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Paramutation: From Maize to Mice

Vicki L. Chandler[1,*]
[1]BIO5 Institute and Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA
*Correspondence: chandler@ag.arizona.edu
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Paramutation is the epigenetic transfer of information from one allele of a gene to another to establish a state of gene expression that is heritable for generations. RNA has recently emerged as a prominent mediator of this remarkable phenomenon in both maize and mice.

In the 1950s, Alexander Brink defined paramutation as an interaction between alleles of genes that leads to heritable changes in gene expression (reviewed by Chandler et al., 2000). Paramutation has three key features: (1) the newly established expression state is transmitted to subsequent generations even though the allele or sequences originally issuing the instructions are not transmitted; (2) the altered locus continues to issue similar instructions to homologous sequences; and (3) there are no associated DNA sequence changes in the affected allele or sequences, indicating that the instructions and memory are mediated through epigenetic mechanisms.

The existence of paramutation challenges traditional paradigms for how genes are regulated and inherited. How do homologous sequences communicate to establish distinct states of expression? How are the new expression states maintained through subsequent mitotic and meiotic divisions in the absence of DNA sequence changes? What are the heritable molecules? In several maize systems the altered expression states are not simply on/off states but represent discrete levels of expression set early in development and maintained through mitosis and meiosis (reviewed in Chandler et al., 2000). The newly established levels of transcription also vary between individuals, suggesting that paramutation operates as a rheostat rather than as a switch. What mechanisms establish and maintain this transcriptional rheostat? Although these questions remain to be answered, recent work in both maize (Alleman et al., 2006; Woodhouse et al., 2006) and mice (Rassoulzadegan et al., 2006) suggest prominent, yet different, roles for RNA in paramutation.

RNA in Maize Paramutation

One classic example of paramutation is the *b1* locus in maize (Figure 1A). The *b1* locus encodes a transcription factor that promotes the biosynthesis of purple anthocyanin pigments. Plants homozygous for the *B-I* allele have high expression of *b1* and are dark purple as a consequence, whereas plants homozygous for the weakly transcribed *B′* allele are lightly pigmented. In contrast to conventional genetic alleles that are defined by differences in DNA sequence, the sequences of the *B-I* and *B′* "epialleles" are identical. The mode of inheritance and dominance also differs between these epialleles and conventional genetic alleles. In plants heterozygous for the two alleles, the *B-I* allele is converted (that is, paramutated) to *B′*. Importantly, this new *B′* allele (designated *B′**) is equally capable as the parental *B′* allele of paramutating *B-I* to *B′* in subsequent generations (Coe, 1966).

It has been possible to identify *trans*- and *cis*-acting components for the paramutation at *b1* because the *B′* paramutation is extremely stable and has 100% penetrance. The key sequences required for paramutation are tandem repeats of noncoding DNA located ∼100 kb upstream of the *b1* transcription start site (Stam et al., 2002). Although the *B-I* and *B′* alleles are identical in sequence, the DNA of *B-I* has a different pattern of methlylation and its chromatin is in a more open state relative to *B′* (Stam et al., 2002).

We have recently reported that an RNA-dependent mechanism is critical for paramutation in maize. We found that transcription occurs on both strands of the tandem repeats upstream of *b1* (Alleman et al., 2006), which may lead to the production of double-stranded RNA (dsRNA). Also, an RNA-dependent RNA polymerase (RdRP) called *mediator of paramutation1* (*mop1)* (Alleman et al., 2006) is absolutely required for silencing of *B-I* by *B′* and for paramutation at several other maize genes (Dorweiler et al., 2000). Although transcription of the repeats may be necessary for paramutation, it is not sufficient—the number of repeats is also a critical factor. In previous work we showed that the tandem repeats are required for both paramutation and high expression of *B-I* (Stam et al., 2002). Yet, transcription of the repeats was equivalent in the *b1* genotype with a single copy of the repeat to that in *B′* and *B-I*, which have seven copies (Alleman et al., 2006). More recently, 25 nt small interfering RNAs (siRNAs) from the repeats were detected in all three genotypes but not in the mutant lines lacking *mop1* (M. Arteaga-Vasquez and V.L.C., unpublished data). Thus, although it is clear that *mop1* is required for the generation of siRNAs from the tandem repeats, these findings suggest that siRNAs alone are not sufficient for paramutation (see also Review by M. Zaratiegui et al., page 763 of this issue).

Thus, in the current model for *b1* paramutation, RNA mediates the communication between the *B-I* and *B′* alleles to establish distinct chromatin states within the repeats. RNA is then required to maintain those

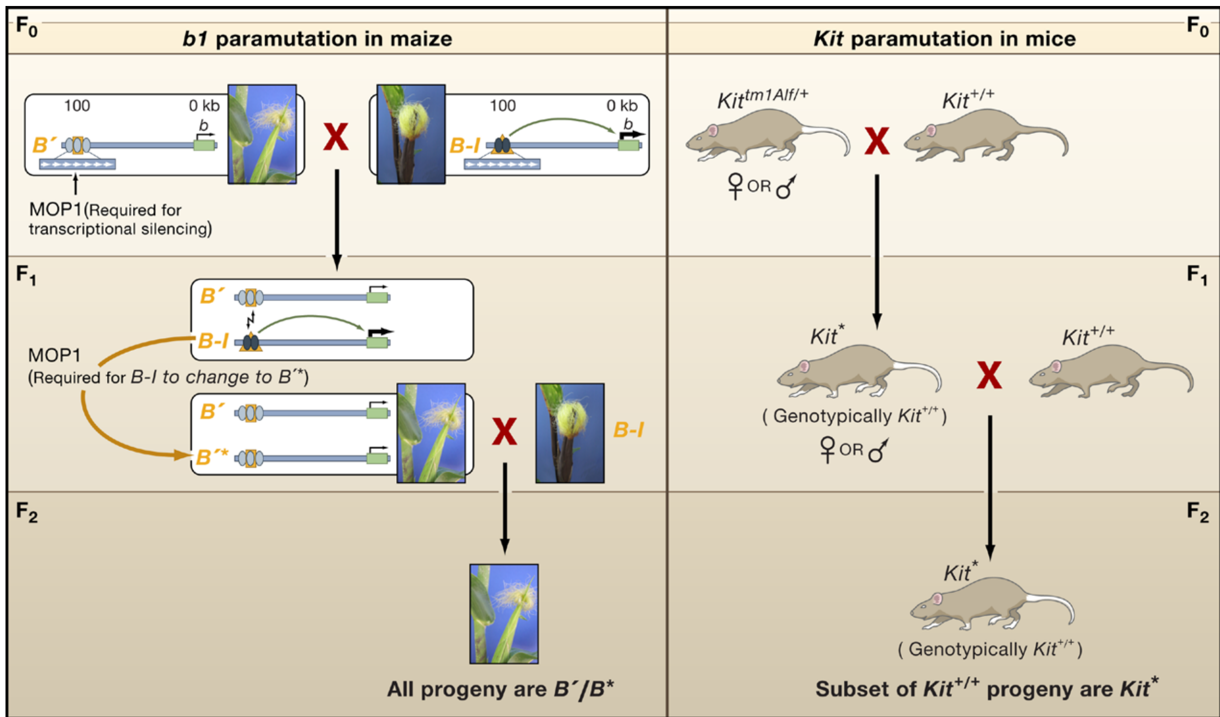

Figure 1. Paramutation in Maize and Mice

(Left) In maize, the *b1* locus encodes a transcription factor that promotes the biosynthesis of purple anthocyanin pigments. The highly transcribed *B-I* allele of *b1* produces dark purple plants (right), in contrast to the weakly transcribed *B′* allele (left). Both *B′* and *B-I* have seven tandem repeats of a 853 bp sequence unique to this location within the maize genome (black arrows). The orange square and triangle in the model represent the repeats in the two alleles, which are identical in sequence, but show differential DNA methylation and DNaseI hypersensitivity (Stam et al., 2002). The potential association of different proteins with the repeats in each allele is indicated by light and dark blue circles. The repeats are hypothesized to contain an enhancer that can only induce expression of the *b1* gene (green arrow) when in the *B-I* chromatin state. RNA is hypothesized to be involved in the allele communication in the F_1 progeny as both an RNA-dependent RNA polymerase *mop1* and the transcribed repeats are required for *B-I* to be changed into *B′** (see text for further discussion). mop1 is also required to maintain the reduced level of transcription associated with *B′*. However, the absence of *mop1* does not heritably change *B′* to *B-I* (Dorweiler et al., 2000).

(Right) Mice heterozygous for the Kit^{tm1Alf} allele, which produces no *Kit* protein, have white tail tips and white feet. When heterozygotes are crossed with wild-type mates, many of the genetically wild-type progeny ($Kit^{+/+}$) showed white tips and reduced *Kit* mRNA levels similar to the heterozygous parent. Progeny with this paramutant phenotype were designated *Kit**. The frequency of *Kit** was not 100% and varied depending on the cross; the extent of the white regions was variable between individuals. Although the white tip phenotype was observed in a second generation of outcrosses of *Kit** with $Kit^{+/+}$, the frequency was lower and the phenotype progressively disappeared in subsequent generations.

states to determine whether the *b1* gene is transcribed at the high (*B-I*) or low (*B′*) level (Figure 1A). However, many questions remain. What is the nature of the RNA that triggers paramutation? Why are tandem repeats required? How does *B-I* stay transcriptionally active in spite of its repeats producing siRNAs? What are the heritable molecules or marks? Perhaps most importantly, why does paramutation exist and is it rare?

Which RNA Mediates Paramutation?

There are a number of examples in which siRNAs are associated with *trans*-silencing. For instance, in *S. pombe*, increasing the levels of siRNAs (by deletion of the *eri1* nuclease that degrades them) is needed for silencing to occur in *trans* (Buhler et al., 2006). In plants, the generation of siRNAs from transcribed inverted repeat sequences correlates with silencing and DNA methylation of homologous promoter sequences (reviewed in Matzke and Birchler, 2005 and M. Zaratiegui et al., page 763 of this issue). In contrast, the transcription of repeats and generation of siRNAs are not sufficient for *b1* paramutation in maize. It is also not sufficient for *trans*-silencing of the *Arabidopsis FWA* gene (regulating the timing of flowering), which involves RNA-directed DNA methylation of two tandem repeats. In the *FWA* system siRNAs are produced by both methylated and nonmethylated repeats, but only lines with methylated repeats are able to induce methylation in *trans* of the repeats within an introduced transgene (Chan et al., 2006). One explanation suggested by Chan et al. (2006) is that the recruitment of the silencing machinery by siRNAs to the introduced transgene is only a prerequisite for silencing; whether DNA methylation and silencing occurs depends on the chromatin state of the *FWA* locus.

An example of RNA-dependent silencing in which siRNAs are produced but are not required for silencing is provided by the *Mutator* system of maize. The *Mutator* system con-

sists of both autonomous elements that encode the transposase (*MuDR*) and nonautonomous elements (*Mu* elements) that do not encode transposase but have terminal inverted repeats with high similarity to *MuDR. Mu* killer (*Muk*) is a locus that efficiently silences *MuDR.* An inverted duplication of part of *MuDR* produces a long dsRNA that triggers the processing of the *MuDR* transposase transcript into siRNAs, followed by DNA methylation and transcriptional inactivation of *MuDR* (Slotkin et al., 2005). Silencing of *MuDR* then results in methylation of the nonautonomous *Mu* elements. *MuDR* siRNAs are not detected in a *mop1* mutant background, indicating that *mop1* is required for the generation of detectable quantities of the siRNAs, yet *mop1* mutations do not prevent *Muk* from silencing *MuDR* (Woodhouse et al., 2006). The *mop1* gene is required to maintain the DNA methylation in the terminal inverted repeats of *MuDR* and *Mu* elements, as this methylation is lost in *mop1* mutants (Lisch et al., 2002). This suggests that the siRNAs are involved in maintaining the methylation, yet removal of the methylation is not sufficient for activation as it takes several generations in a *mop1* mutant background for *MuDR* to become active (Lisch et al., 2002).

This finding contrasts with the role of *mop1* in the silencing of *B-I* by *B′* and in establishing paramutation at two other maize loci, *r1* and *pl1* (Dorweiler, et al., 2000). One possibility for this difference, suggested by Woodhouse et al. (2006), is that *B′* (and by analogy the *r1 and pl1* loci) unlike *Muk* does not generate a dsRNA hairpin transcript in absence of *mop1*. However, transcription on both strands of the tandem repeats and the presence of repeat siRNAs suggests that dsRNA molecules are being produced from the repeats. Thus, another possibility is that the level of dsRNA in not sufficient to trigger paramutation and that *mop1* is required to produce a higher threshold of dsRNA (or another type of RNA) that mediates paramutation.

A second difference between *Mutator* silencing and *B′* paramutation is that reducing expression of maize orthologs of nucleosome assembly protein 1 (NAP1) prevents *Muk* from silencing *MuDR* (Woodhouse et al., 2006) but has no affect on the ability of *B′* to paramutate *B-I* (K. McGinnis and V.L.C., unpublished data). Other evidence suggests that NAP1 orthologs only have a role in establishing *MuDR* silencing, not in maintaining it. The loss of maize NAP1-like proteins did not lead to reactivation of previously silenced *MuDR* elements (Woodhouse et al., 2006) nor to loss of *B′* silencing (K. McGinnis and V.L.C., unpublished data), It is possible that *MuDR* silencing and paramutation are established by different mechanisms, and that only *MuDR* requires the maize NAP1 proteins. Alternatively, silencing in the two systems might occur at different times in development. Paramutation occurs early in embryogenesis (Coe, 1966), and it is possible that the reduction in *nap1* expression occurs at a later developmental time. If the NAP1 proteins are not required to maintain the silenced state, once the developmental window for establishment has passed, loss of NAP1 would have no effect on paramutation.

Why Are Repeats Required?

An intriguing question is why the tandem repeats upstream of *b1* induce silencing, whereas a single copy does not (Stam et al., 2002), and similarly why two tandem repeats are needed for the RNA-directed DNA methylation of *FWA* transgenes (Chan et al., 2006). Robert Martienssen has proposed a hypothesis for why tandem arrays are important for maintaining silencing in centromeric heterochromatin (Martienssen, 2003), which can be applied to *b1* paramutation. Multiple rounds of RdRP and dicer-like activity with tandem repeats as templates would sustain increased pools of siRNA priming throughout the sequence. In contrast, with a single copy sequence subsequent rounds of amplification would produce shorter and shorter dsRNAs. Another model is that a larger RNA synthesized from the repeats is responsible for silencing, which cannot be generated from a single copy sequence. A third idea is that the unique junction fragments created by tandem repeats have specific properties that enable silencing.

How Is Transcriptional Activity Maintained?

It is a puzzle why the *B-I* allele, which produces siRNAs from its tandem repeats, does not autosilence at high frequency. Although whatever is preventing autosilencing at *B-I* is not foolproof—*B-I* is unstable, changing to *B′* with a frequency of 0.1%–10% (Coe, 1966 and V.L.C., unpublished data). One possibility is that specific proteins might actively prevent silencing. For instance, McGinnis et al., (2006) recently showed that chromatin states can become “immune” to silencing. They describe two mutants defective in paramutation (including *mop1-1*) that were able to reactivate a silent transgene such that it stayed transcriptionally active for multiple generations, even after the wild-type proteins were reintroduced through outcrosses. Another possibility is that the tandem repeats in the *B-I* state are localized to a different nuclear compartment where the silencing machinery is unable to function. These two possibilities might also explain the lack of silencing in single copy alleles as well. Recent work from *Arabidopsis* shows that key components of siRNA chromatin-modification pathways are concentrated in specific locations within the nucleus, which may be silencing factories (Li et al., 2006; Pontes et al., 2006).

Paramutation in Mice: Is RNA the Heritable Molecule?

Recently, paramutation has been reported in mice at the *Kit* locus (Rassoulzadegan et al., 2006). The *Kit* locus encodes a tyrosine kinase receptor that functions in melanogenesis, germ cell differentiation, and hematopoiesis. Whereas mutant mice lacking *Kit* die shortly after birth, heterozygotes with one wild-type allele and one allele that produces no protein have white tail tips and white feet (Figure 1B). Remarkably, when heterozygotes are crossed with each other or with wild-type mice, many of the genetically wild-type progeny had

white tail tips and feet and reduced levels of *Kit* mRNA similar to the heterozygous parent. Progeny with this paramutant phenotype were designated *Kit**.

Here, too, transmission of the paramutant state is thought to involve RNA. However, its role in mice appears very different from how it functions at the *b1* locus in plants. Rassoulzadegan et al. (2006) propose that RNA molecules are transmitted through gametes and that these trigger degradation of *Kit* mRNA in the paramutant individuals. In both the paramutant *Kit** and heterozygous progeny there is a 2-fold decrease in *Kit* mRNA relative to wild-type. The authors also observed an accumulation of nonpolyadenylated RNA molecules of abnormal sizes in heterozygous *Kit* mice. In the sperm of heterozygous males there is much more *Kit* mRNA than is found in homozygous wild-type males (similar experiments were not reported for *Kit**). To test whether the increase in *Kit* RNA might be responsible for inducing the paramutant phenotype, the investigators injected RNA from individuals either heterozygous or homozygous for wild-type *Kit* into wild-type one-cell embryos. Of those that came to term, the "white tip" phenotype was more frequently observed in embryos injected with RNA from heterozygotes. Although the white tip phenotype was also observed to a lesser extent in control injections of RNA from wild-type *Kit* mice, the phenotype was rarely transmitted to progeny. By injecting microRNAs (miRNAs) that degrade *Kit* mRNA they were also able to generate the white tip phenotype at frequencies higher than injections with nonspecific control miRNAs. The ability to produce the white tip phenotype by microinjection of RNA and miRNA into an embryo led the authors to postulate that the epigenetic inheritance was associated with zygotic transfer of RNA molecules.

The other two reported cases of paramutation-like inheritance in mice are also allele specific and are associated with engineered alleles (Rassoulzadegan et al., 2002; Herman et al., 2003; reviewed in Chandler and Stam, 2004). The engineered *Kit* allele studied by Rassoulzadegan et al. (2006) produces a unique mRNA with the β-galactosidase coding region under the control of the *Kit* promoter and regulatory sequences. The authors report that another engineered locus (in this case containing a *GFP-neo* cassette in the first intron of the *Kit* gene) also generated paramutated progeny, whereas a classical point mutation in *Kit* did not. Does this engineered *Kit* allele, but not the point mutation, also overproduce aberrant RNA? Further comparison of transcription and DNA structure of the alleles that induce paramutation with those that show Mendelian inheritance should provide hypotheses for further testing.

It remains an open question as to whether the *Kit* system in the mouse employs a fundamentally different mechanism to achieve paramutation than in maize, with gene silencing occurring at the posttranscriptional rather than transcriptional level. Similarly, it is an open question as to what type of molecules mediate heritability. In the *B′* system there are clear differences in chromatin between *B′* and *B-I*, and given the precedence for RNA-mediated changes in chromatin there is no need to invoke RNA as the heritable molecule. However, there are no experiments in maize that rule out RNA transmission. For the *Kit* system, it is reasonable to suggest that transfer of RNA through male and female gametes leads to degradation of wild-type transcripts. However, the data are also consistent with a model in which injected RNA (or RNA produced from the engineered allele) establishes a chromatin state that results in a reduction in transcription in *Kit** mice. Posttranscriptional versus transcriptional silencing should be distinguishable by nuclear run-on assays on *Kit** mice.

Even if miRNA has the ability to degrade *Kit* mRNA, it is not clear that miRNAs are mediating paramutation at the engineered *Kit* alleles; there may be multiple ways to achieve the 2-fold reduction in *Kit* mRNA in progeny with the white tip phenotype. The authors did investigate chromatin modifications but observed no detectable differences in DNA methylation or histone modifications between wild-type, heterozygous, and paramutated animals within the minimal *Kit* promoter. However, as they did not examine any other regions within or around the *Kit* gene, they noted that a role for chromatin could not be excluded.

Why Does Paramutation Exist and Is It Rare?

It is possible that paramutation represent rare accidents, in which normal regulatory processes, such as those regulating centromeric and other heterochromatin, or defense pathways designed to regulate viruses act by mistake on rare euchromatic genes or transgenes containing foreign DNA. Given the prevalence of siRNA pathways (see M. Zaratiegui et al., page 763 of this issue), why is paramutation not observed at more loci? The *FWA* system illustrates this paradox. The endogenous *FWA* locus can adopt either of two stable epigenetic states: either the tandem repeats in the promoter are methylated and the gene is silenced, or the repeats are unmethylated and the gene is active. The *b1* and *FWA* systems share the presence of tandem repeats, which are transcribed and generate siRNAs, yet the methylated, silenced *FWA* allele does not paramutate the unmethylated active allele; heterozygotes transmit the two alleles unchanged. This is in spite of the fact that when an unmethylated transgene with the tandem repeats is introduced, it becomes methylated. How is the "natural" active allele protected from this silencing that the transgene is sensitive to? Are most genes protected from this type of silencing? *B-I* may represent an extreme example of an allele that is highly sensitized to becoming silenced because it is mistakenly seen as foreign DNA by a cellular defense system.

It is also possible that paramutation does occur more frequently than currently appreciated. One feature of several paramutation systems is that subtle variation in levels of expression are sensitively revealed with

visual markers, such as regulatory genes affecting plant pigments or mouse coat color. It should also be emphasized that the extreme penetrance and heritability of *B′*, which is impossible to ignore, is an unusual case (reviewed in Chandler and Stam, 2004). Most other paramutations are more like the observations with *Kit* where the phenotype is not fully penetrant and is lost after several generations of outcrosses. The low penetrance and instability of the engineered *Kit* allele studied by Rassoulzadegan et al. (2006) may explain why it was generated over a decade ago, yet its ability to induce non-Mendelian inheritance was only recently reported. In summary, the combination of few powerful genetic markers, variability and instability of events, and the observation that paramutation only occurs with specific alleles may all contribute to a failure to recognize other examples. The exploitation of genomic markers and examination of allele-specific expression patterns across generations would be one approach to search for paramutation on a genome-wide scale.

Whether or not paramutation arose from cellular defense mechanisms, I favor the view that paramutation now represents a fundamental mechanism of gene regulation and heredity. There are several potential roles for allele- or homology-dependent transfer of epigenetic information to progeny: it might provide an adaptive mechanism for transferring favorable (environmentally induced?) expression states to progeny or could be used to establish functional homozygosity in polyploids and might partially explain reduced fitness associated with inbreeding (reviewed in Chandler and Stam, 2004). Paramutation-like phenomena could also contribute to the low penetrance and non-Mendelian inheritance frequently associated with complex human diseases.

The next few years promise to be exciting. Not only will we obtain a more mechanistic understanding of paramutation in the current systems, I anticipate that heightened awareness will lead to the discovery of many new examples of epigenetic mechanisms imparting information that regulates gene expression across generations.

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