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Paramutation: an encounter leaving a lasting impression

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Paramutation is the result of heritable changes in gene expression that occur upon interaction between alleles. Whereas Mendelian rules, together with the concept of genetic transmission via the DNA sequence, can account for most inheritance in sexually propagating organisms, paramutation-like phenomena challenge the exclusiveness of Mendelian inheritance. Most paramutation-like phenomena have been observed in plants but there is increasing evidence for its occurrence in other organisms, including mammals. Our knowledge of the underlying mechanisms, which might involve RNA silencing, physical pairing of homologous chromosomal regions or both, is still limited. Here, we discuss the characteristics of different paramutation-like interactions in the light of arguments supporting each of these alternative mechanisms.

Paramutation: history and definition

In 1915, William Bateson and Caroline Pellew reported the strange behavior of 'rogue' (inferior) individuals among garden pea (*Pisum sativum*) plants. Plants with this phenotype always gave rise to other rogue progeny when crossed with normal-looking plants, whereas the non-rogue phenotype was permanently lost after being combined with rogues [1]. It was many years before this kind of non-Mendelian behavior was reported in other experimental systems. After extensive genetic analysis of similar observations in maize involving plant pigmentation genes in the 1950s, Alexander Brink named the phenomenon 'paramutation' (see Glossary) to reflect both the similarities and the differences between this phenomenon and true genetic modification [2]. Paramutation resembles a genetic mutation in that it is a heritable change but differs from it in its high frequency, potential reversibility and non-random occurrence. Paramutation does not cause a change in DNA sequence but rather a change in DNA methylation and chromatin structure, and is therefore a classical example of an epigenetic modification. Paramutation has been observed in several plant species and recently also in other kingdoms [3,4]. It is now defined as a *trans* inactivation between homologous alleles that leads to a high frequency of heritable changes in the gene expression of one of the alleles. The changes in epigenetic state induced by paramutation can also be associated with changes in transposition frequency [5], transposition mechanism [6] or recombination [7]. In addition, the term paramutation was recently applied to *trans* inactivations between non-allelic homologous sequences [8–10] but, for simplicity, we here use the term 'alleles' for the interacting loci.

Paramutation alleles come in pairs of one 'paramutable' allele and one 'paramutagenic' allele (Figure 1b). The paramutagenic allele provokes the change and conveys its own expression state, whereas the paramutable allele undergoes the epigenetic change and becomes a paramutated allele, in genetic nomenclature often marked with a prime symbol (for example, R-r'). Once paramutated, some (but not all) alleles exhibit secondary paramutation: they can paramutate naive paramutable alleles in a subsequent encounter (Figure 1b). A given 'set' of paramutable and paramutagenic alleles can consist of genetically identical pairs [11,12] or of different but homologous alleles [7,8,13]. Alleles that participate in paramutation are exceptional: most alleles at a given locus are neutral to paramutation and are neither paramutable nor paramutagenic (Figure 1a). Paramutation has become a biological phenomenon of widespread interest as a result of the growing number of paramutation-related phenomena recognized in eukaryotes, the links between paramutation and other instances of epigenetic gene regulation, and the potential for paramutagenic alleles to spread rapidly within populations. In this review, we provide examples of paramutation-like phenomena, discuss possible mechanistic models in the light of

Glossary

Epigenetics: stable changes in gene expression or DNA compaction determined by factors other than DNA sequence (e.g. cytosine methylation and histone modification). Epigenetic traits can be stably transmitted through many cell divisions but are potentially reversible.
Paramutation: a *trans* inactivation between homologous alleles that leads to reproducible and heritable changes in gene expression at one of the alleles with a high frequency.
RNA silencing: the post-transcriptional or transcriptional loss of gene expression induced by RNA-mediated, sequence-specific degradation of RNA transcripts or modification of homologous DNA.
RNA-directed DNA methylation: sequence-specific methylation of DNA induced by homologous double-stranded RNA and its derivatives.
***trans* inactivation:** an epigenetic change induced by the presence or configuration of another sequence at an allelic or ectopic position.
Transcriptional gene silencing: loss of transcriptional activity from a gene – frequently associated with modification of DNA and histones and formation of heterochromatin.

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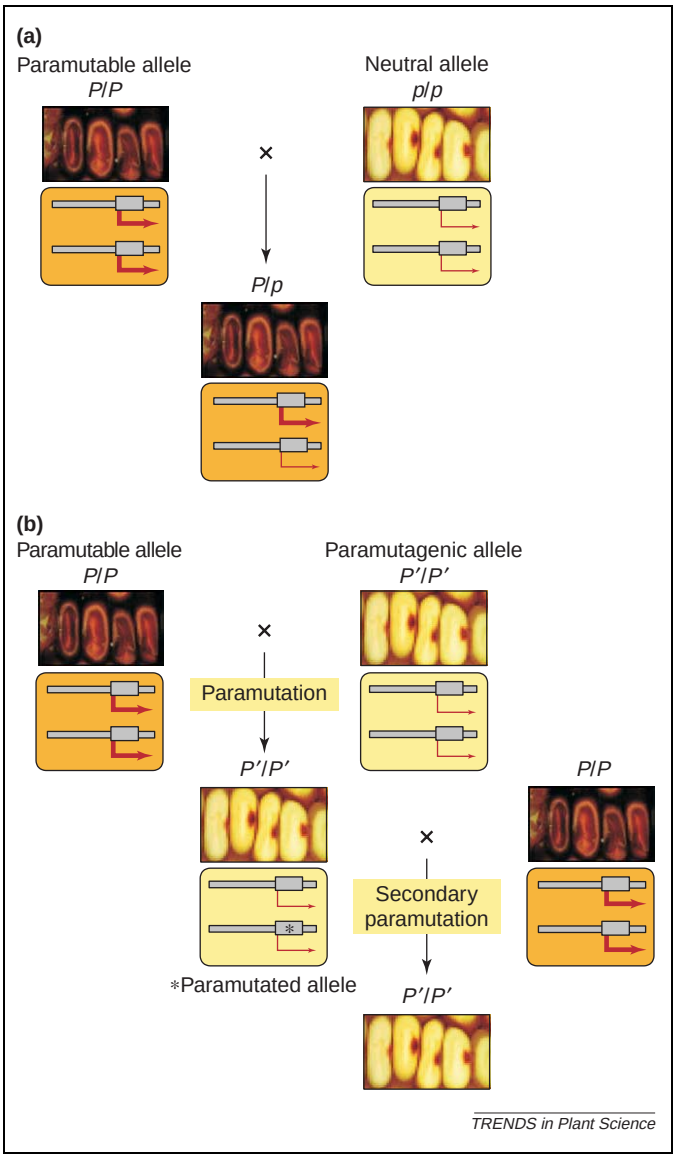

Figure 1. The principle of paramutation. **(a)** A paramutable, highly expressed allele (*P*) maintains the active state when combined with a neutral, non- or weakly expressed allele (*p*), with both alleles separating unchanged afterwards. **(b)** When combined with a paramutagenic allele (*P′*), the same paramutable allele is paramutated and expression is reduced. This change in expression is heritable and the modified allele can now paramutate a newly introduced paramutable allele (secondary paramutation). The illustrations show the effects of *p1* paramutation on the phenotype of maize kernels (*p1* controls kernel pigmentation).

similarities and differences between different paramutation systems, and describe ongoing efforts to understand the lasting impressions caused by this genetic encounter.

Paramutation phenomena

Changes in pigmentation are easy to see and usually not harmful to plants. Therefore, investigations of paramutation have been based primarily on the behavior of genes involved in color formation. The maize *r1*, *p1*, *b1* and *pl1* loci [2,9,14,15], the snapdragon *nivea* locus [5], and the *Petunia an3* [6] and transgenic *A1* loci [11] determine the levels of red and purple plant pigments in various tissues. The paramutagenic *sulfurea* locus controls green pigmentation in tomato leaves [16,17]. More recently, loci conferring an indirectly visible phenotype have also become the subjects of paramutation studies. These are mostly transgenic loci and include those encoding β-galactosidase, green fluorescent protein and phosphoribosylanthranilate isomerase (PAI), and antibiotic resistance genes [7,8,18–21]. Paramutation is not a plant-specific phenomenon – it also affects mice and humans [7,22–25] (for an extensive list, see Ref. [4]).

Models for paramutation and their basis

The outcome of paramutation – usually transcriptional gene silencing at the paramutated allele [10,11,21,26–29] – is similar to that of other epigenetic phenomena and is therefore expected to involve common epigenetic modifications. Indeed, analysis of paramutated loci often reveals the presence of cytosine methylation and other signs of inactive chromatin [7,9,11–13,19,24,30–32]. On this basis, two models are currently proposed to explain the various features of paramutation on a molecular level [4]. The first model (Figure 2a) posits that paramutation is mediated by special RNAs derived from the paramutagenic locus that affect transcription at the paramutable locus *in trans*. This mechanism resembles RNA-induced transcriptional silencing [33–36] and would not require direct physical contact between the paramutation alleles. In the second model (Figure 2b), the paramutagenic locus is proposed to transfer its own transcriptionally inactive state onto the paramutable counterpart via pairing of homologous sequences [4,37]. In this model, pairing triggers the formation of silent chromatin at the previously active allele. These two models are not mutually exclusive and neither production of a paramutagenic RNA nor physical contact would need to occur permanently, provided they last long enough to trigger the heritable change.

Given the role that both coding and non-coding RNAs play in epigenetic regulation [34], transcripts are likely to have some role in paramutation. An RNA signal seems to be involved in at least two paramutation-like phenomena. A sequence-specific transmittable silencing factor mediates *trans* silencing in the fungus *Phytophthora infestans*, suggesting RNA involvement [10]. A double-stranded RNA (dsRNA) derived from the *PAI1–PAI4* inverted repeat (IR) locus in *Arabidopsis* controls *trans* silencing of the homologous single-copy *PAI* loci [38,39]. However, small RNAs, characteristic of many cases of RNA silencing, were not found in the *PAI* system, suggesting that either dsRNA itself or undetectable levels of small RNAs serve as the *trans*-acting signal.

Direct interaction with a target locus appears to play a role in other silencing phenomena [37,40–43], such as the transfer of DNA methylation from a methylated to an unmethylated allele during meiosis in *Ascobolus* [43]. Polycomb group (PcG) proteins can mediate *trans* interactions [44,45] and have been shown to be involved in pairing-dependent silencing and co-suppression in *Drosophila* [46,47]. Methods to analyse long distance physical *cis* interactions, such as the 3C method [48], might be applicable to examine possible *trans* interactions during paramutation.

A combination of both models also finds precedents in other silencing phenomena, such as meiotic silencing in *Neurospora* [42] or the silencing of transgenic arrays in *Drosophila* and *Caenorhabditis elegans* [49]. For example,

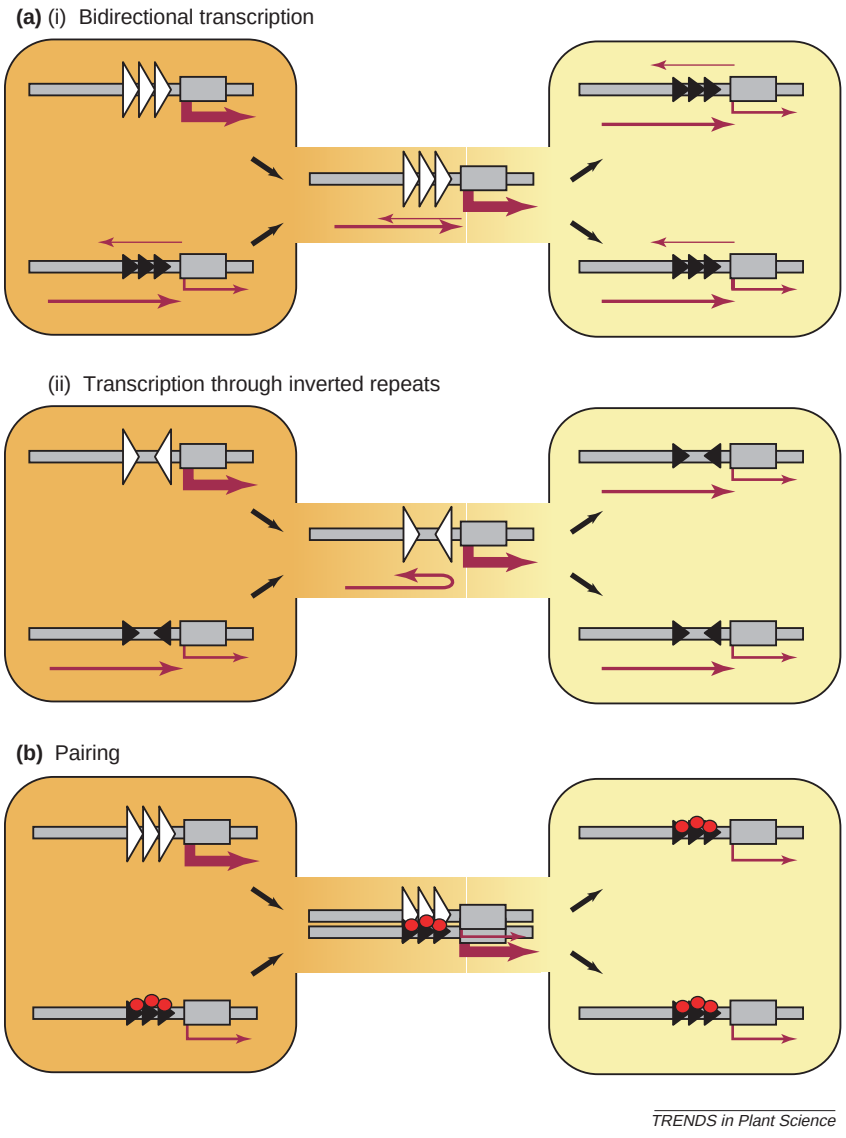

Figure 2. Possible mechanisms of paramutation. The specificity of the interaction indicates that a certain degree of nucleotide sequence homology is required. Therefore, modification of gene expression at the paramutable allele can occur via **(a)** an RNA signaling mechanism or **(b)** pairing with the paramutagenic partner. Genomic elements, in some cases shown to be upstream of the affected genes, could induce (black triangles) or be the target of (white triangles) paramutation. (a) RNA-induced paramutation could be caused by the formation of double-stranded RNA (dsRNA), produced either by **(i)** bidirectional transcription or **(ii)** transcription through regions with inverted repeats. In both cases, the dsRNA (either directly or after further processing) could modify transcription and chromatin structure at the target sequence. Alternatively, paramutation is achieved via physical interaction of the two partners resulting in an exchange of chromatin proteins (red circles). In all cases, the previously active gene is transcriptionally downregulated and inherits this expression state throughout somatic and sexual propagation. The currently known paramutation systems provide arguments for both mechanisms; the two models are not mutually exclusive.

RNA binding by a specific PcG protein of *C. elegans* is essential for its localization and function [50]. Similarities and differences between the various paramutation systems indicate that the mechanisms underlying these phenomena might contain elements of both models to different degrees.

Secondary paramutation

Once paramutated, paramutable alleles can become paramutagenic. This ability is called secondary paramutation. Most paramutable alleles show efficient secondary paramutation [1,11,15,28], whereas, for others, secondary paramutation was not analysed, has not been reported or does not occur. In the cases where secondary paramutation [19,24,27] has not been found this might be because the paramutable loci, although possessing sequence homology, have a different sequence organization than the corresponding paramutagenic locus and therefore lack the features required to become paramutagenic (see below).

Stability of the epigenetic state and dosage-dependent paramutation

Paramutable and paramutagenic alleles are usually stable [9,14,17,21,51]. However, some paramutable and paramutagenic alleles can spontaneously change to the other state, for example, 'ear rogue', *b1*, *A1*, *pl1* and Spr12F-*spt* [1,11,14,15,18], and *pl1* [15], respectively. In addition, some paramutation alleles (*sulf*, *A1*, *pl1* and *p1*) also show intermediate epigenetic states [9,11,15–17]. The

stability of paramutation alleles can depend on the nature and presence of the homologous allele. For example, the paramutable *r1*, *b1*, *pl1* and Spr12F-*spt* alleles show more spontaneous paramutation in a homozygous situation than when heterozygous with a neutral allele or hemizygous with a deletion allele [15,18,52–55]. This increase in frequency of spontaneous paramutation is greater than twofold and therefore not caused merely by the dosage of the paramutable alleles. In the other direction, the paramutagenic *Pl′* state frequently reverts to a less paramutagenic *Pl* state when heterozygous with a neutral allele or hemizygous with a deletion. Remarkably, this reversion is only heritable in the presence of the neutral allele, suggesting that allelic pairing might be involved in fixing the epigenetic state [53].

The frequency of *trans* inactivation can be influenced by the ploidy level [21]. *Trans* inactivation of an active hygromycin phosphotransferase allele (*HPT*) by its silenced counterpart was observed in tetraploid but not in diploid plants, and was observed only in progeny resulting from self-fertilization of plants heterozygous for the active and inactive *HPT* allele. Small RNAs could not be detected in the affected plants (O. Mittelsten Scheid, unpublished). Polyploids endure a more demanding sorting and pairing of the multiple homologous chromosomes during meiosis [56,57]. Given that *HPT* paramutation occurs only in tetraploid plants and seems to require that active and inactive alleles go through meiosis together, a pairing-based *trans* inactivation seems most likely in this case. If RNA was involved in *HPT trans* inactivation, one would expect paramutation already to have occurred in the F1 generation. Polyploidy has also been shown to affect paramutagenicity of the *sulfurea* locus in tetraploid tomato plants [16].

Repeated sequences

Repeated sequences are involved in several paramutation phenomena [6,9,12,13,18,19,58], although not in all [7,8,11,23,29]. Multicopy genes or repetitive intergenic regions are a major trigger for the formation of silenced chromatin [59–62]. Repeated sequences, whether inverted or direct, can give rise to the production of dsRNA, an important trigger for RNA silencing as well as heterochromatin formation [34,63]. However, repetitive sequences are also able to associate physically with their homologs in non-meiotic cells [64,65] (A. Pecinka *et al.*, unpublished). Furthermore, in yeast, inverted repeats create hotspots for mitotic interchromosomal recombination with homologous single copy sequences [66]. In either case, differences in stability of the paramutable and paramutagenic states might be caused by the different sizes and numbers of repeats involved.

Directly repeated sequences are required for the paramutagenic *r1* alleles and for paramutation of *b1*, *spt* and *p1* [9,12,13,18,58]. They are also present in two other examples of paramutation, although their role in *trans* inactivation has not yet been investigated [21,22]. A few cases of paramutation-related interactions involve IRs. All paramutable *r1* alleles contain an IR [13]; two transposons in an inverted orientation in the *an3* gene result in a paramutation-like change in transposition mechanism [6]. Remarkably, repeats located 100 kb upstream of the *b1* transcription start are required not only for paramutation but also for the high expression levels displayed by the paramutable *b1* allele. Similarly, the repeated sequences required for *p1* paramutation contain enhancer activity [9,12].

However, single copy sequences are also competent, in several cases, to induce paramutation [7,8,11,23,29]. Transcription of single copy sequences in both sense and antisense directions could give rise to dsRNA. Alternatively, inactivated single copy chromatin regions might produce a low level of improperly processed or prematurely terminated transcripts that can act as templates for RNA-dependent RNA polymerase to produce dsRNAs [67]. Pairing appears to be less efficient for single copy sequences than for repetitive regions (A. Pecinka *et al.*, unpublished); however, single copy sequences might be tethered together through specific protein binding sites. Single copy Polycomb responsive elements (PREs), for instance, appear to be sufficient for PcG-dependent *trans* inactivation [47].

Chromatin and DNA modifications

Silent genes and heterochromatin are characterized by specific chromatin structures, histone modifications and DNA methylation [60,67]. Similarly, in several paramutation systems, a positive correlation is observed between paramutagenicity and DNA hypermethylation [7,9,11–13,19,24,25,32]. However, the presence of DNA methylation alone is not sufficient for paramutation to occur because hypermethylated, inactivated *SUPERMAN* alleles in *Arabidopsis* are clearly recessive and do not exert *trans* inactivation [68]. In some cases, the change in DNA methylation appears to be a late event in the paramutation process [12,21], suggesting that chromatin-based silencing mechanisms act upstream of DNA methylation. Furthermore, there are cases of paramutation with no obvious correlation to DNA methylation [10,18]. Nevertheless, DNA methylation could be important for the maintenance or reinforcement of the paramutated state. Therefore, it might not be coincidental that paramutation has not yet been described in organisms such as *Drosophila*, *C. elegans*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, which lack extensive DNA methylation. Additional chromatin parameters of paramutation alleles, such as histone modifications, have not yet been sufficiently well analysed to allow comparison with other silencing phenomena. The only indication that paramutation involves a modification in chromatin structure comes from the decreased nuclease sensitivity at the paramutagenic *b1* and *A1* loci compared with that at their highly transcribed paramutable counterparts [12,31,69]. These differences in nuclease accessibility were confined mostly to regions also displaying differences in DNA methylation [12,70].

Mutations affecting paramutation

Plants are well suited to easy, forward-directed screens to find new components that interfere with epigenetic regulation, as well as to reverse approaches to determine the role of previously identified components. The

application of both approaches is therefore expected to reveal whether paramutation is mechanistically similar to, or distinct from, other epigenetic phenomena.

Maize

Several mutations affecting paramutation have been isolated from maize, including *mop1-1* (*mediator of paramutation 1-1*) [71] and *rmr1* and *rmr2* (*required for maintenance of repression 1* and *2*) [72]. The effects of these mutations on the various maize paramutation systems differ, suggesting mechanistic differences between the systems. The *mop1-1* mutation interferes with establishing paramutation in the *b1*, *pl1* and *r1* system [71]. Furthermore, *mop1-1* elevates the transcript levels of the *B′* and *Pl′* paramutagenic alleles but not that of a paramutated *r1* allele (J. Kermicle and V. Chandler, personal communication). The *rmr1* mutation raises *B′* and *Pl′* transcript levels, although the increase in the *B′* transcript level is not as dramatic as in a *mop1-1* mutant background [72] (C. Belele, M. Stam and V.L. Chandler, unpublished). The *Pl′* transcript level is also increased by *rmr2*. Affects of *rmr2* on *b1* and *r1* paramutation have not yet been reported.

The paramutagenic *pl1* state can heritably revert to a paramutable state when present in a *mop1-1*, *rmr1* or *rmr2* mutant background, although it requires multiple generations in the case of *mop1-1*. The paramutagenic *b1* and *r1* states are affected only transiently in a *mop1-1* mutant background. After outcrossing the *mop1-1* mutation, *b1* and *r1* alleles immediately regain their paramutagenic state [71]. These data are in line with the observations that the epigenetic states of *pl1* are less stable than those of *b1* and *r1*.

In a homozygous *mop1-1*, *rmr1* and *rmr2* mutant background, *Mutator* (*Mu*) transposons have lower levels of DNA methylation [73] (D. Lisch and J. Hollick, personal communication). Furthermore, previously silenced *Mu* elements become somatically active after multiple generations of exposure to the *mop1-1* mutation. Therefore, paramutation and transposon regulation seem to share mechanistic features, although the kinetics, sensitivity and composition of the regulatory complexes can differ. Unlike in *ddm1* mutants (one of the most dramatic *Arabidopsis* gene silencing mutants [74]), general DNA methylation levels at ribosomal and centromeric repeats do not change in *mop1-1* mutants. The *mop1-1* mutation results in pleiotropic developmental effects but the *rmr1* and *rmr2* mutations do not, suggesting that MOP1 has a more general role than RMR1 and RMR2 [71,72].

Genetic mapping experiments indicate that *mop1-1* is not a homolog of any of the well-known *Arabidopsis* mutations affecting gene silencing [4]. Because the *mop1*, *rmr1* and *rmr2* genes have not yet been identified, they cannot at present contribute to the refinement of paramutation models.

Arabidopsis

Mutations affecting classical paramutation systems in organisms other than maize have not been described; the involvement of existing mutations in paramutation-like interactions in the model plant *Arabidopsis* is currently under investigation. Two mutations have been tested for their effects on the maintenance of silencing of the silent *HPT* locus in diploid plants [21]. A mutation of MOM1, a nuclear protein with an incomplete SWI2/SNF2-like ATPase helicase motif, releases transcriptional gene silencing from repetitive target loci without affecting their DNA methylation status [75]. The *mom1* mutation does not reactivate the inactivated *HPT* locus, which is paramutagenic in tetraploid plants [21], suggesting that the *HPT* locus is silenced by a different mechanism. A mutation in the *DDM1* gene that codes for a SWI2/SNF2-like chromatin remodeling factor [76] affects CpG and non-CpG DNA methylation and reactivates several repetitive targets and (to a lesser extent) single copy sequences [77]. A strong *ddm1* mutant allele has been shown to release silencing of the paramutagenic *HPT* locus; this release of silencing was partial and occurred only after multiple generations in a homozygous *ddm1* background [21].

The *trans* inactivation of single copy *PAI* loci by DNA methylation is dependent on transcription of the *PAI* IR. This resembles RNA-directed DNA methylation (RdDM), during which promoters are turned off and their DNA is methylated by a homologous dsRNA trigger [33,35,36]. The effect on *PAI trans* inactivation of mutations in *MET1* (a CpG maintenance methyltransferase) and *DDM1*, both of which affect RdDM [33], was tested. After multiple generations in a *ddm1* or *met1* mutant, the DNA methylation level at the paramutable single copy loci was reduced, whereas that of the paramutagenic, transcribed IR was significantly reduced only in a *met1* mutant [78]. This indicates that, as in the case of another RdDM-mediating IR-silencing locus [33], maintenance methylation of the *PAI* IR is DDM1 independent. Two other genes required for RdDM can be tested for their effect on *PAI trans* inactivation: *HDA6*, encoding a putative histone deacetylase [79,80], and *DRD1*, encoding a plant-specific putative SNF2-like chromatin-remodeling factor [81]. Mutations in *HDA6* affect histone acetylation and, to a lesser extent, CpG and C(N)G methylation levels at particular repetitive sequences, whereas *drd1* mutants lack non-CpG methylation at RdDM targets.

A forward mutant screen using the *PAI* system has identified two additional genes, *CMT3* and *KYP*/*SUVH4*. CMT3 is a chromomethylase required for maintenance of non-CpG methylation in general and at the single copy and IR *PAI* loci [68,82]. *KYP*/*SUVH4*, a SET domain protein with histone H3Lys9 methyltransferase activity, affects the maintenance of DNA methylation indirectly, mainly in a non-CpG context [83–85]. It might act downstream of CpG methylation by reinforcing chromatin silencing [86]. Maintenance of DNA methylation at the single copy *PAI* genes requires H3K9 histone methylation, whereas maintenance methylation of the *PAI* IR and IR-induced *de novo* methylation of single copy *PAI* genes are independent of H3K9 histone methylation. Although there is good evidence that *PAI trans* inactivation is dependent on a specific dsRNA, various mutations in genes involved in the production and amplification of dsRNAs did not affect maintenance methylation of any of the *PAI* loci [38]. This indicates that the *PAI* IR-derived dsRNA itself is capable of triggering *trans* inactivation,

either directly or via the production of transiently expressed or undetectable levels of small RNAs.

Outlook

All cases of paramutation share the feature that allelic or ectopic interaction results in heritable epigenetic changes at one of the partners involved, but they also have additional distinct properties. The mechanisms underlying the *trans* inactivations are not known in any of the classical examples of paramutation. To reveal these mechanisms and the extent to which they overlap with those of other epigenetic phenomena, it will be crucial to clone the genes involved and to provide a detailed molecular and biochemical characterization of their gene products. The isolation of mutants in plants is relatively easy and has proved to be beneficial. Because current screens are not likely to be saturated, they should be continued. The complexity and diversity of epigenetic regulation indicates that analysis of any single experimental system will provide incomplete answers. Therefore, it is important that screens are performed in a range of systems and that mutations are thoroughly characterized for effects beyond those queried in the screens. This should include analysis of their effects on genome-wide expression levels using cDNA and genomic microarrays, and the application of recently developed techniques to analyse sequence-specific chromatin features and global chromatin organization. To determine whether the various mutations act in the same pathway, double mutants should be examined for epistasis or synergism between the genetic factors. In addition, it will be important to test the role of the maize orthologs of the *Arabidopsis* genes involved in transcriptional and post-transcriptional silencing for their role in classical paramutation systems. The requirement for repeated sequences at several paramutation alleles indicates that it could be rewarding to explore other structural distinctions, genomic localization or spatial arrangements.

The existence of only a few well-documented cases of paramutation makes it appear to be an exotic rather than a general epigenetic regulatory mechanism. This is largely because the examples analysed have been limited to easily detectable visible phenotypes. If paramutation is indeed more common than was previously assumed, transcriptome and proteome analyses of parents and their offspring should provide a wealth of new examples.

Does paramutation exist incidentally or does it have biological functions and evolutionary implications? For a more elaborate discussion, see Refs [4,69]. Paramutation might be part of the defense system against invasion by foreign DNA. It might also be involved in creating a balance between different chromosomes upon polyploidization and hybrid formation. Last, but not least, the existence of different epigenetic states of an allele might enable organisms to adapt relatively easily and in a heritable but reversible manner to changes in the environment. The rate of genetic mutations is too low to allow quick adaptation of gene expression levels. Whatever its function, the recent discovery of related phenomena beyond the plant world shows that the mechanisms underlying paramutation are conserved throughout all kingdoms. Paramutation might therefore have more widespread implications than previously anticipated.

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