,29,30,34–40,48]
pl1 (*purple plant1*)	*Pl1-Rhoades*	*Pl′*	No	Yes	Yes	24-nt	RPD1, RP(D/E)2a, RDR2, RMR1, RMR2	None identified	[17,33–41]
p1 (*pericarp color1*)	*P1-rr*	*Prr′*, *p1* enhancer-based transgenes	No	Yes	Yes	24-nt	RP(D/E)2a, RDR2	5meC, H3K9me2	[18,19,49,67]
Mus musculus									
rasgrf1	*Rasgrf1*	*Rasgrf1*$^{tm3.1pds}$	Paternal imprinting required	Yes	Yes	None identified	N.D.	N.D.	[73]
kit	*Kit*$^{+}$	*Kit*^{tm1Alf}; cellular RNA, miRNA and oligo-ribonucleotides	Yes	Unclear	Yes	RNAs, miRNA and oligo-ribonucleotides	Dnmt2	N.D.	[75,76]
kit	*Kit*$^{+}$	*Kit*^{copGFP}	Yes	Unclear	Yes	piRNA and miRNA	Drosha Mov10l1	N.D.	[79]
sox9	*Sox9*	*miR-124*- and *Sox9*-based oligo-ribonucleotides	No	Unclear	Yes	miRNA and oligo-ribonucleotides	Dnmt2	H3K9me2, H3K9me3	[76,78]
cdk9	*Cdk9*	*miR-1* targeting *Cdk9*, *Cdk9* oligo-ribonucleotides	No	Unclear	Yes	miRNA and oligo-ribonucleotides	N.D.	N.D.	[77]
Drosophila melanogaster									
Transgene	*BX2* array and progenitors	*T-1*, *BX2** and *P-1152* cytoplasm	Maternal only	No	Yes	piRNA	AUB	N.D.	[89]
Caenorhabditis elegans									
Transgene	*gfp::csr-1 (RNAa)*	*gfp::csr-1 (RNAe)*	Yes	N.D.	N.D.	piRNA, 22-nt	HRDE-1, PRG-1, CSR-1, RDE-3, HPL-2, MUT-7, MES-3, MES-4	H3K9me3	[93,94]
Transgene	*spn4::mcherry::h2a::par-5*	*dpy-30::mcherry::gpd2/3::gfp::par-5* (germline silent)	Yes	No	N.D.	N.D.	N.D.	N.D.	[97]

N.D., not determined.

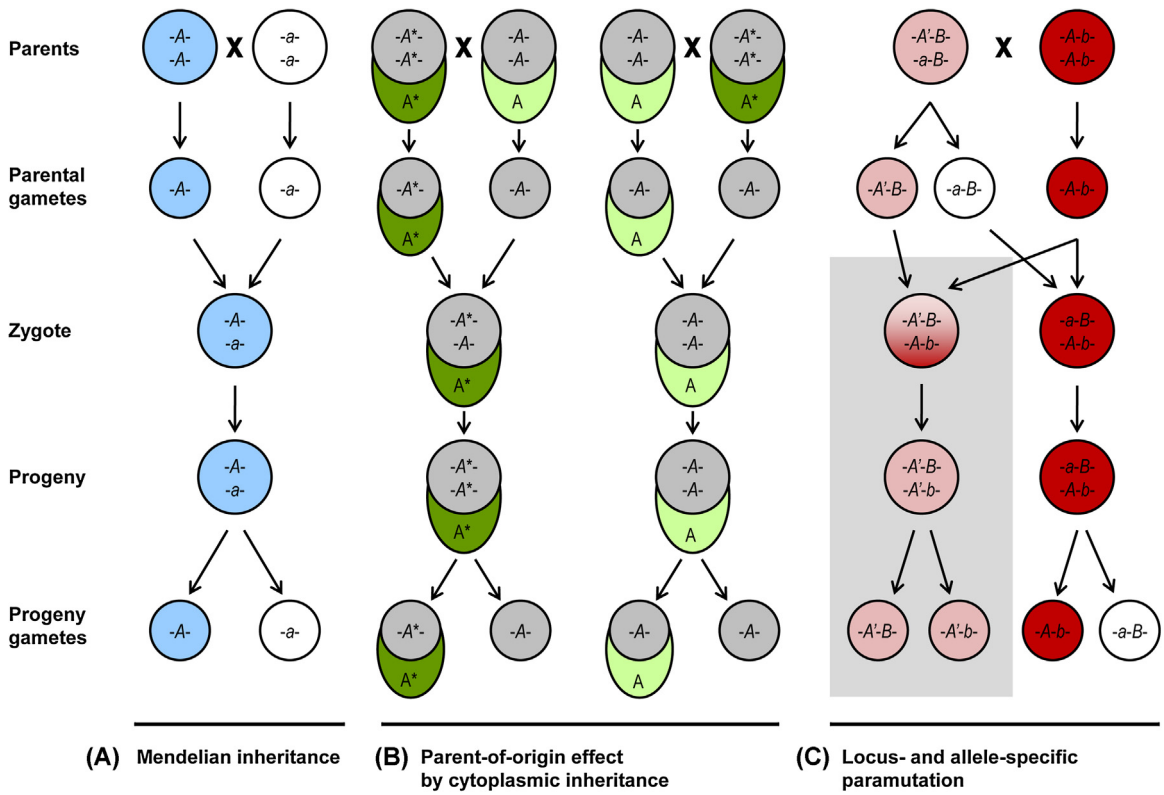

Fig. 1. Inheritance patterns. (A) Mendelian inheritance conforms to the law of segregation. Dominant and recessive alleles retain this relationship through mitosis and are sexually transmitted unchanged. (B) Inheritance patterns are dependent on a parent-of-origin, as exemplified by cytoplasmic inheritance. If nuclear gene expression depends on presence or absence of a corresponding cytoplasmic factor, preferential or exclusive contribution of cytoplasm from one parent can determine whether A^* or A are transmitted. (C) Inheritance patterns typifying paramutation (gray box). Here, allele A' is dominant to both A and recessive a. Regardless of parental origin, only progeny inheriting A' from parents show *trans*-dominant silencing. Alleles of a linked locus (b) are indicated to emphasize that only A' forms are transmitted from A'/A plants yet linked b alleles show Mendelian inheritance from $A'B/Ab$ individuals.

with the acquisition of paramutagenic properties. Although the trigger(s) for these spontaneous events remain unknown, transgenes comprised of these enhancer sequences either in tandem arrays (413 bp subfragment of the *b1* TR) [29], inverted hairpin (full *b1* TR) [30], or unknown organizations (*p1* enhancer) [18] can initiate similar instability.

Plant color is a convenient readout of both *b1* and *pl1* functions. When the paramutable *B1-Intense* allele (*B-I*) is combined with its paramutagenic derivative (B'), plant color is nearly equivalent to that of B'/B' individuals [25]. Mosaic analyses show the paramutable *B-I* state in *B-I*/B' plants remains unchanged in pigmentation potential up to developmental time points near initiation of meiosis. These results are consistent with a form of *trans*-homolog repression occurring during somatic development that becomes irreversible only during latter stages of plant development, at meiosis, during gametophyte (haploid phase) development, or during early embryogenesis in the next generation [25]. Based on these results and similar mosaic studies carried out at *pl1* (J. Hollick, unpublished results), it is the sexual transmission of an acquired *trans*-repression behavior that distinguishes a paramutation event. For example, neutral *Pl1-W22* is repressed in *trans* by the paramutagenic Pl' allele but *Pl1-W22* is transmitted unchanged from Pl'/*Pl1-W22* heterozygotes [31].

More recently, a potential example of paramutation occurring at the maize *low phytic acid1* (*lpa1*) locus was described in which an ethyl methanesulfonate-derived mutant allele (*lpa1-241*) shows *trans*-repression of endogenous alleles found in several inbred lines [32]. The *lpa1* allele from the B73 inbred appears to be spontaneously unstable and is inherited in a repressed state following exposure to *lpa1-241*. However, evidence of secondary paramutation remains ambiguous. This example hints that paramutation behaviors in maize are unlikely confined to genes controlling flavonoid biosynthesis.

3.2. *Paramutation* trans-*acting components*

3.2.1. *Genetic screens and the RNA-directed DNA methylation pathway*

Mutations define at least 14 distinct loci affecting repressed expression states of Pl'/Pl' individuals [33–36] (J. Hollick, unpublished results). Cloned loci (Table 1) encode proteins [36–41] potentially orthologous to components of an *A. thaliana* RNA-directed DNA methylation (RdDM) pathway [42] providing a working model invoking 24-nucleotide (24-nt) sRNAs as THI mediators (Fig. 2A).

In *A. thaliana*, RdDM uses two plant-specific DNA-dependent RNA polymerases, Pol IV and Pol V [42] to establish and maintain 5meC patterns. Pol IV and RNA-DIRECTED RNA POLYMERASE2 (RDR2) together produce double stranded RNA transcripts from non-coding DNA templates [43] that are processed into 24-nt sRNAs. Specific Argonaute proteins loaded with these sRNAs associate with Pol V and its nascent transcripts; together recruiting *de novo* DNA methyltransferases to establish 5meC patterns. In maize, there are at least two Pol IV and three Pol V subtypes [44] that add potential regulatory complexity to this ancestral RdDM mechanism [45].

Separate genetic screens for *mediators of* [B'] *paramutation* (*mop*) and components *required to maintain repression* (*rmr*) of Pl' states identified 24-nt sRNA biogenesis proteins. These include the largest subunit of Pol IV (rna polymerase d1; RPD1) [39], one of three

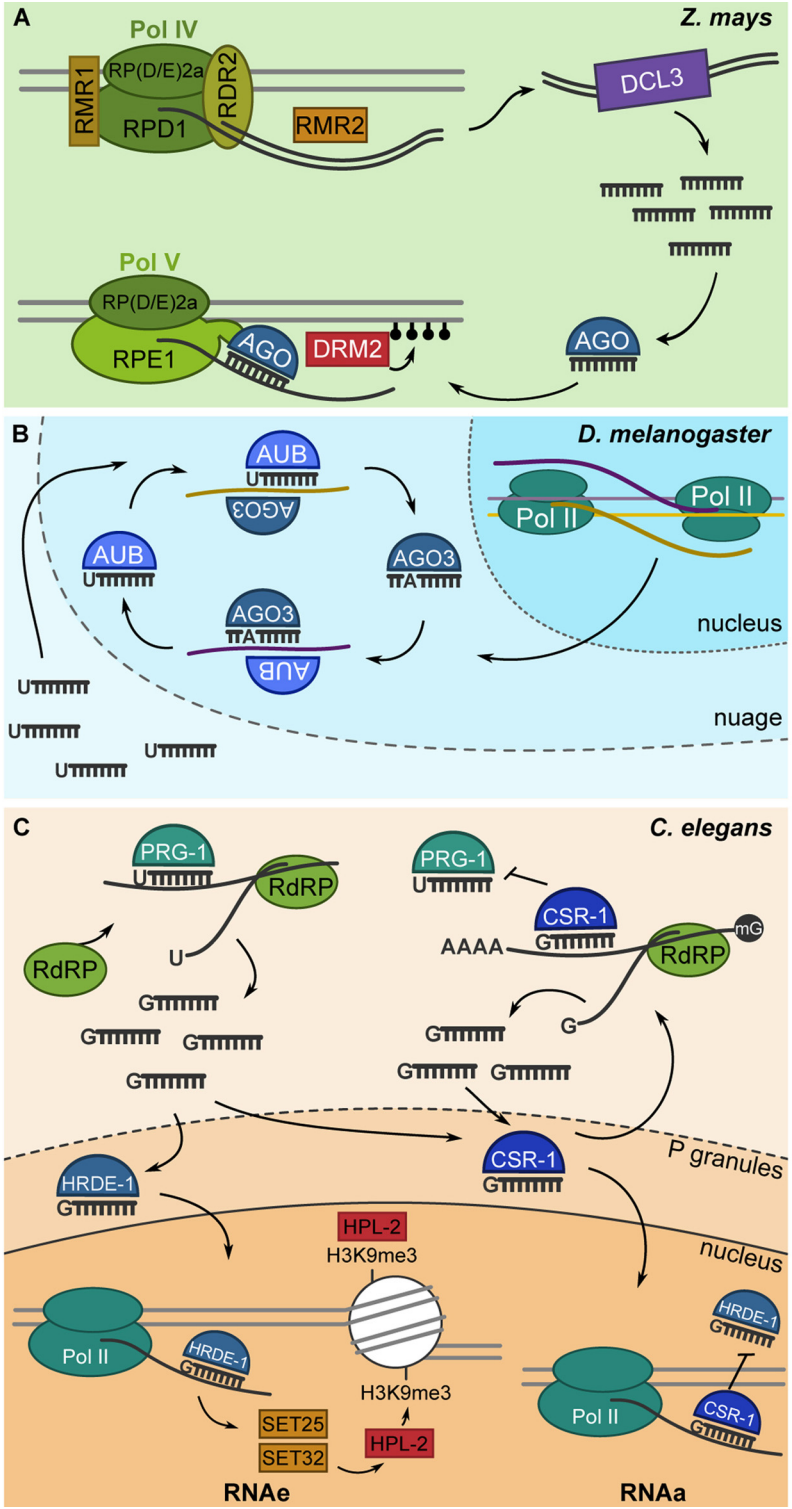

Fig. 2. Small RNA biology implicated in non-Mendelian inheritance. (A) Maize RdDM-type components are combined with presumed *A. thaliana* orthologs to illustrate a working model (see 3.2.1 for details). With RMR1, RMR2 and RDR2, Pol IVa produces double stranded RNA (black lines) that is processed by a presumed dicer-like3 (DCL3) into 24-nt sRNAs. These sRNAs presumably guide argonaute (AGO) proteins to Pol V nascent RNAs and effect recruitment of *de novo* cytosine methyltransferases (here the presumed ortholog to DOMAINS REARRANGED METHYLTRANSFERASE2; DRM2) to add methyl groups (black lollipops). (B) In the *D. melanogaster* germline, maternally-inherited piRNAs seed a ping-pong cycle requiring both sense (gold lines) and antisense (purple lines) RNAs (see 4.2.1 and 4.2.2 for details). Within the nuage, Aubergine (AUB) antisense piRNAs direct slicing of sense transcripts, and 5′ ends of these sliced RNAs bound to AGO3 direct slicing of antisense transcripts. (C) In *C. elegans* germline cells, sRNAs silence or license transcription of sequences represented among piRNAs (represented with 5′ U) (see 4.3.1 and 4.3.2 for details). Primary antisense piRNAs direct Argonaute PRG-1 to cytoplasmic RNA targets and prime RNA-dependent RNA polymerases (RdRPs) that produce 22-nt secondary and tertiary sRNAs (represented with 5′ G). Within P-granules, these sRNAs are loaded into one of at least two nuclear Argonautes, HRDE-1 and CSR-1. HRDE-1 effects RNA-induced epigenetic silencing (RNAe) by presumably recruiting histone methyltransferases SET25 and SET32 to nascent RNA scaffold transcripts to create H3K9me3 modifications bound by HPL-2. CSR-1 associates with nascent Pol II transcripts to effect RNA-induced gene activation (RNAa) and may compete with HRDE-1 for nascent RNAs. CSR-1 may also compete with PRG-1 for cytoplasmic mRNAs (5′ mG and 3′ poly A tail) to inhibit secondary and tertiary sRNAs from HRDE-1 loading.

potential second largest Pol IV subunits (rna polymerase (d/e)2a; RP(D/E)2a) [36,40], RDR2 [37], RMR1 (a Rad54-like ATPase) [38], and RMR2 (a small pioneer protein) [41]. Proteomic profiles show that all but RMR2 exist in one or more Pol IV complexes [44], and genetic experiments indicate that Pol IVa (defined by RP(D/E)2a) is a functionally distinct subtype [36]. None of the downstream RdDM-type effectors of Pol IV-derived sRNAs have yet been identified, potentially reflecting redundant or possibly essential functions for these maize orthologs.

3.2.2. Small RNAs and mutational analyses

Targets of maize Pol IV subtypes, mostly non-coding and transposable element (TE) sequences, are inferred from sRNA profiles of developing cobs (female inflorescences bearing haploid egg sacs) from RDR2-deficient plants [50]. RDR2 also produces 24-nt sRNAs from the unique *b1* TR sequences even though Pol II transcribes these repeats in both sense and antisense orientations [30]. The functional importance of these *b1* TR sRNAs is unclear given that neutral *b1* alleles produce similar sRNA profiles [30]. Nonetheless, RDR2 and RPD1 are required to establish paramutations at *r1*, *b1*, and *pl1* [34,35], strongly indicating a functional role for sRNAs in mediating the THI.

Mutant analyses identify differences between the various examples of paramutation. All five proteins identified so far are required to maintain the repressed pigment phenotype seen in *Pl′*/*Pl′* homozygotes but these phenotypes do not show whether paramutation is reversed or inhibited at *pl1* or any other locus. Genetic requirements for inducing paramutations are evaluated by combining paramutagenic and paramutable alleles in mutant backgrounds and scoring the behavior of these two alleles in resulting progeny. Results of such tests are complicated at *pl1* and *r1* as both paramutagenic *Pl′* and *R-r′* can revert back to paramutable reference states in certain mutant backgrounds [33–35,38]. In contrast, *B′* states never revert [51]. By tracking specific chromosomes carrying paramutable and paramutagenic versions, both reversion and induction frequencies can be assessed [34]. Accordingly, paramutable *Pl-Rh* changes to *Pl′* in the absence of RMR1 and the sRNAs dependent on RMR1 action [38]. RPD1, RDR2, and RMR1 are all required to maintain meiotically heritable *Pl′* states [33–35,38] but only RPD1 [34], and possibly RDR2 [35], are required to induce heritable changes of *Pl-Rh* to *Pl′* in *Pl-Rh*/*Pl′* plants. Induction of both *b1* and *r1* paramutation also require RPD1 [34] and RDR2 function [35]. In the absence of RP(D/E)2a, induction of paramutation still occurred at *b1* but only if *B′* was inherited through the female [36] – a potential parent-of-origin effect that deserves more experimental repetitions. RMR2 is partly required for induction of paramutation at *pl1* but not at *r1* [41] and its requirement at *b1* remains unknown. Collectively, these mutant analyses implicate a general requirement for one or more Pol IV subtypes containing RDR2 to induce meiotically heritable paramutagenic states at all three loci, yet clearly locus-specific requirements exist.

3.3. Sequences affecting paramutation

3.3.1. Transcriptional control regions

Sequences mediating or affecting paramutation have been functionally defined by mutation, recombination, and/or transgenesis at all four maize loci [18,27–30,46,47,52–54]. These sequences affect transcription of the respective gene and some correlations exist between repeat numbers and paramutagenic strength.

3.3.2. b1 *locus*

At the *B1-Intense* allele, the seven upstream TRs, composed of a unique 853 bp sequence, confer both high transcription levels and maximum paramutagenicity [27,52]. A five-repeat derivative has nearly equivalent pigment levels and is still fully paramutagenic, three repeats confer slightly weaker paramutagenicity, and alleles having only a single copy – including neutral alleles – are weakly expressed and incapable of acquiring paramutagenic function [27]. Transgenic tests showed that tandem repeats of the 5′ 413 bp of the TR are sufficient to both acquire and impart paramutagenicity [29]. The changes to *B-I* induced by these TR transgenes are unstable, unlike those induced by *B′*, indicating that other features of the endogenous *b1* TR are necessary for full paramutagenicity [29].

3.3.3. r1 *locus*

At *Rst* and *Rmb*, paramutagenic strength correlates with the number of intact *r1* genes found in these compound haplotypes [53,54]. At *R-r*, a specific inverted duplication of two *r1* genes separated by a 387 bp promoter largely consisting of a *doppia*-type DNA TE fragment [55] is the feature affected by paramutation. Because deletions of this promoter interfere with [46], or abolish [47], the capacity for secondary paramutation, either this small promoter sequence or perhaps the action of transcription itself is required for paramutation at *R-r*.

3.3.4. pl1 *locus*

At the *Pl1-Rhoades* allele, both enhancer and paramutation-essential sequences reside >12 kb 3′ of the coding region [28]. *Pl1-Rhoades* also has a promoter-proximal *doppia* fragment whose 5meC patterns are defined by RPD1, RDR2 and RMR1 [38]. This fragment may be necessary, but is insufficient, for paramutation as some neutral alleles retain this feature [28,31,56]. Most, if not all, *rmr* mutations could have been identified using these neutral *pl1* alleles raising the possibility that effects of these mutations on paramutation are indirect [28].

3.3.5. p1 *locus*

At *P1-rr*, the *p1* coding region is flanked by a 5.2 kb direct repeat each containing two internal tandem direct repeats of ~1.2 kb. The most 5′ of these repeats harbors a *hAT*-type DNA TE insertion, and a 1.2 kb fragment of the adjacent downstream repeat has both enhancer function and paramutation-inducing function as assayed by transgenesis [18]. Part of this enhancer sequence is unique to the *p1* locus but also contains a *Mutator*-like (*MULE*) DNA TE fragment [19].

3.3.6. Transposons

In all cases, the sequences facilitating paramutation behaviors represent endogenous features required for strong pigment phenotypes. There are currently no motifs or otherwise unifying characteristics of the *b1*, *p1*, and *r1* enhancer sequences implicating a role for specific DNA binding factors. A role for allele-specific TEs cannot be overlooked [28,57] and it remains possible that the variable results obtained with specific *b1* and *p1* enhancer transgene insertions [18,29] might be influenced by local TEs. Given that TEs comprise nearly 85% of the maize genome [58], many TE-related controls are possible. Certain examples of *Suppressor-mutator* (*Spm*) TE behaviors described by McClintock [59] reflect behaviors similar to those of paramutation [60,61]. In this context, perhaps paramutations in maize represent competitions between cellular mechanisms maintaining Pol II transcriptional competence at largely unique sequences and Pol IV transcription at repetitious feature. Growing evidence supports direct competitions between Pol II and Pol IV for both types of templates [57,62].

3.4. Nature of the meiotically heritable mark

Three sources of meiotically-heritable information adjunct to primary DNA sequence are considered here; sRNAs, 5meC, and specific histone modifications.

3.4.1. Small RNAs

Parent-of-origin-independent inheritance patterns defining locus-specific paramutation (Fig. 1C) are conceptually inconsistent with models requiring parental transmission of sRNAs. Unlike metazoans, plant meiotic products (spores) undergo independent mitotic divisions as haploid gametophytes to produce sperm (two per pollen grain) and egg (one per egg sac) cells. In *A. thaliana*, and most likely in all plants, the vegetative nucleus of the pollen grain produces 21-nt sRNAs that direct silencing of sperm cell targets [63,64]. Similarly, the accessory central cell produces sRNAs that may affect silencing in the egg [65]. Paramutation-specific sRNAs could therefore be produced in gametophyte accessory cells and passed to either sperm or egg, although two observations argue against this idea. First, mutations defining all 14 *rmr* loci are recessive. Thus haploid expression from these loci is not required. Second, non-equivalent sperm cells produced from single pollen grains only transmit paramutagenicity if the sperm cell contains a *Pl′* allele [66]. Therefore, no pollen-specific or sperm sRNAs by themselves can induce paramutation. Although sRNAs may mediate the THI required for *trans*-repression and/or the creation of a meiotically heritable mark, there is currently no support for models in which sRNAs serve as meiotically heritable agents.

3.4.2. Cytosine methylation

Cytosine methylation as an RdDM outcome provides the most parsimonious meiotically heritable paramutation mark. Using methyl-sensitive restriction enzyme (MSRE) digestion, the *b1* TR has higher 5meC levels in *B′* versus *B-I* states [48] correlated with DNaseI sensitivity [27]. In *B-I*/*B′* plants, 5meC levels increase throughout development and reach *B′*/*B′*-like levels by leaf 10 [48] – a point coincident with genetic mosaic results [25] that establish a time for irreversible changes. At the *p1* enhancer, bisulfite sequencing results show a similar correspondence between 5meC levels and paramutagenicity [19]. MSRE analyses at *R-r* also point to increased 5meC levels coincident with acquisition of paramutagenicity [47]. However, in both examples of *b1* and *p1* induction, reductions in the paramutable allele function precede the 5meC changes [19,48] implying that more immediate alterations affect transcription and that 5meC changes are subsequently acquired.

3.4.3. Histones and modifications

Relatively limited nucleosome profiles are available for comparisons with paramutagenic action. At the *b1* TR, comparisons of adult husk tissues found active (H3Ac and H3K4me2) and repressive (H3K9me2 and H3K27me2) marks associated with husk-specific expression of *B-I* and *B′* respectively [48]. At the *p1* enhancer, increased H3K9me2 levels are also associated with paramutagenic *p1* states in tissues where *p1* is normally expressed [67]. In contrast, no histone modifications at the *b1* TR were well correlated with *B′* or *B-I* states in seedlings – a tissue where *b1* is not expressed – indicating these marks reflect tissue-specific regulation. At the 5′ *b1* untranslated region, higher H3K27me2 levels were correlated with *B′* states in both husk and seedling tissues potentially implicating this as a meiotically heritable mark [48].

In *A. thaliana*, a positive feedback exists between H3K9me2 and non-CG context 5meC marks [68] making it possible that both 5meC and H3K9me2 collaborate in maintaining repression through meiosis and somatic development. Recently, reversible H3K27 methylation was implicated in erasing the epigenetic program of vernalization at the alternation of generations [69] and H3K27me1 has been hypothesized to be the mark that recruits Pol IV [43]. H3K27me1 is a candidate for a heritable mark as it can be propagated through replication-dependent depositions of a histone variant H3.1 monomethylated by H3K27 methyltransferases [70]. These histone marks and nucleosome cores remain to be profiled in maize.

3.5. Summary

The examples representing paramutation in maize provide excellent paradigms for understanding its molecular basis (3.1). Their commonalities help define the conceptual framework and their differences highlight the mechanistic diversity acting at each locus (3.2.2). Although the current working model to explain the THI mediating paramutation is based on the *A. thaliana* RdDM pathway (3.2.1), it remains to be seen if 5meC is the meiotically heritable mark (3.4.2). If so, then careful consideration needs to be given to ascribing a genomic context to the current genetic definition of paramutation (see 2.2 and 2.3). Paramutation-like behaviors occurring in fungi and plants [71] and in metazoans (see 4 and other contributions to this volume) not explained by 5meC pose one of many problems in generalizing a molecular definition.

4. Paramutation-like behaviors in metazoans

In perhaps the first example in animals, human epidemiological data indicated that disposition to type I diabetes attributed to insulin allele *814* is influenced by being heterozygous with another insulin allele in patients' fathers [72]. More recent examples are detailed in other contributions to this volume. Here we summarize these studies and compare them to paramutation as described in maize.

4.1. Mus musculus

4.1.1. Rasgrf1 *locus*

Paramutation-like behavior in mouse was induced by a knock-in allele in which an imprinting control region (ICR) of the *rasgrf1* locus was swapped with the *Insulin-like growth factor2 receptor* ICR (Table 1). When paternally inherited, this allele (*Rasgrf1*$^{tm3.1Pds}$) *trans*-activated a normally imprinted maternal *Rasgrf1* allele that was then capable of silencing a paternally inherited *Rasgrf1* allele in the next generation [73]. This example illustrated inheritance of a meiotically-heritable change induced by a specific allele and showed evidence of secondary THI behavior. More recently, it was found that the TRs upstream of *Rasgrf1* that control ICR methylation initiate transcription of an embryonic testis-specific non-coding RNA targeted by Piwi-interacting RNAs (piRNAs) that may act as a recruitment site for *de novo* DNA methyltransferases similar to plant RdDM [74]. Whether this transcript plays a role in paramutation-like behavior [73] remains unknown.

4.1.2. Kit *locus and sRNA*

Two *kit* knock-out (KO) alleles facilitate changes to endogenous *Kit*$^{+}$ alleles (Table 1). The *Kit*^{tm1Alf} allele is homozygous lethal, but heterozygotes display increased incidence of white tail tips (WTT) and paws [75]. Similar phenotypes were manifest in all progeny of *Kit*$^{+/tm1Alf}$ mice bred with *Kit*$^{+/+}$ dams or sires. The authors infer that *Kit*$^{+}$ changes to a paramutagenic state (*Kit**), but this result could be due to an extragenic inducing material. Indeed, microinjections of *kit*-specific microRNAs (miRNAs) [75] and *Kit*$^{+}$ oligoribonucleotides [76] into zygotes induced the WTT phenotype. Injections of both miRNAs and oligoribonucleotides targeting at least two other genes (*Cdk9* and *Sox9*) provided additional proof that zygotic and/or embryonic sRNAs can heritably affect the regulation of endogenous genes [77,78]. While the mechanism responsible for these effects remains unclear, mutant analyses show both *Kit*^{tm1Alf}-induced WTT inheritance and *Sox9*-induced effects via microinjections require an oocyte-expressed methyltransferase (Dnmt2) that normally modifies tRNAs [76].

Heterozygotes for the *Kit*^{copGFP} KO allele also have WTT, and inheritance of this phenotype is similar to that seen with *Kit*^{tm1Alf} [79]. Injected RNAs from both *Kit*$^{+/copGFP}$ sperm and oocytes also

induced the WTT phenotype, but this trait was not transmitted to progeny. Mutant analyses showed higher transmission frequencies of the WTT phenotype in the absence of oocyte miRNAs and piRNAs, leading the authors to speculate that these sRNAs help clear the zygote and/or developing embryo of any paternally transmitted silencing materials [79].

4.1.3. Summary

The behaviors documented in mice represent fascinating examples of non-Mendelian inheritance implicating roles of sRNA molecules in modifying gene regulation (Table 1). The parent-of-origin effects, extragenic inheritance, and high frequencies of reversibility are, however, unlike the paramutation examples described in maize, and evidence for secondary paramutation is lacking.

4.2. Drosophila melanogaster

4.2.1. Maternal piRNAs

In the female germline, piRNA amplification occurs via a ping-pong cycle (PPC), in which Argonautes Aubergine (AUB) and Argonaute3 (AGO3) slice both sense (AUB) and antisense (AGO3) RNAs in a subcellular region (nuage) peripheral to the nucleus (Fig. 2B). The 5′ ends of sliced RNA transcripts are incorporated into the alternate Argonaute and the cycle continues to degrade mature RNAs and produce additional piRNA pairs having characteristic 10-nt 5′ end overlaps relative to each other (a PPC signature).

Maternally inherited piRNAs trigger PPCs when both sense and antisense RNA targets are present [80,81]. The genomic 42AB cluster – consisting of mostly TE fragments – is one source of bidirectionally transcribed RNAs that feed the PPC in developing oocytes [80]. In follicle cells surrounding developing oocytes, the other PIWI-type Argonaute (PIWI) helps process primary antisense piRNAs from the 42AB cluster and other discrete genomic clusters [82]. PIWI is nuclear localized and can direct histone modifications via piRNA-specific recruitment to nascent RNAs [83–85]. However, only cytoplasmic AGO3 is expressed in developing and mature oocytes [86] and these are the cells in which examples of *lacZ* silencing are meiotically heritable and potentially paramutagenic (Table 1).

4.2.2. Trans-silencing effects and working models

The *P-1039* transgene conferring strong *lacZ* staining in follicle cells, nurse cells, and oocytes can be silenced *in trans* by other *lacZ*-containing transgenes including *P-1152* [87,88]. This *trans*-silencing effect (TSE) is manifest as variegated *lacZ*-staining specifically inside developing egg chambers, not in surrounding follicle cells. The frequency of females exhibiting *P-1152*-induced TSE is strongly influenced by a parent-of-origin effect (0.89 versus 0.1 when *P-1152* is contributed from the mother or father, respectively) and induction of TSE by the *T-1* transgene array is exclusively dependent on maternal inheritance [89]. Both observations implicate cytoplasmic functions and correspond with transgene-specific ovary piRNAs and PPC signatures [89].

T-1 consists of seven tandem *P*-element-based *lacZ* transgenes and represents an X-ray-induced derivative of the *BX2* array [87,90]. The *BX2* TR nature was sequentially built by transposition events from an initial *P*-element integration [87,91]. *BX2* and its progenitors acquire TSE function when paternally transmitted to all eggs laid from hemizygous *T-1* mothers [89]. This acquired capacity of *BX2*, denoted *BX2**, is coincident with the appearance of *BX2*-specific piRNAs and is transmissible to naive *BX2* alleles only via maternal inheritance [89,92]. Maternally inherited TSE acquisition is also supported by all *BX2* progenitor transgenes, though the efficiencies of TSE and *trans*-generational propagation appear correlated with the number of *P*-elements found in the arrays [89].

The piRNAs associated with induced TSE have distinct PPC signatures implying both sense and antisense RNAs are produced from *T-1*, *BX2*, and progenitor transgenes. Levels of *BX2* RNA remain unchanged between *BX2* and *BX2** states and propagation of this behavior is genetically dependent on female AUB function [89]. These results indicate that the transgene arrays act as bidirectionally transcribed piRNA loci that feed a PPC that is primed by maternally inherited piRNAs and is responsible for post-transcriptional turnover of *lacZ*-containing, *P-1039*-derived, transcripts. It is possible that no heritable changes occur at these arrays themselves though the fate of their RNAs are influenced by maternally-inherited sRNAs.

4.2.3. Summary

These examples of non-Mendelian inheritance are analogous, both genetically and mechanistically, to that seen in hybrid dysgenesis in which maternally inherited piRNAs are thought to prime and propagate innate immunity to TEs indexed among their endogenous piRNA clusters [80,81]. The parent-of-origin effect coupled with the dual requirement for both inherited sRNAs and endogenous loci distinguish these behaviors from paramutation as it occurs in maize.

4.3. Caenorhabditis elegans

4.3.1. Self versus non-self recognition

Monitored by germ cell expression of transgene-encoded GFP and/or mCherry, several non-Mendelian inheritance patterns have been described (Table 1) [93–97] that reveal the action of a transcript recognition system initiated by 21-nt piRNAs bound to the PIWI-type Argonaute PRG-1 [93–96]. Heritable silencing of "non-self" germline-expressed transgenes occurs by a mechanism coined RNA-induced epigenetic silencing (RNAe) [93] that requires the Argonaute protein HRDE-1 [93,96,98] to presumably recruit histone methyltransferases (SET-25 and SET-32) and Heterochromatin Protein Like2 (HPL-2) via nascent transgene RNAs (Fig. 2C) [96]. Persistence of germline transcription for genes classified as "self" requires the CSR-1 Argonaute, and reactivation of previously silenced transgenes via CSR-1 is referred to as RNA-induced activation (RNAa) [94,95]. In a particularly compelling experiment, tethering of a modified CSR-1 to box-b hairpins present in the transgene transcript reactivated an RNAe GFP transgene [95]. For transgenes consisting of both endogenous and foreign sequences, there is presumed competition of RNAe and RNAa mechanisms acting on nascent and/or cellular RNAs to ensure either heritable silencing (non-self-recognition) or activation (self-recognition) [93,94,99].

4.3.2. Trans-silencing and trans-activation: examples and working models

The first transgenes displaying paramutation-like behaviors are derived from *gfp*::*csr-1* fusions placed into the genome via a single-copy insertion technique at random locations (Table 1). Transgenes at *neSi8* and *neSi10* insertion sites were silenced (RNAe) but the *neSi9* insertion was expressed (+) [93]. Combined with either *neSi8* or *neSi10* RNAe transgenes, *neSi9* (+) became silenced and no subsequent progeny showed GFP expression [93]. Because *neSi10* and *neSi9* are on separate chromosomes and *neSi10* was selected against in the F2 generation, this represents a clear example of heritable *trans*-silencing. Reciprocal crosses indicate this *trans*-silencing is less efficient when the RNAe transgene is transmitted through the father, hinting at some parent-of-origin effect [93]. Heritable maintenance of the RNAe state was associated with 22-nt secondary RNAs having *gfp* sequence identity and dependent on HRDE-1, RNA processing proteins RDE-3 and MUT-7, components of Polycomb and Trithorax complexes MES-3 and MES-4, and HPL-2 [93]. In

a similar screen for piRNA-induced heritable transgene silencing, additional requirements were identified for H3K9 methyltransferases SET-25, SET-32, and three novel proteins NRDE-1, NRDE-2 and NRDE-4 [96]. Thus RNAe appears to represent a mechanism in which sRNAs produced from "non-self" entities can enforce silencing *in trans* to sequences previously recognized as "self" [93,99].

A recent example found that a chimeric *gfp::mcherry* transgene silenced *in trans* by another silent transgene (a piRNA-triggered silencing of a *gfp*-containing transgene) was able to confer heritable silencing on a third transgene having only *mcherry* homology (Table 1) [97]. This demonstration clearly shows evidence of a secondary silencing event. Based on cosegregation, this silencing occurs in the absence of inheriting the *gfp::mcherry* transgene thus implicating a heritable extragenic agent responsible for RNAe.

Initiation of RNAe requires PRG-1 [93]. Primary 21-nt piRNAs represent genome target sequences with exact matches and, by allowing two mismatches, all genome transcripts [96] including the *gfp* portion of transgenes targeted for RNAe [93]. An emerging model consistent with profiles of secondary 22-nt RNAs and both HRDE-1 and CSR-1 function is presented in Fig. 2C. In this model, antisense 22-nt RNAs are produced from RNA-dependent RNA polymerases acting on germline-expressed transcripts identified by PRG-1-bound piRNAs. There is also evidence of so-called tertiary 22-nt RNAs that reflect apparent spreading of sRNA biogenesis following initial amplification via secondary 22-nt RNAs [97]. CSR-1 and HRDE-1 are somehow selectively loaded with these 22-nt RNAs in a manner consistent with their presumed nuclear functions promoting or inhibiting transcription. One idea envisions a spatial separation of CSR-1 and HRDE-1 loading such that the CSR-1 slicing function may essentially filter most expressed RNAs before they arrive in an area outside the P-granule where HRDE-1 22-nt RNAs are produced and loaded [99].

4.3.3. Summary

Germline-expressed sequences are either targeted for RNAe silencing or for active licensing. Depending on the sequences in question, a competition between these alternatives can promote either heritable activation or repression of similar or identical sequences in *trans*. The syncytial nature of germ cell maturation makes it likely that both piRNAs and secondary 22-nt RNAs produced from all meiotic products provide a cytoplasmic inheritance of both silencing and licensing signals to the next generation [93–96,99,100]. Given only four cell divisions separate the zygote and initial primordial germ cell, it is possible that such sRNAs themselves specify *trans*-generational regulatory information.

The *C. elegans* examples provide strong evidence of heritable *trans*-silencing and *trans*-activation in manners reminiscent of paramutation as described in maize, yet the heritable silencing function appears cytoplasmic [97]. Mechanistically, the parallels between emerging RNAe and RdDM models are striking even though the genesis, maintenance, and presumed inheritance of the sRNA effectors are seemingly very different.

5. Conclusion

Cytoplasmic inheritance of RNAs appears to distinguish the paramutation-like behaviors seen in metazoans and paramutation as defined in plants. Perhaps this distinction reflects differences in reproductive biology. Metazoan primordial germ cells are set aside early in development and presumably rely on inherited sRNAs to model regulatory patterns throughout the genome. In plants, germlines are initiated from somatic lineages far removed from the initial embryo. Thus any sRNA information from previous generations must be amplified over extensive cell divisions prior to acquiring reproductive potential. The RdDM pathway provides just such a locus-specific amplification system coupled with the maintenance of potentially heritable 5meC patterns. Regardless of the mechanistic differences, the recognition that sRNA biology can mediate non-Mendelian inheritance in all these systems promises to heavily influence our future approaches to improving both agriculture and human health.

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