Chromatin conversations: Mechanisms and implications of paramutation

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CHROMATIN CONVERSATIONS: MECHANISMS AND IMPLICATIONS OF PARAMUTATION

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Paramutation is a widespread epigenetic phenomenon that was first described in pea and then extensively studied in maize, whereby combining two specific alleles results in a heritable change in the expression of one of the alleles. Far from being restricted to endogenous plant genes, paramutation-like interactions have been described in several kingdoms, in which they can occur between homologous transgenes or between transgenes and homologous endogenous genes at allelic or non-allelic positions. In this review, we discuss potential mechanisms underlying paramutation, compare paramutation to several other *trans*-sensing phenomena, and speculate on the potential roles and evolutionary implications of these intriguing homology-sensing mechanisms.

MENDEL'S FIRST LAW
This states that in the process of the formation of the gametes the allelic pairs separate, one going to each gamete, and that each gene remains completely uninfluenced by the other.

EPIGENETICS
Describes a heritable effect on chromosome or gene function that is not accompanied by a change in DNA sequence. It is accompanied by modifications of chromatin or DNA.

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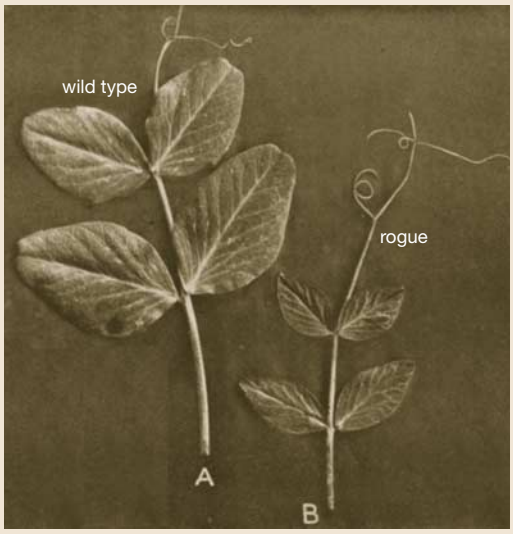
About 50 years ago, Brink[1] observed that an interaction between two maize alleles caused a mitotically and meiotically heritable change in the expression of one of the two alleles. When the *R-r* allele, which confers dark purple seeds, was crossed to *R-stippled*, which confers purple stippled seeds, the *R-r* allele was heritably changed such that it conferred lightly pigmented seeds (designated *R-r′*), whereas the *R-stippled* allele segregated unchanged (for detailed reviews, see REFS 2,3). This phenomenon violated MENDEL'S FIRST LAW, because heterozygotes segregated alleles that had been influenced by the presence of the other allele[2]. Brink called this phenomenon 'paramutation' because the altered alleles were not behaving as typical mutations[4]; the frequency of the change was higher and the stability of the change was lower.

Subsequently, paramutation was observed at several other maize loci and at a few loci in a number of other species, including mouse and human (BOX 1 presents a timeline of research in this field). The first examples of paramutation involved genes controlling phenotypes that are easily scored, such as pigment levels, morphological changes or drug resistance (TABLE 1). More recently, examples of paramutation have been found that involve expression differences that can be monitored using molecular tools. It is now clear that paramutation involves communication between homologous sequences that are present *in trans*; this communication establishes heritable changes in chromatin structure that often correlate with alterations in DNA methylation. So, the change of *R-r* into *R-r′*, induced by *R-stippled*, that was observed by Brink represents an EPIGENETIC change, and *R-r* and *R-r′* are EPIALLELES.

Paramutation-like phenomena challenge established models of heredity and evolution and have fascinated geneticists for a long time. Within the past decade or so, these various *trans*-acting phenomena have attracted the interest of other research fields because of the realization that epigenetic changes are more widespread than previously appreciated and that they are involved in a number of human diseases and affect the ability to efficiently engineer plants and animals.

In this review, we discuss in detail two well-characterized paramutation systems in maize and a paramutation-like phenomenon in mice, which together illustrate the diversity of these processes. In the context of paramutation models, we discuss similarities and differences between these systems and draw parallels to certain other epigenetic phenomena (BOX 2). We also discuss potential roles for and consequences of homology-dependent transfer and transmission of epigenetic information.

Box 1 | **Timeline of paramutation research**



A phenomenon that fits the definition of paramutation was first reported in 1915 (REF. 76)**. Unusual 'rogue' phenotypes with narrower leaves and petals were noticed within crops of the garden pea *Pisum sativum* (see photo). Hybrids between normal plants and rogues showed an intermediate phenotype at the base of the plant. The hybrids became more rogue-like during development and only transmitted the rogue phenotype to progeny. Similar phenomena were later described using the terms 'paramutation', 'mass somatic mutation', 'somatic conversion', 'conversion' and 'conversion-type phenomenon'. Examples include the *cruciata* character, which refers to the smaller cross-shaped flowers seen in hybrids of several *Oenothera* species (evening primrose)[77]; certain alleles of the *r1* (REF. 1) and *b1* (REF. 78) loci of *Zea mays* (maize), which control production of the purple anthocyanin pigment; and the *sulfurea* locus of *Lycopersicon esculentum* (tomato)[79], which contributes to chlorophyll accumulation. In 1968, at the International Congress of Genetics in Tokyo, Brink, Coe and Hagemann proposed that a single term — 'paramutation' — should be used to emphasize the similarities among the various phenomena.**

The advent of molecular tools allowed the study of several paramutation systems that involved endogenous genes[22,80,81] or transposons[82]. *Trans*-interactions were described between two homologous transgenes[57,83,84], and between transgenes and endogenous genes[60,62]. Where tested, transcription was shown to be altered[57,80,84,85] and the alleles or homologous sequences often showed differences in chromatin, such as DNA methylation and/or NUCLEASE HYPERSENSITIVITY[3,24,83,86]**. We now know that paramutation involves *trans*-interactions between homologous sequences that establish distinct heritable chromatin changes and that paramutation is not limited to plants; similar phenomena have been described in organisms from several other kingdoms** (TABLE 1)**. Photo reproduced with permission from** REF. 76 **© (1915) Indian Academy of Sciences.**

Paramutation: important features

The diverse phenomena compiled in TABLE 1 share a common theme that serves as a broad definition of paramutation; *trans* interactions between homologous sequences — whether they are two alleles of the same gene, or two transgenes, or one transgene and one endogenous gene —set up distinct epigenetic states that are heritable. Below, we define several terms and discuss features that are needed to interpret the similarities and differences among these phenomena.

Loci that participate in paramutation can have three different types of allele: those that do not participate in paramutation are referred to as either neutral or non-paramutagenic; sensitive alleles are termed paramutable; and alleles inducing the change are paramutagenic. Once changed, paramutable alleles are termed paramutated and are often designated with an apostrophe (for example, *R-r'*). We refer to the initial transfer of epigenetic states as the establishment of paramutation, and the heritable transmission of these states through mitosis and meiosis as maintenance of paramutation.

The PENETRANCE of establishing the new epigenetic state varies from 100% to less than 10%, depending on the particular phenomenon. In most cases, the altered state is seen in F_1 progeny or in the first generation in which a transgene and homologous endogenous gene are together; however, in a few cases the altered state is only seen in the F_2 progeny.

In many examples, the paramutated state is unstable (although still heritable), returning towards its original state in subsequent generations. The stability of the paramutated state sometimes depends on the other allele; for example, the state is less stable when an allele is *in trans* with a neutral allele. In other examples, the paramutated state is extremely stable. Frequently, the newly altered allele becomes paramutagenic — that is, it gains the ability to alter a paramutable allele.

Communication *in trans* between homologous sequences that establishes distinct, heritable chromatin states raises many interesting questions driving research in this field. These include: What is the nature of the communication and how does communication transfer specific epigenetic states? How are specific chromatin states maintained through mitosis and meiosis? Which gene products mediate paramutation? How common are these phenomena, and what are their evolutionary consequences?

Genetic studies of paramutation

One intriguing issue in paramutation research is to explain how epigenetic states are transferred *in trans* between alleles or between non-allelic homologous sequences at very high frequencies. Genetic and molecular studies are beginning to build a picture of the genes participating in, and potential mechanisms contributing to, paramutation. In maize, paramutation has been described for four genes (*r1*, *b1*, *pl1* and *p1*), all of which encode transcription factors that activate the biosynthesis of flavonoid pigments in plant or seed tissues. In this review, we highlight the two systems for which the

EPIALLELES
An epigenetic variant of an allele. The activity of an epiallele is dependent on epigenetic modifications (for example, histone deacetylation and cytosine methylation) and not on nucleotide changes.

NUCLEASE HYPERSENSITIVITY
The presence of chromosomal sites that are highly accessible to cleavage by nucleases such as deoxyribonuclease I (DNAseI). Such sites indicate the binding of proteins to specific sites, and tend to be associated with transcriptional activity of the nearby gene.

PENETRANCE
The frequency with which individuals that carry a given gene or epiallele will show the manifestations associated with the gene or epiallele.

Table 1 | **Paramutation-like phenomena**

Organism	Gene(s) affected	Phenotype affected	Frequency of effect	Extent heritable	Acquisition of paramutagenicity	Refs
Pisum sativum (garden pea)	Unknown	Narrower leaflet and petals	100%; progresses during development	100%	Yes	76
Oenothera (multiple species)	*cr (cruciata)*	Cross-shaped flowers of reduced size	Variable	100%	Yes	77
Zea mays (maize)	*r1 (red1)*	Reduction of seed pigment	100%; only seen in F_2 progeny	Variable; depending on allele	Variable; depending on number of generations with paramutagenic allele	1,2
Zea mays (maize)	*b1 (booster1)*	Reduction of plant pigment	100%	100%	Yes	32,78
Lycopersicon esculentum (tomato)	*sulf (sulfurea)*	Yellow (speckled) cotyledons and leaves	Variable; progresses during development	Variable	Yes	79,100
Antirrhinum majus (snapdragon)	*niv (nivea)*	Reduction of transposition	Variable,depending on allele and direction of cross	100%	Not reported	82,101
Nicotiana tabacum (tobacco)	*nptII (neomycin phosphotransferase II)* transgene	Loss of kanamycin resistance	Variable, depending on allele	100%	Not reported	83,102
Petunia hybrida (petunia)	*a1* (*anthocyanin1*) transgene	Reduction of flower pigment	Variable,depending on direction of cross	90–100%	Not reported	57
Zea mays (maize)	*pl1 (purple1)*	Reduction of pigment in anthers	100%	Variable; depending on allele	Yes	81
Arabidopsis thaliana	*PAI (phosphoribosyl-anthranilate-isomerase)*	Gain of blue fluorescence	Variable, depending on position of target locus	Nearly 100%	No	17,103
Nicotiana tabacum	*hpt (hygromycin phosphotransferase)* transgene	Loss of hygromycin resistance	100%	100%	No	84
Ascobolus immersus (filamentous fungus)	*b2 (brown spore 2)*	Spore colour	Variable; 9% reported	Variable; 60–90%	Low	61
Homo sapiens (humans)	*Insulin* minisatellite *vntr (variable number tandem repeats)*	Protection against type 1 diabetes	See *	Not reported	Not reported	72
Mus musculus (mouse)	*U2af1-rs1* (see ‡)	Loss of paternal allele expression; gain of DNA methylation	~100% methylation of parental allele DNA; see ‡	11%; see §	No	96
Nicotiana tabacum (tobacco)	*spt (streptomycin phosphotransferase)* transgene	Loss of streptomycin resistance	~60% of plants	~100 %	Not reported	23
Petunia hybrida (petunia)	*an3* (*anthocyanin 3)* with transposon	Transposition mechanism is altered	Nearly 100%	Not applicable	Not applicable	104
Phytophthora infestans (potato blight fungus)	*inf1 (major elicitin of P. infestans)* transgene and endogenous gene	Reduction of INF1 protein levels	Variable; effect seen in heterokaryon	100%	Not reported	60
Zea mays (maize)	*p1 (pericarp colour 1)* transgene and endogenous gene	Reduction of seed and cob pigment	Variable; 40–60%	100%	Yes	62
Arabidopsis thaliana	*bal* and *cpr1-1 (constitutive expressor PR genes 1)*	Reversion of dwarf phenotype morphology	Effect only seen in F_2 progeny; 20%	Intermediate	Not reported	105
Arabidopsis thaliana	*GFP-COP1 (green fluorescent protein constitutively photomorphogenic 1)* transgene	Reversion of dwarf; reduction of *GFP* expression	100%	See ‖	Yes	106
Mus musculus (mouse)	*loxP* transgene and wild-type insertion site	Inactivation of recombination; increased DNA methylation	Nearly 100%	100%	Yes	58
Mus musculus (mouse)	*Rasgrf1* (see ¶) transgene and endogenous gene	Induction of expression; increased DNA methylation	See ¶	Heritable: % not reported	Yes; see #	95
Arabidopsis thaliana	*hpt (hygromycin phosphotransferase)* transgene	Loss of hygromycin resistance in tetraploid	See **	100%	Not reported	19

The list includes phenomena that the authors suggest might be explained by a paramutation-like mechanism, as well as several examples that we believe could be mechanistically related. The studies are listed in order of publication date. *No effect in the father; in progeny of the father, paternally inherited class I allele does not predispose to disease when it has been allelic to the class III allele in the father. ‡*U2af1-rs1* (U2 small nuclear ribonucleoprotein auxillary factor) is normally paternally expressed, maternally methylated and silenced. A multicopy *U2af1-rs1* transgene locus results in methylation and repression of the paternal allele; methylation was close to 100%; expression was not quantified. §Silencing is only heritable for one generation in absence of the transgene. ‖Heritability of morphology is 100%; *GFP* expression is variable. ¶*Ras protein-specific guanine nucleotide-releasing factor 1*. Wild-type maternal *Rasgrf1* allele gets activated and methylated when the paternal allele has its imprinting control region replaced by that of Igf2R (region 2). #Maternally inherited activated allele is associated with silencing of the wild-type paternal allele in the next generation. **No effect in F_1; in F_2, 100% of plants become affected during development.

Box 2 | **Definitions for paramutation and other epigenetic phenomena**

We have defined paramutation as *trans*-interactions that lead to heritable changes in a phenotype (see TABLE 1 for all phenomena that we know fit this definition). In this review, we introduce other epigenetic phenomena that provide models for paramutation. Although potentially mechanistically related, these phenomena are either not heritable through meiosis or they have only been shown to act *in cis*, so do not meet our paramutation definition. This list is not comprehensive; rather, it contains in alphabetical order the phenomena that are discussed in the context of potential mechanisms of paramutation.

Centromere silencing
Centromeric heterochromatin is essential for centromere function. Recent experiments have shown that the RNA interference (RNAi) machinery is required to maintain the epigenetic state at centromeres[49] and that the epigenetic state can spread *in cis* using mechanisms that are reminiscent of chromatin transcriptional silencing[87].

Genomic imprinting
Imprinted genes are genes that are expressed from only one of the two homologues, depending on whether the chromosome was inherited from the egg or the sperm. This parental origin-specific gene regulation is controlled by epigenetic modifications of DNA and chromatin (for reviews, see REFS 88,89).

RNAi (RNA interference)
A mechanism by which dsRNA induces the degradation of homologous RNAs, producing siRNA (small interfering RNA). The RNAi machinery and siRNA have been shown to also be associated with DNA methylation, heterochromatin formation and translational repression (for a review, see REF. 37).

RNA-directed DNA methylation
In plants, RNA that is transcribed from transgenes[40,90] and endogenous genes[41] can serve as a signal that triggers *de novo* DNA methylation of homologous sequences — specifically, of DNA regions that are complementary to the directing RNA.

***Trans*-inactivation**
The *brown* (*Dominant*) (*bw*(*D*)) allele in *Drosophila melanogaster* contains a large insertion of heterochromatin that mediates *trans*-inactivation of the wild-type allele in *bw*(*D*)/*bw*(+) heterozygotes. Silencing correlates with the localization of *bw*(+) to a region of the interphase nucleus containing centric heterochromatin[26].

Trans*-silencing by arrays of transgene repeats in *D. melanogaster
Tandemly repeated transgenes can promote silencing in a copy-number-dependent manner and the arrays can also show long-range interactions *in cis* and *in trans* with other sequences[25].

Transvection
This phenomenon was first defined at the *bithorax* complex in *D. melanogaster* by E.B. Lewis and is a pairing-dependent and non-heritable influence on gene expression, most often involving the action of enhancers *in trans* (for a review, see REF. 91).

X-inactivation
A dosage compensation mechanism for X-linked genes in mammals that involves an extremely stable inactivation and heterochromatinization of one of the two X chromosomes in females (for a review, see REF. 53).

regulatory sequences required for paramutation have been identified — *b1* (BOX 3) and *p1* (BOX 4). These two examples also illustrate variation in penetrance and stability, and the types of genes (endogenous genes versus transgenes) that can be involved.

Mutations that affect paramutation. In several systems that exhibit paramutation in maize, the easily visualized pigment phenotypes, the strong penetrance of the paramutation and the ability to screen large numbers of individuals allow a genetic approach to dissecting the underlying mechanisms. Genetic screens using two paramutation systems — *b1* and *pl1* (both of which control the production of purple anthocyanin pigment in seedlings and mature plant tissues) — have identified three genes that are required for paramutation (REFS 5,6). The screen design is similar in both systems. F_1 plants — generated from crosses with one mutagenized parent (either the paramutable allele or the paramutagenic one) and the corresponding wild-type parent — are self-pollinated to generate F_2 progeny; essentially all progeny are lightly pigmented because paramutation at the *b1* and *pl1* loci occurs with 100% penetrance. Rare, darkly pigmented F_1 plants potentially carry dominant mutations that prevent the establishment of paramutation; rare F_2 families segregating darkly pigmented plants might carry recessive mutations that fail to maintain the low expression levels that are associated with paramutation and could affect maintenance of the paramutagenic state.

We limit our discussion to the best-characterized gene, *mop1* (*mediator of paramutation 1*), which was originally identified as a recessive mutation that increased the transcription of *B′* (see BOX 3). Subsequent experiments showed that the wild-type *mop1* allele is required for maintaining the low expression states that are associated with the paramutagenic *b1* and *pl1* alleles, but is not required for maintaining the low expression state that is associated with *R-r′* (J. Kermicle and V.L.C., unpublished observations). However, it is absolutely required to establish paramutation at three loci: *b1*, *pl1* and *r1* (REF. 5). Experiments to test *mop1* effects on *p1* paramutation are ongoing (L. Sidorenko and V.L.C., unpublished observations). Intriguingly, *mop1* mutants do not affect the maintenance of the paramutagenic

MUTATOR
A specific class of maize transposable element. A transposable element is genetic material that is capable of changing its location in the genome of an organism.

HERITABILITY
Classically defined as the proportion of the variation in a given characteristic or state that can be attributed to genetic factors. In the context of epigenetics, it means that the epigenetic state is transmitted to progeny.

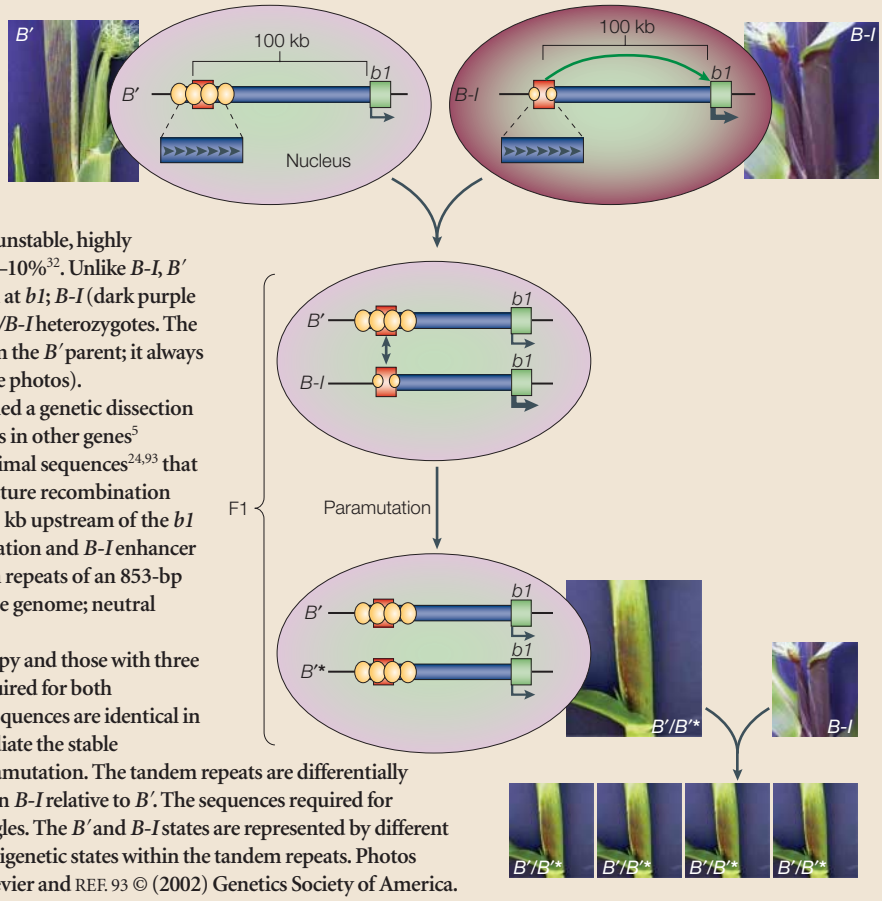
PERICARP
The ovary wall that forms the seed coat.

state. Although the *B′ mop1* mutant plants look like they are in the *B-I* paramutable state (see BOX 3) based on expression levels, *B′* is not heritably changed in *mop1*-mutant backgrounds; following outcrosses to wild type, the light phenotype and full paramutagenic activity are restored. The *mop1* gene is also required for maintaining the extensive DNA methylation levels that are associated with silenced MUTATOR transposons[7]; however, *mop1* mutations do not cause global hypomethylation of repeated sequences at centromeres and rDNA loci[5]. The *mop1* mutation also reactivates some, but not all, of several transcriptionally silent transgenes tested (V.L.C., unpublished observations).

The observation that *mop1* does not affect all aspects of paramutation at each locus tested, and that it does not reactivate all transcriptionally silent transgenes tested, indicates that multiple mechanisms might be involved in these different examples of silencing. This hypothesis is consistent with a recent study in *Arabidopis thaliana* that uncovered distinct pathways that mediate transposon silencing[8]. In that study, mutations in several genes (*ddm1*, *met1*, *cmt3*, *kyp* and *ago1*), which affect DNA methylation, histone H3Lys9 methylation and RNA interference (RNAi; see BOX 2), were tested for their ability to heritably reactivate six distinct families of silent transposable elements. The chromatin changes associated with, and the HERITABILITY of reactivation of the transposable element families differed in the various mutant backgrounds. In this context, it is attractive to hypothesize a role for RNAi in paramutation as *mop1*, like RNAi, participates in transposon and transcriptional transgene silencing[8–11].

There are two examples in which *A. thaliana* mutations that are involved in epigenetic silencing were tested for their involvement in paramutation-like phenomena. Mutations in *ddm1* (REF. 12), *met1* (REF. 13) and *cmt3* (REF. 14), which affect DNA methylation patterns through distinct mechanisms, were tested for their ability to reduce methylation and increase expression of the silenced *PAI* loci in *A. thaliana*[15,16]. The *PAI* inverted repeat induces DNA methylation and silencing of *PAI2*, an unlinked expressed singlet locus[17] (TABLE 1). All three mutations caused partial demethylation and increased expression of the singlet *PAI* locus. However, there were intriguing differences: the *cmt3* mutation affected methylation of both the inverted repeat and the singlet locus; and the *ddm1* mutation affected the singlet gene more strongly than the inverted repeat, whereas *met1* affected the inverted repeats more strongly than the singlet gene. Mutations in a SET domain protein with histone H3Lys9 methyltransferase activity, *SUVH4*, showed reduced cytosine methylation on the singlet

Box 3 | **Paramutation at the *b1* locus of maize**



The *b1* locus encodes a basic helix–loop–helix transcription factor that activates the anthocyanin biosynthetic pathway and results in the presence of purple pigment throughout the plant[92]. Two *b1* alleles are involved in paramutation, *B′* and *B-I*: these two variants have a 10–20-fold difference in transcription, but do not differ in DNA methylation in the promoter-proximal or coding regions[80]. The paramutagenic *B′* state arises spontaneously from the unstable, highly expressed paramutable *B-I* state at frequencies of ~0.1–10%[32]. Unlike *B-I*, *B′* is extremely stable. The figure illustrates paramutation at *b1*; *B-I* (dark purple plant) is always changed to *B′* (light purple plant) in *B′/B-I* heterozygotes. The newly paramutated allele, *B′**, is indistinguishable from the *B′* parent; it always paramutates *B-I* in subsequent crosses (as shown in the photos).

The full penetrance and extreme stability of *B′* enabled a genetic dissection of *b1* paramutation. This analysis identified mutations in other genes[5] (V.L.C., unpublished observations), as well as the minimal sequences[24,93] that are required for paramutation at this locus. Fine-structure recombination mapping delimited a 6-kb region, which is located 100 kb upstream of the *b1* transcription start site, that was required for paramutation and *B-I* enhancer activity[24]. In this region, *B-I* and *B′* have seven tandem repeats of an 853-bp sequence (black arrows) that is otherwise unique in the genome; neutral alleles only have one copy of this sequence.

A comparison of recombinant alleles with a single copy and those with three or five tandem repeats showed that the repeats are required for both paramutation and high levels of transcription. DNA sequences are identical in *B-I* and *B′*, indicating that epigenetic mechanisms mediate the stable transcriptional silencing that is associated with *b1* paramutation. The tandem repeats are differentially methylated and have greater DNaseI hypersensitivity in *B-I* relative to *B′*. The sequences required for paramutation are indicated in the figure by red rectangles. The *B′* and *B-I* states are represented by different numbers and sizes of ovals to symbolize the distinct epigenetic states within the tandem repeats. Photos reproduced with permission from REF. 65 © (2002) Elsevier and REF. 93 © (2002) Genetics Society of America.

VARIEGATION PROPERTIES
The object showing variegation properties can show a variety of phenotypes within somatic sectors, usually reflecting the clonal patterns of cell division in the tissue. Typically, these involve easily visualized differences in pigment.

INTERSTITIAL HETEROCHROMATIN
Heterochromatic regions that are situated in the body of a chromosome (regions other than centromeric and telomeric heterochromatin). The term heterochromatin is widely used for the densely-staining regions of the nucleus that generally contain condensed, transcriptionally inactive regions of the genome.

gene *PAI2*, but did not affect methylation on the *PAI* inverted repeat[18]. Establishment was tested with the *suvh4* and *cmt3* mutations, yielding different results. An unmethylated singlet *PAI2* gene was not methylated *de novo* in *cmt3*, whereas it could be methylated *de novo* in *suvh4*. These results indicate that *SUVH4* is involved in maintenance but not establishment of the inverted-repeat-induced methylation, whereas *cmt3* is involved in both.

The second example involves a paramutation-like phenomenon that occurs between transgene alleles in tetraploid but not in diploid plants[19]; the effects of *mom1* (REF. 20) and *ddm1* (REF. 12) on this phenomenon were examined. The *mom1* mutation had no effect. The *ddm1* mutation showed a subtle effect on maintenance — the silent transgene was derepressed only after several generations, in contrast to changes being observed more quickly in the *PAI* system. Establishment effects were not tested with *ddm1* and *mom1*. Because *ddm1* and *mom1* can reactivate silenced repetitive transgenes in diploids[20,21], it seems likely that they do not function directly in the *trans* phenomenon that is observed in tetraploids.

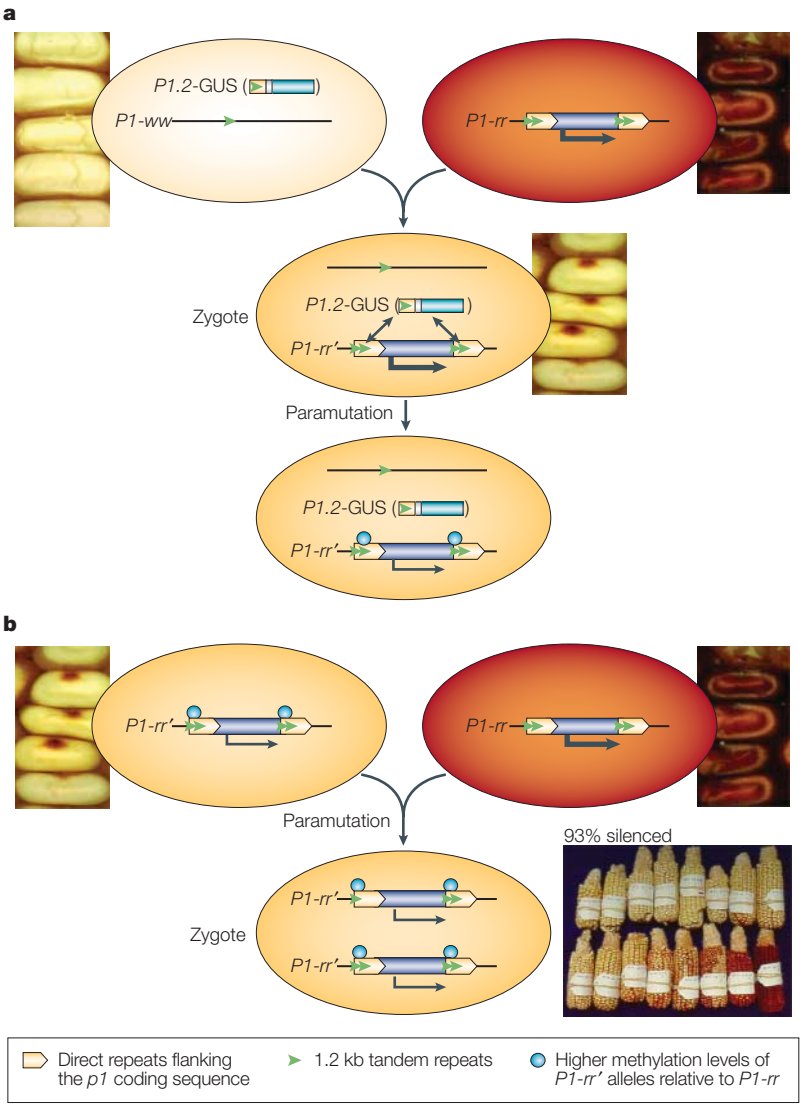
Although the molecular nature of *mop1* is not yet known, mapping studies have established that it is not the maize orthologue of *ddm1*, *mom1* or any of the DNA methyltransferases. Comparison of the *mop1* map position to that of candidate genes that are homologous to genes involved in chromatin and epigenetic silencing in other species has eliminated the ~140 genes that have been mapped so far as being *mop1* (chromatin gene data from K. Cone, University of Missouri; see The Plant Chromatin Database in online links box), including *ddm1*, *mom1* and DNA methyltransferase orthologues in maize.

A more detailed understanding of the genes that mediate paramutation will be crucial for distinguishing among the mechanistic models discussed below. A top priority will be to determine the gene products that are

Box 4 | **Paramutation at the *p1* locus of maize**

The *p1* locus encodes a myb transcription factor that activates the phlobaphene pigment pathway, producing orange–red pigments in the cob and PERICARP tissues[94]. Paramutation was described in the progeny of transgenic maize plants that carried a specific region of the *P1-rr* promoter (which normally confers red pericarp and red cob)[62]. Transgenic lines containing a *GUS* reporter gene fused to the basal *p1* promoter and distal P1.2 enhancer fragment from the *P1-rr* allele (*P1.2:GUS*) were crossed with *P1-rr* plants; as a result, expression of *P1-rr* was reduced (the new allele is designated *P1-rr′*) and seeds were lighter and patterned (see part a). The *P1-rr′* phenotype was only observed in plants carrying the transgene, not in siblings that did not carry the transgene. No reduced expression of *P1-rr* was observed in progeny from crosses with transgenic lines containing two other promoter-proximal enhancer fragments[62]. Three independent *P1.2:GUS* transgene loci were tested and all showed the effect (brackets around the transgene in the figure indicate that the exact structure of the multicopy transgene loci is unknown).

In subsequent crosses, the *P1-rr′* phenotype was heritable in the absence of the transgenes and the allele was paramutagenic, but not fully penetrant. As illustrated in the bottom figure (see part b), in crosses where plants with naive *P1-rr* alleles are combined with non-transgenic *P1-rr′* plants, *P1-rr* was changed to *P1-rr′* at a high frequency[62]. Most of the F1 ears (15 out of 16) showed reduced pigmentation relative to *P1-rr*, but the degree of silencing varied among and within the ears. Molecular analysis of *P1-rr′* indicated a tight correlation between reduced pigment, increased DNA methylation and decreased transcript levels[62] relative to *P1-rr*. Photos reproduced with permission from REF. 62 © (2001) American Society of Plant Physiologists.



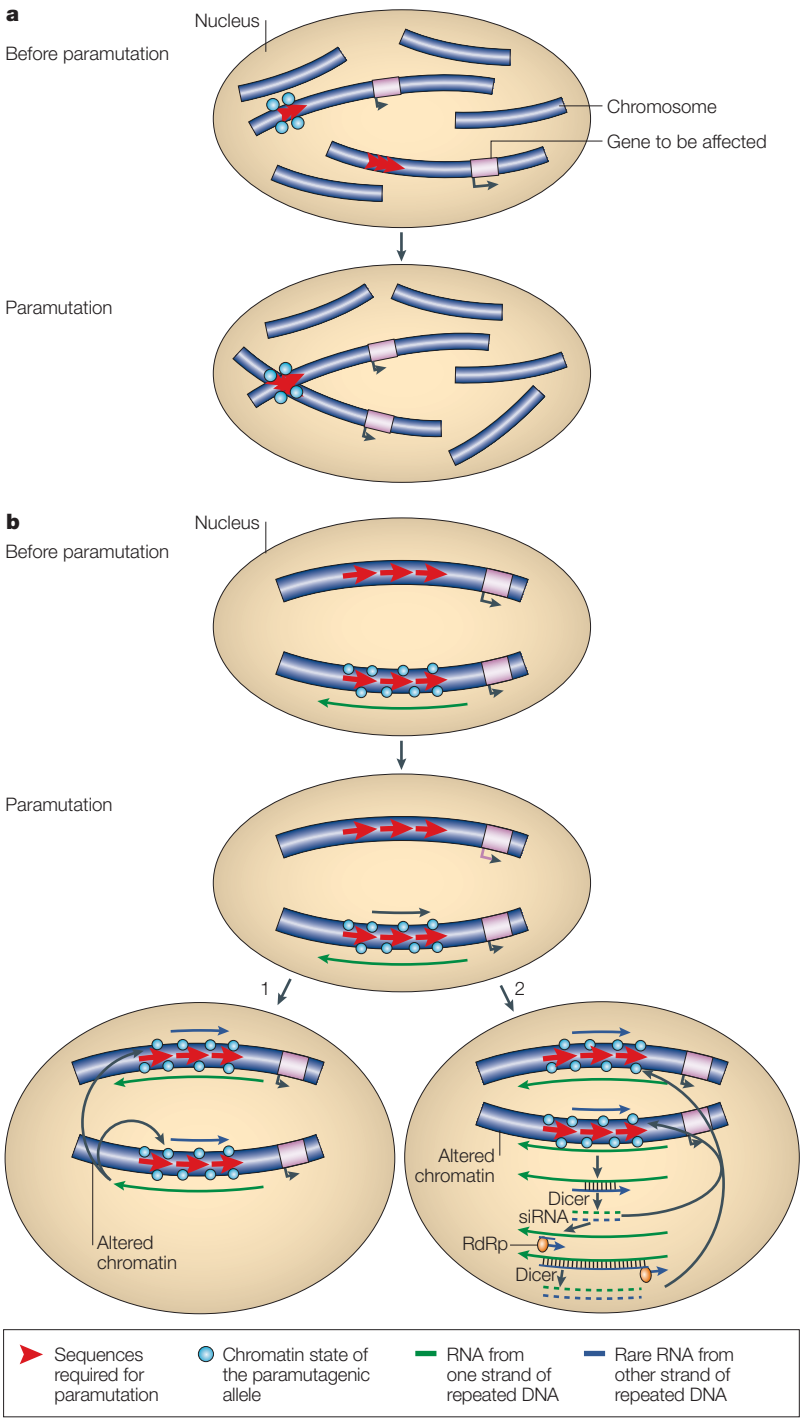


Figure 1 | **Models for paramutation.** For simplicity, only allele interactions are illustrated, but these models could also explain interactions between non-allelic homologous sequences. **a** | Pairing model for *trans*-induction of chromatin changes. Pairing between repeats is hypothesized to reduce gene expression by changing the subnuclear localization of the repeats, establishing a distinct chromatin structure, or both. **b** | RNA-mediated *trans*-induction of chromatin. Two mechanisms are illustrated. 1) Long non-coding RNAs from one or both strands are postulated to induce altered chromatin *in cis* and *in trans*. 2) A second possibility is a role for small interfering RNA (siRNA). The dsRNA that is formed by transcription from the two strands of the repeated DNA is a target for Dicer, which produces siRNA (dashed lines in figure). The siRNA is then postulated to mediate chromatin changes, which in turn alters the expression of the adjacent gene. Possible mechanisms include, but are not limited to, RNA-directed DNA methylation[40,41,97,98] and RNA-directed histone modification[8,49,99]. RdRP activity, which synthesizes complementary strand RNA using siRNA primers, results in increased amounts of siRNAs from throughout the repeats (for a detailed model, see REF. 51).

encoded by the mutations that have been isolated in maize. Because the maize screens are far from saturated, additional genes are likely to be identified in further screens. It is interesting that the genes that have been identified so far are not the main genes involved in gene silencing that have been identified in *A. thaliana* (*ddm1, met1, cmt3* and *mom1*). Therefore, it will be interesting to test whether mutations in the maize orthologues of these genes affect paramutation. There are also ~100 other maize genes that are potentially involved in chromatin changes and RNAi, which are reasonable candidates for the paramutation mutations; therefore, mapping these genes should be informative as well.

Molecular models of paramutation

Two models have been proposed to explain the *trans* communication that occurs during paramutation: one suggests that epigenetic states are altered by direct interactions between chromatin complexes (the 'pairing model'; FIG. 1a), whereas the other invokes the participation of RNA-mediated chromatin changes (the '*trans*-RNA model'; FIG. 1b). Our current understanding of paramutation is consistent with either or both models contributing to establishment or maintenance of one or more of the phenomena in TABLE 1. Below, we discuss these two models in more detail and the reasons that we believe paramutation could involve more than one mechanism.

Pairing model. A simple way to envision *trans* communication is through direct physical interactions between homologous sequences. Tandem repeats, which are required for paramutation at three genes in plants[22–24], mediate physical interactions *in trans* in *Drosophila melanogaster* and *Saccharomyces cerevisiae*. Tandem repeats of *D. melanogaster* transgenes are prone to silencing in a copy-number-dependent way. These transgene-repeat arrays also show long-range interactions with other sequences *in cis* and *in trans*. They also show VARIEGATION PROPERTIES that are similar to those seen in naturally occurring blocks of INTERSTITIAL HETEROCHROMATIN, such as that at the *brown(Dominant)* (*bw(D)*) allele, which contains a large insertion of heterochromatin[25] (BOX 2). The extent of gene silencing within the tandem repeats depends on two factors: their physical distance from heterochromatin on the same chromosome, or on pairing with heterochromatin on the homologue. The transgene arrays also cause pairing-dependent silencing of an expressed insertion at the same position on the homologous chromosome. More recent studies indicate that location, distance and gene-specific differences all influence the susceptibility of genes to *trans*-silencing[26].

Non-meiotic pairing has also been observed in other species. In *S. cerevisiae*, allelic and ectopic tandem repeat arrays of *LAC* or *TET* OPERATORS associate with similar frequencies in non-meiotic cells[27,28]. Fluorescence *in situ* hybridization (FISH) analysis in wheat revealed associations between widely separated homologous, repetitive transgene integration sites during interphase[29]. In *A. thaliana*, a considerable number of interphase nuclei show perfect alignment of homologous euchromatic chromosomal territories, indicating physical interactions

between homologous sequences[30]. In human T lymphocytes, a temporal and spatial association between maternal and paternal chromosomes 15 was observed; the association occurred specifically during the late S phase of the cell cycle at the 15q11-q13 regions, which contains several imprinted genes that are under epigenetic control[31].

The involvement of pairing is often tested by asking whether chromosomal translocations, which should disrupt pairing, interfere with the phenomenon under investigation. None of the translocations that have been examined for *b1* and *r1* paramutation disrupted paramutation (reviewed in REFS 2,3,32). However, the translocation breakpoints were not adjacent to the loci; so, pairing might still occur. In addition, once the epigenetic state is established, it is heritable in the absence of the inducing allele; so, brief physical interactions, rather than stable SYNAPSIS, might be all that is required for paramutation.

How might pairing between repeats promote homologue pairing and cause the transfer of an epigenetic state? Arrays of repeat sequences might increase the local concentration of bound proteins, conferring distinct chromatin properties that mediate pairing of homologous sequences or association of regions with similar heterochromatic properties[33], resulting in altered subnuclear localization[34,35]. Pairing would enable the transfer of chromatin complexes from one sequence to another, similar to that hypothesized for the communication between complexes at enhancers and RNA polymerase initiation sites that result in transvection[36] (BOX 2).

Trans-RNA model. Another way to envision communication is through an intermediate molecule; given the homology requirement for paramutation, RNA is a strong candidate. We discuss the potential involvement of two types of RNA: small interfering RNAs (siRNAs) and long non-coding RNAs (FIG. 1b). The production of dsRNA can trigger RNAi, which in turn can result in: degradation of homologous mRNA (reviewed in REF. 37); altered chromatin states associated with DNA methylation (reviewed in REF. 38); or, potentially, inhibition of translation[39]. Intriguing examples of RNA-directed DNA methylation that is mediated by inverted repeats have been demonstrated for inverted repeat transgenes[40] and the endogenous *PAI* genes[41]. In the transgene studies, an inverted repeat of a nopaline synthase promoter (NOSpro) that is transcribed by a heterologous promoter results in the production of NOSpro dsRNA. This dsRNA was able to induce DNA methylation and silencing of promoters that are homologous to the inverted repeat[40]. The *PAI* genes are arranged as an inverted repeat plus two singlet genes at unlinked loci (only one of the singlet genes encodes a functional protein)[17]. The predominant *PAI* transcript initiates at a novel unmethylated promoter that lies upstream of one of the inverted repeat *PAI* genes. Suppression of transcription from the upstream promoter reduces methylation on the singlet *PAI* genes, but not on the inverted repeat, which is consistent with an RNA methylation signal acting *in trans* on the singlet loci[41].

Silencing is not limited to inverted repeat loci, as efficient silencing of tandemly repeated transgenes has been observed in several species[42–47]. Tandem repeats at centromeric loci are involved in maintaining proper chromatin structure that is necessary for centromere function (reviewed in REF. 48). Interestingly, components of the RNAi machinery in *Schizosaccharomyces pombe* process transcripts that are derived from centromeric tandem repeats and mediate the formation and maintenance of silent chromatin, which is essential for centromere function[49,50]. RNAi is also required for heterochromatin formation and for the silencing of tandemly repeated transgenes in *D. melanogaster*[47].

How could silencing within tandem repeats be established and maintained? Once dsRNA is generated from the repeats by sense and rare antisense transcription, the production of siRNAs could be efficiently amplified from tandem array transcripts by RNA-dependent RNA polymerase (RdRP) and Dicer, as the siRNA primers can prime from an upstream repeat, replenishing siRNA for the whole repeat sequence. By contrast, siRNAs from single-copy sequences or dispersed repeats would eventually become depleted through subsequent rounds of priming by RdRP with further downstream primers[51]. Applying this model to *b1* paramutation, we speculate that the tandem repeats that are required for *b1* paramutation could produce enough siRNAs to stably maintain paramutation. If a paramutagenic locus produces a dsRNA that leads to silencing of a paramutable locus, how does the paramutable locus itself become paramutagenic? One hypothesis is that the chromatin change associated with paramutation results in transcription, which leads to production of the dsRNA. Although it might seem counterintuitive, heterochromatin can be transcribed. For example, the repeats at centromeres are transcribed even though they are heterochromatic[49]. Although there are no reports of an association with siRNA, one model for chromatin changes being associated with production of antisense RNA comes from the mouse *Air* locus; here, differential methylation of the Air promoter controls expression of the antisense *Air* transcript[52], which is required for the silencing *in cis* of several autosomal imprinted genes.

Alternatively, longer RNAs might mediate the *trans*-induction of chromatin changes. *Xist* RNA is necessary and sufficient to confer a chromatin-based mechanism of inactivation on adjacent sequences during X-inactivation in mammals (reviewed in REF. 53). *Xist* action is repressed by the antisense gene, *Tsix*, the full-length RNA product of which is complementary to *Xist* RNA in mice[54]. Expression of the large non-coding RNA, *Air*, on the paternal chromosome is required for silencing *in cis* of three imprinted protein-coding genes that lie within a 400-kb region[52]. So, the expression of large non-coding RNAs (sense and/or antisense) can mediate altered chromatin formation. Theoretically, such RNAs could function *in trans* in some systems; alternatively, chromatin states established through RNA mechanisms *in cis* could be communicated to the other allele through pairing interactions.

LAC OPERATOR
A DNA regulatory element that is derived from the *E. coli lac* operon that interacts with the *lacI* repressor.

TET OPERATOR
A control element of the tetracycline-resistance operon from *Escherichia coli*. The *tet* operator interacts with the *tet* repressor.

SYNAPSIS
The pairing of homologous chromosomes along their length; synapsis usually occurs during prophase I of meiosis, but it can also occur in somatic cells of some organisms.

spt::*Ac* TRANSGENE
A transgene that carries the streptomycin phosphotransferase (*spt*) gene. It carries the maize transposable element *Activator* (*Ac*) that is inserted into the 5′ untranslated leader of the *spt* gene.

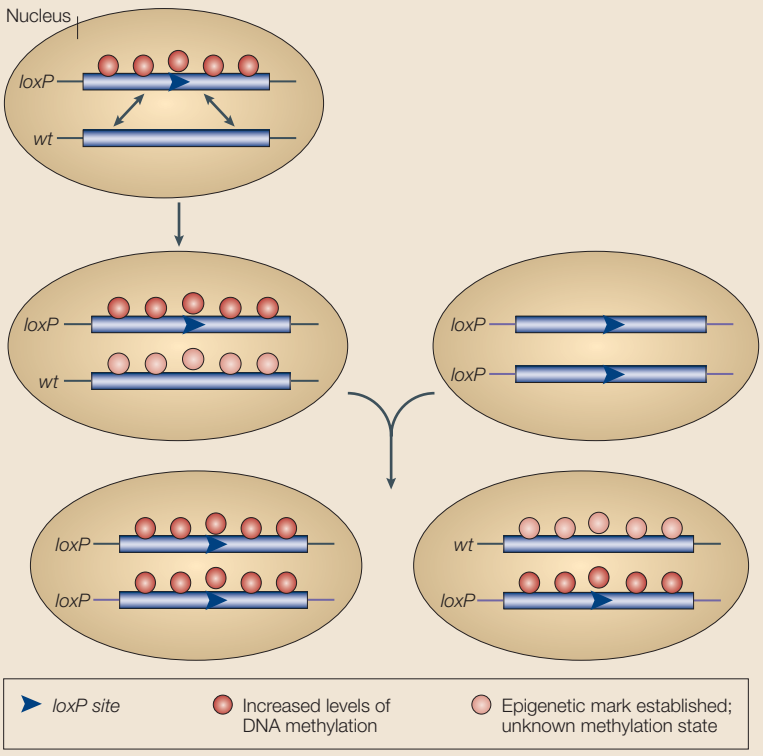
POLYCOMB-GROUP PROTEINS
A class of proteins — originally described in *Drosophila melanogaster* — that maintain stable and heritable repression of a number of genes, including the homeotic genes, with which they are associated.

Involvement of repeats. Repeats are often, but not always, associated with paramutation. The frequent correlation with repeats is consistent with either the pairing or *trans*-RNA models. The *PAI* system requires an inverted repeat to silence an unlinked singlet locus[17]. Tandem repeats are required for paramutation at three loci: *r1*, *b1* and the *spt*::*Ac* TRANSGENE[22–24]. For example, at *b1* (BOX 3), seven or five tandem repeats are highly paramutagenic, whereas one copy is completely inert (neutral allele). Interestingly, an allele with three repeats is paramutagenic, but unstable and less penetrant[24]. These results indicate a cellular mechanism for sensing the number of repeats.

The types of tandem repeat are different in the three examples, and could contribute to distinct mechanisms. At *r1*, the repeats are large (each repeat is at least 13 kb[55] or 26 kb[56]) and include the promoter and transcribed regions, whereas at *b1* (BOX 3) the repeats are less than 1 kb, are located 100 kb upstream of *b1* and share no homology with the promoter or transcribed *b1* sequences. In the *spt::Ac* transgene example in tobacco, the repeats flank the *spt* gene and are 4.5 kb each[23]. In other paramutation examples, sequences are within complex transgene arrays but, because allelic series have not been reported, the requirement for repeats has not been rigorously demonstrated.

Not all examples of *trans*-silencing involve tandem or inverted repeats. For example, in *Petunia* a simple, single copy of the *a1* transgene mediates paramutation[57], and a single *loxP* site can induce the *loxP trans*-silencing in the mouse[58] (BOX 5). The *cis* and *trans* pairing that is mediated by the binding of the POLYCOMB GROUP (PCG) PROTEINS to Polycomb response elements (PREs) provides a useful example of the pairing model. PcG proteins that are bound to one polynucleosome template can recruit a second template from solution; this leads to transcriptional repression and provides an explanation for how long-range *cis* and *trans* interactions could mediate changes in epigenetic chromatin states[59].

Box 5 | ***Trans*-interactions involving mouse transgenes**



Two paramutation-like phenomena involving loci with integrated *loxP* sites have been described in the mouse[58]. One involves a locus with an integrated transgenic reporter gene that is flanked by *loxP* sites and the other a locus containing a single *loxP* site. When the *loxP* elements were combined in a cross with a *Cre* recombinase gene that is only expressed in meiotic spermatocytes, recombination between *loxP* elements occurs at high efficiency in the first generation. However, in subsequent generations, recombination of newly introduced *loxP*-flanked transgenes was completely blocked and the *loxP* sequence, the other transgene sequences and the adjacent chromosomal sequences were hypermethylated. Recombination was inhibited for the several generations tested. Intriguingly, both the methylated *loxP* alleles and their corresponding wild-type alleles were paramutagenic, as illustrated in the figure. When either of the two alleles — wild-type or *loxP*-containing — were heterozygous with an unmethylated *loxP*-containing locus, the latter became methylated. Although this phenomenon was called transvection when it was described (see BOX 2), it is very reminiscent of *p1* transgene-induced paramutation in maize (BOX 4), except that here the affected process is recombination rather than gene expression.

Other paramutation-like phenomena reported in the mouse include alterations in gene expression involving interactions between a *Rasgrf1* allele containing transgenic sequences and its wild-type counterpart on the homologous chromosome[95], or the endogenous *U2af1-rs1* gene and an homologous multicopy transgene locus integrated at a non-allelic position[96]. The *Rasgrf1* example offers two interesting parallels with the plant systems: the paramutable allele becomes paramutagenic (like the *loxP* system) and the epigenetic state of the wild-type *Rasgrf1* allele is controlled by a regulatory region with tandem repeats[95].

Is paramutation at single copy loci also consistent with the *trans*-RNA model? If single-copy loci continuously generate sense and antisense transcripts, tandem repeats are not necessary for maintaining the silenced state; the resulting dsRNA would be cleaved into siRNAs, which would then establish the altered chromatin state. Paramutation in *Phytopthora infestans*[60] is most consistent with a *trans*-RNA model. Silencing of an endogenous gene by an homologous transgene occurs when the genes are in different nuclei in the heterokaryon, indicating a mobile *trans*-acting signal.

Trans-silencing might also occur through recombination mechanisms. The *loxP*-mediated silencing in the mouse[58] (BOX 5) is reminiscent of the transfer of methylated sequences that occurs during recombination in *Ascobolus immersus*[61]. Here, transfer of DNA methylation from a methylated allele to an unmethylated allele of the *b2* spore-colour gene occurred when the two alleles were together in meiotic cells. The transfer always accompanied GENE CONVERSION, indicating that methylation transfer and recombination are mechanistically linked. In the mouse system, the connection to recombination is less clear. Methylation transfer occurs between loci with single *loxP* sites, which do not recombine. The presence of the Cre recombinase was required to establish the epigenetic state that is associated with DNA methylation, but Cre was not required for the subsequent spreading or for the paramutation-like transfer of DNA methylation between alleles.

Enhancer sequences. The sequences that are required for paramutation at the *b1* and *p1* loci co-localize with sequences that are required for enhancer activity[24,62] (BOXES 3,4). One intriguing model for how enhancers might be involved in *trans*-interactions comes from mammalian studies that examined how a functional enhancer can suppress transgene silencing[63]. Results indicate that enhancers might maintain transgene expression by preventing localization of the transgene near centromeric heterochromatin and/or by recruiting the transgene to a nuclear compartment in which transcription is favoured and is stably heritable. Therefore, the *b1* and *p1* enhancer sequences might mediate sequestration into distinct compartments that are compatible with high expression or low expression, depending on whether alleles are in paramutable or paramutagenic states, respectively.

An intriguing question is whether the enhancer sequences are directly involved in mediating the *trans* interactions, in addition to their role in mediating long-distance *cis* regulation. Further identification of the minimal sequences that are sufficient for enhancer activity and those that are sufficient for paramutation in both the *b1* and *p1* systems should address this question and shed light on the mechanisms that underlie this phenomenon.

Possible evolutionary significance

Paramutation could represent one of several homology-dependent gene-silencing mechanisms, which function as host defence strategies to counter foreign or invasive nucleic acids such as transposable elements and viruses (reviewed in REF. 64). Common triggers of gene silencing include DNA repeats and dsRNA. The repeats that are associated with certain paramutation systems could be recognized by the same machinery that evolved to defend the host against viruses. It has also been proposed that paramutation represents the aberrant function of the machinery involved in identifying and maintaining chromatin boundaries between genes and nearby repetitive sequences (for further discussion, see REFS 3,65).

Whatever the mechanistic origin of paramutation, several potential roles for, and consequences of, allele or homology-dependent transfer of epigenetic information to progeny have been proposed. An important unanswered question for evaluating the potential significance of paramutation contributions to evolutionary processes is the number of genes that are affected by paramutation. Only if a large number of genes are affected would paramutation contribute significantly to evolutionary processes.

Transmitting adapted expression states. Paramutation provides a mechanism for transmitting environmentally adapted expression states that were established in somatic cells to progeny (reviewed in REF. 66). Indeed, in two instances paramutation is influenced by temperature: *r1* paramutation in maize[67,68] and *a1* transgene paramutation in *Petunia*[69]. The transfer of environmentally adapted expression states could be especially important for plants, the progeny of which are most often in the same location and similar environment as their parents. The reversibility and the distinct quantitative expression states observed for many paramutation systems provides variation on which selection could act.

Evolution of polyploids. Polyploidy is common in plants and has been significant in the evolutionary history of vertebrates and other eukaryotes. Allele interactions and homology-sensing mechanisms could function differently in diploids than in organisms with higher ploidy. Indeed, epigenetic changes in gene expression are observed in recently formed ALLOTETRAPLOIDS[70,71]. Furthermore, a particular transgene locus undergoes paramutation in tetraploid, but not in diploid *A. thaliana*[19]; which indicates that the *trans*-sensing machinery behaves differently depending on ploidy. Paramutation-like phenomena could quickly establish functional homozygosity in polyploids; if widespread, these phenomena could contribute to the rapid evolution of polyploid species.

Contribution to complex traits. An interesting speculation is that paramutation-like phenomena contribute to the low penetrance and other complexities observed in genetic analyses of many COMPLEX (quantitative) human diseases and other quantitative characters in many organisms. Predisposition to type I diabetes provides one example; the allele-specific

GENE CONVERSION
A specific type of recombination in which the sequence of one DNA strand is used to alter the sequence of the other, resulting in non-reciprocal genetic exchange.

ALLOTETRAPLOID
A polyploid organism that is established from hybridization of two different species. This organism carries four complete sets of chromosomes, two derived from each parental species.

COMPLEX
A measured phenotype, such as disease status or a quantitative character, which is influenced by many environmental and genetic factors, and potentially by interactions within and between them.

VARIABLE NUMBER OF TANDEM REPEAT (*VNTR*) LOCUS
A locus that contains a variable number of short tandemly repeated DNA sequences that vary in length and are highly polymorphic.

HETEROSIS
The greater fitness of a hybrid individual carrying different alleles of genes relative to either of the two corresponding homozygous parents.

MICROSYNTENY
Collinearity in the order of genes and intervening DNA sequences in homologous chromosomal regions of two (sub)species.

OVERDOMINANCE
Describes the greater phenotypic expression seen in the heterozygote compared to that of either homozygote.

effect depends on the identity of the non-transmitted paternal allele. Therefore, information might be transferred to progeny through a paramutation-like mechanism between the non-transmitted and transmitted paternal alleles[72]. Intriguingly, this system involves the *VNTR* (VARIABLE NUMBER TANDEM REPEATS) minisatellite at the 5′-end of the insulin gene. Although the literature does not provide a clear correlation between the repeat number and predisposition to the disease, one interesting speculation is that the presence of the tandem repeats in both alleles might promote communication between them.

Hybrid vigour. This phenomenon (also known as HETEROSIS) has intrigued geneticists and evolutionary biologists for nearly 100 years, but no consensus has emerged about its genetic basis (reviewed in REF. 73). Recent studies have suggested that the lack of MICROSYNTENY between maize inbred lines results in different genes being absent in distinct inbred lines relative to others[74]. The missing genes identified so far are members of multi-gene families and therefore are likely to be non-essential in the inbred line, but combining inbred lines that are missing different combinations of genes could result in complementation and hybrid vigour[74]. Another, not mutually exclusive, possibility is that allele interactions that resemble paramutation occur in hybrids and cause epigenetic changes in gene expression. Consistent with this idea, alleles at the *pl1* locus display single-locus heterosis, or OVERDOMINANCE; darkly pigmented heterozygotes result from combining lightly pigmented paramutagenic and neutral alleles[75]. So, epigenetic as well as genetic mechanisms might contribute to the variation underlying hybrid vigour.

Conclusions

The term 'paramutation' has come to describe many phenomena in which communication between alleles or homologous sequences establishes distinct, heritable epigenetic states. We hypothesize that multiple mechanisms underlie the diverse phenomena and suggest two non-mutually exclusive models; pairing interactions between specific chromatin complexes and *trans*-RNA-based communication.

Plants provide exceptional systems to investigate paramutation because of their powerful genetics, the easily scorable phenotypes associated with several of the genes undergoing paramutation, and the fact that, late in development, somatic tissues give rise to gametes, which often results in somatic variation being transmitted to the next generation. The identification of the sequences that are required for paramutation, and the likelihood of identifying *trans*-acting proteins that are involved in paramutation, should reveal answers to the following fascinating questions. How are homologous sequences sensed and counted? How do homologous sequences communicate; are RNA molecules and/or physical interactions mediating paramutation?

The number of published examples of paramutation is relatively small. Is paramutation an unusual circumstance at rare loci, or do similar interactions occur at many genes? Most examples are at genes that confer visible, quantitative phenotypes, which we suspect facilitated their discovery. We predict that the availability of genome-wide approaches to access gene expression in a large number of organisms will reveal that paramutation is more widespread than currently appreciated. If this prediction is fulfilled, our current models for heredity and evolution will need major modifications.

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Competing interests statement
The authors declare that they have no competing financial interests.

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