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Paramutation in Plants

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Paramutation describes a directed, meiotically heritable alteration of gene regulation influenced by allelic interactions.

Introduction

Paramutation describes a chromosomal event that results in a directed, meiotically heritable change in gene control (Brink, 1956). Gene function is repressed but not eliminated by these changes. Such events are distinguished from classical mutations in their origin, their quantitative variability and, in many cases, their high frequency of reversion. In addition, paramutations reflect epigenetic changes; that is, heritable alterations without deoxyribonucleic acid (DNA) sequence changes (Chandler *et al.*, 2000; Stam *et al.*, 2002). Because the paramutation mechanism is epigenetic in nature, the meiotic heritability of such changes blurs the very definition of genetics. Paramutations, together with other examples of meiotically heritable epigenetic changes in *Drosophila* and *Schizosaccharomyces pombe*, emphasize that eukaryotic chromosomes, not just DNA sequences, can act as fundamental units of inheritance.

Only specific alleles exhibit paramutation and it remains unclear in every case what discrete features define such alleles. With a few possible exceptions, all examples of paramutation have been described in plants and have been identified as heritable alterations in the action of specific genes regulating visible traits. Many examples of unusual inheritance patterns for traits in ornamental and agricultural breeding material are probably due to paramutation; however, the poor genetic resources of those species do not allow confirmation. To fit the description of paramutation, a single genetic locus responsible for the heritable change in phenotype must be identified. This requires sexual crossing, genetic markers, and at least a rudimentary genetic map. Although the extent to which paramutation occurs in plants is unknown, similarities with numerous examples of endogenous gene and transgene silencing suggest that at least some aspects of the paramutation mechanism are widely used in genome control.

The most distinctive feature of paramutation is that the events are defined by chromosome-based allelic interactions. Specific alleles are capable of causing paramutation to occur in *trans* on homologous alleles and the newly repressed alleles can subsequently facilitate a similar event in subsequent generations (Figure 1). In some cases, the alleles capable of causing paramutation (paramutagenic) are directly derived from those alleles that are susceptible (paramutable). By following the sexual transmission of genetic markers flanking these specific alleles it is clear that such events are initiated by the paramutagenic allele and that both chromosomes are properly segregated. The result of this *trans*-silencing behaviour violates Mendel's first law of genetics: that genetic factors remain unchanged in a heterozygote. In at least two experimental cases, maintenance of the repressed expression state requires continued allelic interactions; the repressed state can progressively change to a fully active state when the allele is hemizygous. Although the exact nature of these allelic interactions is unknown, there are two general models (Figure 2); either the interactions require physical homologue pairing, or they are mediated through diffusible materials, presumably ribonucleic acid (RNA). Other experimental examples of *trans*-silencing events provide more detailed molecular models for various types of allelic interactions (see below).

Epigenetic Regulation

Epigenetic refers to the inheritance of information not encoded by primary DNA sequence. Cytosine methylation, nucleosome modifications, chromosome associations with non-coding RNAs or specific proteins, replication timing and nuclear positioning all provide non-DNA-based platforms for templating and replicating both chromosome and nuclear architectures that influence gene expression. These epigenetic mechanisms are used as a general means to control specific genes during development. For example, in *Drosophila*, genes responsible for specifying the adult body plan are spatially activated or repressed during embryogenesis through the transient operation of positive and negative transcription factors. Activated or repressed transcription states are subsequently maintained in clonal descendants of these embryonic precursors by general chromosome-associated proteins of the *Trithorax* or *Polycomb* classes respectively.

Although most epigenetic information is terminally restricted to specific somatic cell types, it can transcend generations through the germline. Genomic imprinting is one example of epigenetic information initiated in one parent that is passed to its progeny. In both animals and plants there is widespread evidence that such imprinting is essential to properly coordinate embryonic development. Gynogenetic (maternal genome only) mouse embryos fail to develop. Maize endosperms lacking male-derived chromosome segments are reduced in size. In the few examples of specific imprinted loci, the epigenetic mark appears to be cytosine methylation, a methyl group addition to the 5' position of cytosine residues. Methylase enzymes acting on hemimethylated substrates (DNA replication products of symmetrical ^{Me}CpG or ^{Me}CpNpG sequences) act to maintain these methylation patterns through successive rounds of cell divisions. Imprinting is a 'parent-of-origin' source of epigenetic information that can be erased or reset every generation. In contrast, alterations occurring via paramutation are passed on from generation to generation irrespective of parent-of-origin transmission.

Gene Silencing in Plants

'Gene silencing' generally refers to epigenetic repression: loss of gene expression in the absence of DNA sequence alterations. Dosage compensation via X-chromosome inactivation in female mammals is an excellent example of such silencing behaviours. Entire chromosome complements, chromosomes, chromosome segments or individual loci may be subject to epigenetic repression. Restricting genetic functions within the context of a eukaryotic nucleus is essential to maintain genome stability and to accomplish developmental differentiation. Occasionally, the occurrence of unexpected gene silencing provides experimental entrées to understanding the molecular mechanisms involved.

Centromeric and telomeric regions appear to be particularly unfavourable environments for transcriptional activity. These regions are composed largely of repetitive retroviral sequences, are associated with the nuclear envelope, are replicated late in S phase, and have a lower gene density. Genes juxtaposed to these regions via chromosome translocations often display silenced or variegated patterns of expression; a phenomenon referred to as position-effect variegation (PEV). Similarly, transgenes integrated into centromeric or telomeric regions often show similar types of silencing. Thus physical proximity to silenced genomic regions alone can result in gene or transgene silencing.

Repeated sequences in general appear to be potent signals for attracting epigenetic silencing. Variant alleles and transgene loci containing duplicated sequences are often repressed. Derivatives that sequentially reduce or increase the numbers of repeated sequences, either through unequal crossing-over or transpositions, demonstrate a strong correlation between repeats and silencing. This repeat-induced silencing mechanism presumably evolved to protect genomes from deleterious actions of autonomous replicating elements. However, certain regions of repetitive sequences, such as those encoding ribosomal DNA, have managed to co-evolve protection from such silencing.

In plants, there are numerous examples of endogenous genetic elements and an ever-expanding array of exogenous transgene constructs that exhibit unexpected gene silencing behaviours. Normal occurrences of gene silencing related to viral immunity are particularly experimentally tractable. Genetic and molecular dissection of these silencing systems has so far highlighted a surprising variety of repression mechanisms with hints of potential crosstalk. These studies have illustrated that silencing events can be triggered by inherent properties of the genetic locus in question (*cis*-inactivation) or they can be triggered through interactions with homologous sequences in *trans* (*trans*-inactivation). 'Cosuppression' is a term reserved for specific examples of *trans*-silencing in which a given transgene may silence homologous endogenous genes.

Transcriptional gene silencing

Silencing due to reduced transcription rate of a given gene, as measured by *in vitro* transcription assays using isolated nuclei, is generally referred to as transcriptional gene silencing (TGS). Other properties of TGS are listed in Table 1. Particularly prevalent in transgenic plant lines, TGS is usually not an inherent property of the transgene construct *per se* but instead is triggered by either the multicopy nature or

the chromosomal position of a given transgene integration event. TGS is generally associated with increased cytosine methylation ($5^{m}C$) in both symmetrical (CpG or CpNpG context) and nonsymmetrical (CpN) sequences of promoter regions, as well as decreased susceptibility to nuclease digestion. These molecular changes presumably reflect chromatin-level alterations that restrict recruitment and/or processivity of transcription complexes. TGS is most commonly triggered by repetitive sequences. Repeated sequences are known to promote the formation of repressive chromatin states in *Drosophila*. Presumably, similar chromatin alterations occur in TGS, as a putative chromatin-remodelling factor (*DEFICIENT DNA METHYLATION1*; *DDM1*) is required for the maintenance of TGS in *Arabidopsis*.

Table 1 Transcriptional (TGS) versus posttranscriptional gene silencing (PTGS)

Characteristics	TGS	PTGS
Inducing agent	Chromosome position / repeated sequences	dsRNA
RNA levels	Decreased	Decreased
Transcription rates	Decreased	Unchanged
Small RNA fragments	None detected	Increased presence
Cytosine methylation	Increased in regulatory regions	Increased in transcribed regions
Nuclease sensitivity	Decreased	Not determined
Systemic silencing signal	None detected	Graft transmissible
Trans-silencing	Meiotically heritable	Not meiotically heritable

Similar to examples of paramutation, chromosomal regions subject to TGS can sometimes induce TGS in *trans* to other homologous sequences. This *trans*-silencing is cell autonomous; the causative agent is not transmissible from one region of a plant to another. In a few cases, endogenous alleles or transgenes affected by TGS can induce TGS on homologous alleles or other transgenes containing as little as 90 bp of promoter homology. These *trans*-silenced alleles or transgenes often retain their silenced state following meiotic segregation away from the inducing agent. Unlike examples of paramutation, however, these *trans*-silenced alleles or transgenes are not able to facilitate additional *trans*-silencing events. This discrepancy should not be interpreted to mean that there are significant mechanistic differences between TGS and paramutation. In fact, one particular transgenic *Petunia hybrida* (petunia) line exhibits all the genetic and molecular characteristics of TGS-based paramutation (Table 2; Meyer *et al.*, 1993). All other *P. hybrida* lines containing different chromosomal integrations of the same transgene construct do not display this behaviour. This observation emphasizes that only specific chromosome regions are involved in facilitating these types of heritable *trans*-silencing behaviours.

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Table 2 Paramutation examples

Species	Locus	Affected phenotype	Paramutable alleles	Paramutagenic alleles	Paramutagenic element(s)	$5^{m}C$ correlations	Nuclease sensitivity	Reactivation
Lycopersicon esculentum	*sulfurea (suf)*	chlorophyll	*suf +*	*suf*^{para}, *suf*^{variegated}[a]	Unknown	Unknown	Unknown	Yes
Zea mays	*red1 (r1)*	seed colour	*R-r:std*	*R-st*, *R-mb*, *R-sc*[b], *R-scm*[b], *R-r:std*	Duplications	Increases at *S* component of *R-r:std* with silencing	Unknown	Yes
Zea mays	*booster1 (b1)*	plant colour	*B-I*	*B*^{I}	Distal 5′ duplications	Remain unchanged at promoter; decreases at distal enhancer region following silencing	Decreases with silencing	No
Petunia hybrida	$A1^{RLOI\text{-}17\,(c)}$	flower colour	$A1^{RLOI\text{-}17\text{-}R\,(c)}$	$A1^{RLOI\text{-}17\text{-}W\,(c)}$	Chromosome position	Increases with silencing	Decreases with silencing	Yes
Zea mays	*purple plant1 (pl1)*	plant colour	*Pl-Rh*	*Pl*^{I}	Unknown	Remain unchanged	Unknown	Yes
Zea mays	*pericarp colour1 (p1)*	seed colour	*P1-rr*	*P1-rr*^{I}, *P1.2b::GUS*[d]	Repeated regions?	Increases with silencing	Unknown	Yes?

[a] *suf*^{para} and *suf*^{variegated} refer to a paramutagenic *suf* allele having greater or lesser effect on chlorophyll accumulation (Hagemann, 1969).
[b] *R self-coloured* (*R-sc*) and *R self-coloured marbled* (*R-scm*) are respective derivative alleles of *R-st* and *R-mb* that have lost the transposable elements responsible for stippled and marbled pigment patterning.
[c] $A1^{RLOI\text{-}17}$ describes a specific transgene locus containing a single-copy transgene insertion in which the cauliflower mosaic virus promoter for the 35S protein (CaMV35S) controls expression of a *Z. mays anthocyaninless1* (*A1*) coding region. *A1* encodes the biosynthetic enzyme chalcone synthase. $A1^{RLOI\text{-}17\text{-}R}$ and $A1^{RLOI\text{-}17\text{-}W}$ denote unsilenced (red) and silenced (white) states of the CaMV35S-A1 transgene.
[d] P1.2::GUS denotes a transgene containing a 1.2-kb enhancer region of the *P1-rr* allele fused to a basal promoter controlling expression of a bacterial β-glucuronidase (GUS) gene. Transgenic lines containing multicopy arrays of P1.2b::GUS are paramutagenic (Sidorenko and Peterson, 2001).

Posttranscriptional gene silencing

Silencing associated with reduced RNA levels in the absence of transcriptional differences is generally referred to as posttranscriptional gene silencing (PTGS). First identified in plants, this RNA-based mechanism appears to be a major eukaryotic regulatory system for controlling developmental timing of gene expression and for immunity against double-stranded RNA viruses. Specific hallmarks of PTGS are listed in Table 1. RNA molecules with greater than 25 bp of perfect complementary sequences can become targets for a double-stranded RNAase-III enzyme (*Dicer* in *Drosophila*; *CARPEL FACTORY1* in *Arabidopsis*). Short 21–25-nt digestion products are then incorporated into a multisubunit RNA-induced silencing complex (RISC) that targets other related RNAs for degradation. These short RNA degradation products can also act as primers for an RNA-dependent RNA polymerase (encoded by *SILENCING DEFECTIVE1/SUPPRESSSOR OF GENE SILENCING2*; *SDE1/SGS2* in *Arabidopsis*) that can create additional double-stranded RNA (dsRNA) targets.

Both endogenous alleles as well as transgene loci can be susceptible to PTGS and can also cause PTGS-based silencing in *trans*. In practice, these *trans*-silencing properties provide a powerful tool for functional genomics. Any given gene or multigene family can be functionally 'knocked-out' by designing transgenes, or viruses, to specifically produce RNA molecules with double-stranded character. Unintentionally, however, certain transgene loci or even endogenous alleles can fortuitously produce either single RNAs with internal complementarity or both sense and antisense transcripts from opposing promoter activities. Either situation can trigger PTGS. It is conceptually easy to understand how *trans*-silencing can occur via a PTGS mechanism. Small interfering RNAs (siRNAs), products of dsRNA degradation, presumably diffuse throughout the plant and facilitate systemic acquired silencing (SAS). PTGS is not cell-autonomous and can, in fact, occur in previously unsilenced plant grafts. Transgenes or endogenous genes silenced in *trans* via a PTGS-inducing element generally do not maintain the silenced state following meiotic segregation from the inducer.

In some cases, both PTGS and TGS mechanisms conspire to achieve heritable gene silencing. Although not well understood, there is a feedback relationship between PTGS and chromosome alterations. PTGS is associated with increased $5^{m}C$ levels

within transcribed regions (RNA-dependent DNA methylation), implying that some information from the process of dsRNA degradation influences modifications of these genomic regions. Thus, PTGS-inducing transgenes that target specific promoter sequences in *trans* can silence that target gene via a TGS mechanism. Presumably, PTGS causes changes to the promoter regions that then act to recruit TGS components. Although most molecular aspects of known paramutation systems implicate the involvement of TGS-type mechanisms (see below), potential contributions of PTGS-type mechanisms should not be overlooked.

Paramutation

Alexander Brink (1956) first applied the term 'paramutation' to examples of meiotically-heritable silencing of specific *red 1* (*r1*) alleles in *Zea mays* (corn). These examples were relatively simple to study since *r1* encodes a basic helix–loop–helix (bHLH) transcriptional activator of the anthocyanin pigment biosynthetic pathway (see below). Genetic crosses demonstrate that strongly pigmenting *r1* alleles invariably change to a weaker expression state after exposure to other specific *r1* alleles. This newly repressed allele is also endowed with the ability to cause similar changes in naïve, strongly pigmented *r1* alleles.

Other examples of nonmendelian inheritance patterns for novel leaf shapes in pea, petal shapes in primrose, chlorosis in strawberries, leaf crinkling in sweet cherry, and bud failure in almonds (reviewed in Brink, 1973) closely resemble paramutation behaviour. While these probably represent *bona fide* examples, definitive genetic proof is lacking. In contrast, the *Lycopersicon esculentum* (tomato) *sulfurea* (*sulf*) locus is a genetically defined example of paramutation affecting leaf chlorophyll production (reviewed in Hagemann, 1969). A specific *sulf* allele conferring dominant leaf chlorosis was recovered from an X-irradiation experiment and was shown to cause 'conversion-type' inheritance to naïve *sulf*+ alleles, in that primarily chlorosis-inducing *sulf* alleles segregated from heterozygous plants. Although radiation-induced damage was presumably responsible for the genesis of this type of *sulf* allele, normal *sulf*+ alleles are clearly able to mimic the silencing effect. Meiotically heritable alterations in *sulf* closely follow somatic lineages where *trans*-silencing is occurring; flowers borne on strongly chlorotic branches transmit a higher percentage of silenced *sulf* alleles than branches with a variegated chlorotic phenotype. These observations emphasize that the *trans*-silencing mechanism has a somatic basis and displays a degree of cell autonomy in its action.

Remaining examples of paramutation all occur at maize loci encoding transcriptional regulators of flavanoid metabolism. Silencing of these regulators results in visible reduction of colourful pigments (Figure 3). The dispensable nature of this metabolic pathway tolerates the creation of unusual allelic variants, and the visible nature of the pigments contributes to the discovery and study of alleles required for their synthesis. In addition to the *r1* locus, specific alleles of the *booster1* (*b1*), *purple plant1* (*pl1*) and *pericarp colour1* (*p1*) loci also undergo paramutation (Coe, 1959; Hollick *et al.*, 1995; Sidorenko and Peterson, 2001). The *r1* and *b1* loci encode functionally duplicate bHLH factors while *pl1*, *coloured aleurone1* (*c1*) and *p1* all encode *myb*-class transcriptional activators. Genes encoding the enzymes required to produce red phlobaphene pigments are transcriptionally induced through binding of the *P1*

activator to their promoters. Regulation of purple anthocyanin pigment production is slightly more complex. Genes encoding the anthocyanin biosynthetic enzymes are transcriptionally induced via combined actions of one MYB protein (PL1 or C1) plus one of the bHLH proteins (B1 or R1). Wherever the patterns of *pl1/c1* and *r1/b1* expression overlap, anthocyanin pigments can be produced. Intensive human selection for pigmentation variants in maize has captured tremendous allelic diversity among these regulatory loci. Among these alleles are those susceptible to paramutation (paramutable) and those that cause paramutation (paramutagenic). Alleles that are neither paramutable nor paramutagenic are termed 'neutral'.

Paramutation examples

Similarities and differences for all examples of paramutation in plants are summarized in Figure 4 and Table 2). Between the *r1*, *b1*, *pl1* and *p1* loci, no two examples of paramutation are exactly the same. Genetic analyses, however, indicate that certain molecular features are conserved (see below). Differences in allele structures, occurrences of spontaneous paramutation, affected regulatory features and reversion abilities are primary discriminatory behaviours. In some cases, these differences suggest that certain aspects of the molecular mechanisms may be distinct.

b1 paramutation

Most *b1* alleles are expressed at some level in mature plant tissues, and all *b1* alleles characterized have a single coding region (Figure 4). The *B-Intense* (*B-I*) allele is maintained through continued selection for dark purple pigment phenotypes. *B-I* can spontaneously change to a weaker expression state referred to as *B'* (Figure 3). These alterations can manifest somatically (visible as clonal sectors of weaker pigment) or in progenies (entire progeny set or fraction thereof have weak pigment), but they rarely occur when *B-I* is heterozygous with a neutral *b1* allele. The *B'* state is stable; *B'* has never been observed to revert back to the fully expressed *B-I* form. The *B'* state is also strongly paramutagenic; without exception, *B'* / *B-I* heterozygotes display a reduced pigment *B'* phenotype and only *B'* states are transmitted to progeny. Fine scale mapping has shown that repetitive sequences (seven, highly AT-rich, direct repeats) responsible for *B'* paramutagenic activity reside ~100 kb upstream of the *b1* coding region (Stam et al., 2002).

pl1 paramutation

Like *b1*, all known *pl1* alleles have a relatively simple molecular structure (Figure 4) that includes a small 3' duplication. Almost all *pl1* alleles require light cues for induction and are expressed in seedling tissues, mature plant tissues, and anthers. The *Pl-Rhoades* (*Pl-Rh*) allele is maintained through continued selection of dark purple plants or anthers. Unlike other *pl1* alleles, *Pl-Rh* lacks the light-dependent requirement for expression. In a similar manner as *B-I*, *Pl-Rh* can spontaneously change to a weaker, light-dependent regulatory state referred to as *Pl'* (Figure 3). However, unlike the apparent binary switch between *B-I* and *B'* states, a continuum of quantitatively distinct expression states can be attained from *Pl-Rh* (Hollick et al., 1995). Expression levels are direct indicators of paramutagenic strength. *Pl'* states that show slightly less expression than the *Pl-Rh* state are weakly paramutagenic, while *Pl'* states with moderate to low pigmenting activity are strongly paramutagenic; without exception, only strongly paramutagenic *Pl'* states are transmitted from these types of

Pl' / *Pl-Rh* heterozygotes. *Pl'* also differs from *B'* in its stability. Although weaker *Pl'* states are extremely stable in homozygous condition, they can revert back to fully expressed *Pl-Rh* states in some cases. When *Pl'* is maintained in heterozygous combination with neutral *pl1* alleles, hemizygous over a chromosomal deficiency, or in the absence of specific genetic functions (see below), expression levels are heritably increased. *Trans*-suppression of *Pl-Rh* is apparent in the earliest tissues of *Pl'* / *Pl-Rh* seedlings. Reversion properties of *Pl'* states make fine scale genetic mapping of the functional *cis*-acting elements difficult. However, the observation that other *pl1* alleles share nearly identical coding and immediate flanking sequences suggests that distal regulatory elements are responsible for the paramutation behaviour at *pl1*.

r1 paramutation

Unlike *b1* and *pl1* alleles, most *r1* 'alleles' are complex; they contain multiple *r1* coding regions (Figure 4) and are better referred to as haplotypes. Most *r1* coding regions that make up a given haplotype are expressed exclusively in seeds, specifically in the single cell layer of the endosperm (the aleurone layer) directly underlying the seed coat (pericarp). Other *r1* coding regions are expressed in seedlings and anthers. Also unlike examples at *b1* and *pl1*, there exist strongly paramutagenic *r1* haplotypes that are structurally distinct from paramutable *r1* haplotypes. In fact, while paramutable regions such as the seed component (*S1-S2*) of *R-r:standard* (*R-r:std*) do undergo paramutation, the derivative *R-r:std'* state is only weakly paramutagenic to naïve *R-r:std* haplotypes. *R-stippled* (*R-st*) and *R-marbled* (*R-mb*) are strongly paramutagenic; only weak pigmenting *R-r:std'* states are transmitted from *R-st* / *R-r:std* or *R-mb* / *R-r:std* heterozygotes.

Again unlike the *b1* and *pl1* examples, immediate *trans*-suppression of *R-r:std* is not detected in F_1 material. However, this difference may be related to following details of the assay. *R-r:std* pigmenting activity is assayed in the aleurone following its male transmission to a recessive *r1* (no seed color) female. The *R-r:std* haplotype is imprinted at some stage prior to sperm cell development so that it confers a discontinuous, or mottled, pigment phenotype (Figure 3). The imprinted potential for mottling action is measurably less in the *R-r:std'* state relative to *R-r:std*. Additional reductions in mottling action can be achieved following repeated exposures to paramutagenic *r1* haplotypes. Similar to *Pl'*, the activity state of *R-r:std'* can be quantitatively distinct. *R-r:std'* can also be stabilized in a homozygous state or reversed to *R-r:std* levels when maintained in heterozygous combination with recessive (no seed colour) haplotypes or hemizygous for regional deletions.

Paramutagenic activities of the *R-st* and *R-mb* haplotypes are directly correlated with *r*-coding region copy numbers. Intragenic recombinant derivatives containing various numbers and combinations of *r*-coding regions illustrate that no single *r*-coding region by itself is responsible for the *trans*-silencing behaviour. Instead, paramutagenic strength is directly correlated with the numbers of *r*-type coding regions. Single copy derivatives lack detectable paramutagenic activity. At the *S* component of *R-r:std*, deletion derivatives indicate that the small *S1-S2* intergenic region along with 5' *S1* and *S2* transcribed regions are critical for either acquiring, or maintaining paramutagenic activity.

p1 paramutation

While the *p1* allele displaying paramutation (*P1-rr*) has a relatively simple coding region, it is flanked on both sides by large direct repeats that themselves contain a number of subrepeats (Figure 4). *P1-rr* directs strong red phlobaphene pigment synthesis in both the maternally derived seed coats, also known as the pericarp (*P1-rr*), and cob glumes (*P1-rr*). This allele is exceedingly stable but can change to a weaker expression state (*P1-pr*; patterned pericarp) at a very low frequency. However, complex transgene loci composed of β-glucuronidase (GUS) reporter constructs containing a 1.2-kb region of repeated sequences flanking *P1-rr* are able to cause *trans*-silencing of *P1-rr* at about a 20% frequency (Sidorenko and Peterson, 2001). This silenced state is meiotically heritable, has similar phenotypic and molecular features with *P1-pr*, and is weakly paramutagenic toward naïve *P1-rr* alleles. Hence a complex transgene is able to elicit paramutation at *P1-rr* via a small region of defined homology.

Genetics of paramutation

Given the previous descriptions, repeated sequences emerge as common features of both paramutable and paramutagenic alleles or haplotypes. So far it appears that repetitive elements *per se* are the functional sites of repressive activities. How these elements might mediate *trans*-allelic silencing remains unclear (Figure 2). One possibility is that repressive chromatin formation on repeated regions causes localization to transcriptionally unfavourable regions of the nucleus. Through transient or continuous homologue pairing, the previously unsilenced allele could be dragged into an area where establishment of a heterochromatic state is likely to occur (see Stam *et al.* (2002) for discussion). So far, however, there is little evidence for somatic homologue pairing in plants.

Mutational analyses have begun to identify other *trans*-acting genetic factors that either mediate the paramutation interactions (*mediator of paramutation1*; *mop1*) and/or are *required to maintain repression* (*rmr1* and *rmr2*). Recessive mutations of these factors were found in genetic screens where failure to maintain *B'* or *Pl'* silencing was obvious as rare darkly pigmented seedlings. Genetic tests show that MOP1 function is required to facilitate paramutagenicity at *b1*, *pl1*, and *r1*. This result emphasizes that paramutation occurring at distinct maize loci involve a related molecular mechanism. Although *B'* fails to revert back to a nonparamutagenic *B-I* state in *mop1* mutant plants, *Pl'* can revert to a fully stable, nonparamutagenic, *Pl-Rh* state at high frequency. Similar reversion of *Pl'* also occurs in both *rmr1* and *rmr2* mutant plants. These results indicate that the repression mechanism involves multiple players whose combined actions are necessary to maintain meiotically heritable repression states.

Molecular mechanisms of paramutation

Both *b1* and *pl1* paramutation are associated with reductions in transcriptional activity, suggesting a similarity with TGS mechanisms. Transcription analyses for *r1* and *p1* have not been performed and would be technically difficult given the limited tissue sources (aleurone and pericarp). Curiously, there are no local cytosine methylation differences associated with these alternate transcription states at *b1* and

pl1. There is, however, local increased nuclease sensitivity in the *B-I* versus B′ states. In contrast, both *R-r:std′* and *P1-rr′* are associated with increased levels of $5^{m}C$ in their promoter and coding regions. At the repeated sequences responsible for high levels of *B-I* expression and subsequent paramutagenicity of *B′* (100 kb 5′) there is an unusual inverse correlation between transcriptional activity and $5^{m}C$ levels. The seven direct repeats in this distal upstream region display high levels of $5^{m}C$ in the highly expressed *B-I* state relative to the weakly expressed *B′* state. Moreover, this methylated region is relatively sensitive to nuclease digestion; again the opposite of normal TGS correlations. Upon paramutation between *B-I* and *B′*, nuclease sensitivity decreases rapidly in this region, yet decreases in $5^{m}C$ levels appear to occur progressively throughout plant development and continue into the following generation. Thus heritable chromatin structure alterations appear to be primarily responsible for the transcriptional-based silencing seen in *b1* paramutation. Long-range interactions between this important regulatory region and the *b1* transcription unit suggest that major features of chromosome architecture, such as alterations in loop domain boundries, are affected by paramutation (Chandler et al., 2000).

PTGS mechanisms may yet be found playing additional roles in certain paramutation examples. For instance, the inverted orientations of *S1* and *S2* in the *R-r:std* haplotype (Figure 4) is remarkably suited for the production of PTGS-inducing dsRNA species. Perhaps TGS-based silencing of the *S1-S2* promoter, initiated from paramutagenic *r1* haplotypes, leads to a low level of readthrough transcription from flanking promoter regions. The resulting RNAs could then initiate PTGS, owing to their ability to form complementary regions of dsRNA. Given that *S1-S2* promoter sequences encoded by unintended readthrough RNA could also become targeted for dsRNA degradation through action of an RNA-dependent RNA polymerase, the promoter region would become a substrate for RNA-dependent DNA methylation, which could, in turn, recruit or reinforce the action of TGS components. In a similar manner, unintended readthrough transcripts from adjacent promoters could routinely lead to antisense transcripts encompassing both coding and noncoding regulatory regions. Pairing of sense and antisense RNA transcripts could again trigger a PTGS cascade that leads to a meiotically heritable TGS. Molecular identification of MOP1 and RMR proteins should indicate whether or not PTGS-type mechanisms are integral to paramutation.

Evolutionary perspectives

There are hints that certain aspects of the paramutation mechanism are utilized in normal genome functions. Mutations of *mop1* can lead to the reactivation of silenced transposons and can also interfere with proper plant development. The actual extent and types of genomic regions influenced by paramutation-type silencing remain unknown. It is interesting to note, however, that all four maize examples are found at transcriptional regulatory loci. This may implicate fundamental differences in the types of control mechanisms used for regulatory loci versus their downstream targets.

The existence of nonmendelian inheritance systems such as paramutation has significant implications for evolutionary concepts regarding the generation and maintenance of heritable variation. A fundamental assumption of population genetics is that all alleles are static elements. However, it is clear that some alleles are capable of dynamic behaviours. These exceptional alleles complicate existing mathematical models for predicting allele frequencies within a given population, which in turn

affects virtually every predictive model of population genetics. These dynamic alleles also illustrate that continuous variation for a given trait need not be due to the combined action of many alleles with small effects; continuous variation of anther colour can be achieved by a single allele (*Pl-Rh*) existing in distinct epigenetic states (Hollick et al., 1995). Perhaps more significant is that this type of epigenetic variation can be influenced by allelic interactions. For instance, *Pl-Rh / Pl-Rh* genotypes are unstable and display a high frequency of spontaneous paramutation (weak pigment) but *Pl' / pl-neutral* genotypes display a high frequency of reactivation (strong pigment). These types of behaviours closely mimic the breeding behaviours of inbreeding depression and hybrid vigour, respectively.

Paramutation may also have direct adaptive value in plant evolution. Mikula (1995) demonstrated that *r1* paramutation is sensitive to environmental conditions during early stages of seedling development. Different temperature and light conditions, applied during times that tassel progenitor cells are becoming determined, are able to elicit distinct meiotically heritable expression states of *R-r:std'*. Similar environmental effects influence spontaneous TGS silencing of certain transgenes. These results imply that paramutation represents a nuclear system capable of responding to environmental alterations and transmitting these responses to future generations. Rapid adaptive responses using epigenetic sources of heritable variation may precede, and in fact provide an opportunity for, the genesis and fixation of favourable DNA-based variation during organismal evolution (Waddington, 1942).

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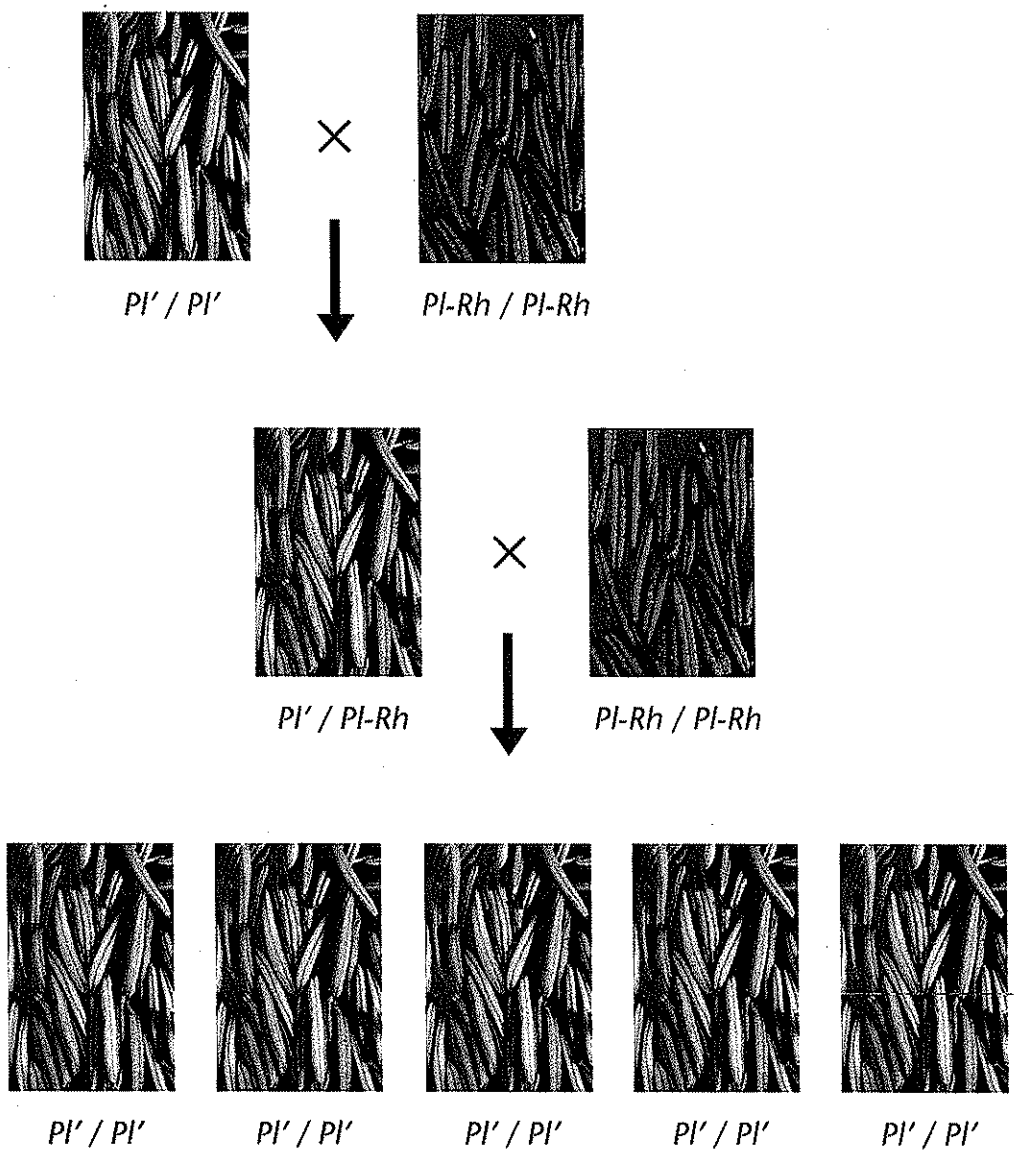

Figure 1#Paramutation behaviour. Anther phenotypes and corresponding diploid genotypes are represented for two sets of genetic crosses that together illustrate the nonmendelian inheritance pattern typifying paramutation. *Pl-Rhoades* (*Pl-Rh*) and *Pl′* alleles control anthocyanin pigment production in the anthers (see text).

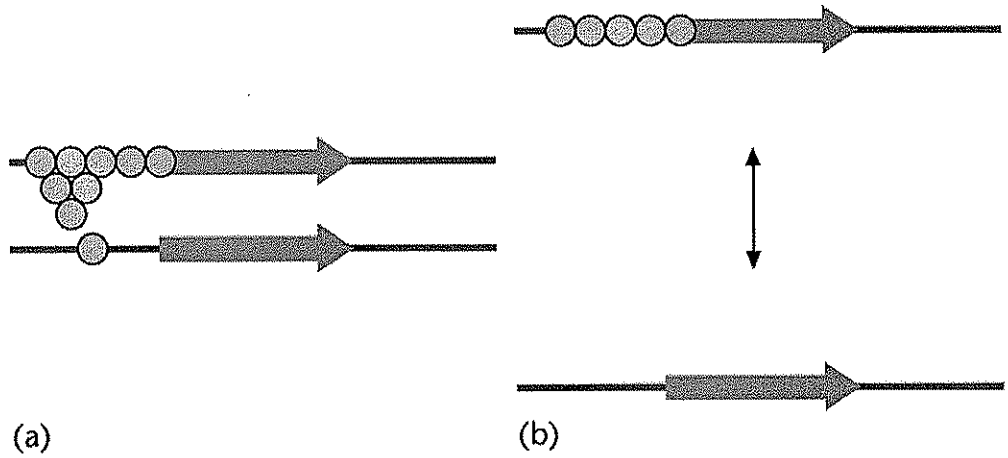

Figure 2#Allelic interaction models. Two general models of allelic interactions mediating *trans*-silencing. Solid horizontal arrows represent gene sequences and grey dots represent chromosome materials unfavourable to gene transcription. Bidirectional arrow indicates a diffusible substance. (a) Allelic interaction involving transfer of chromosome materials via physical pairing of homologous sequences. (b) Allelic interaction involving transfer of a diffusible substance.

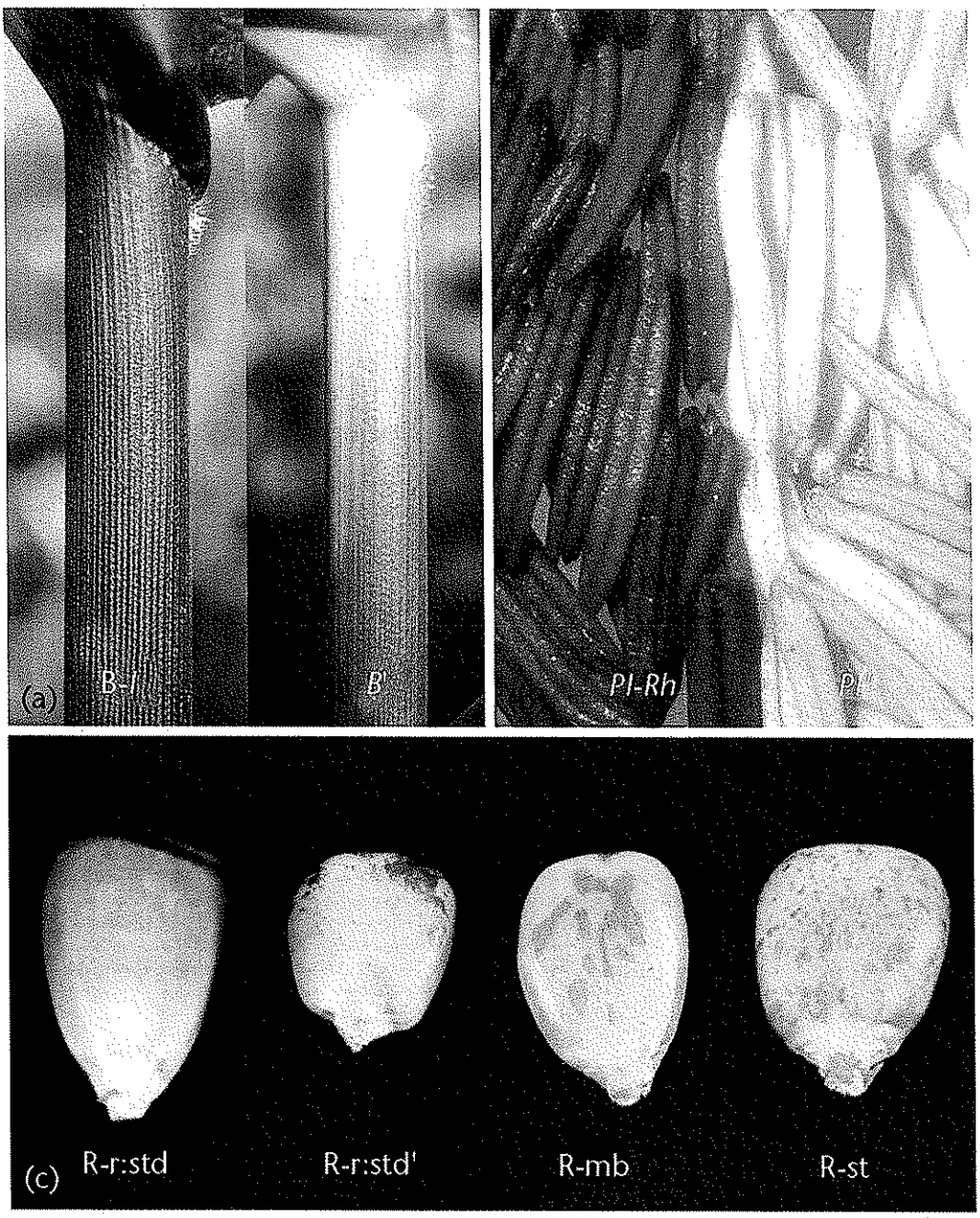

Figure 3#Paramutation phenotypes. Visible pigment phenotypes conferred by paramutable and paramutagenic alleles or haplotypes are displayed. (a) *B-I* versus *B'* leaf sheath phenotypes. (b) *Pl-Rh* versus *Pl'* anther phenotypes. (c) *R-r:std*, *R-r:std'*, *R-st* and *R-mb* kernel phenotypes.

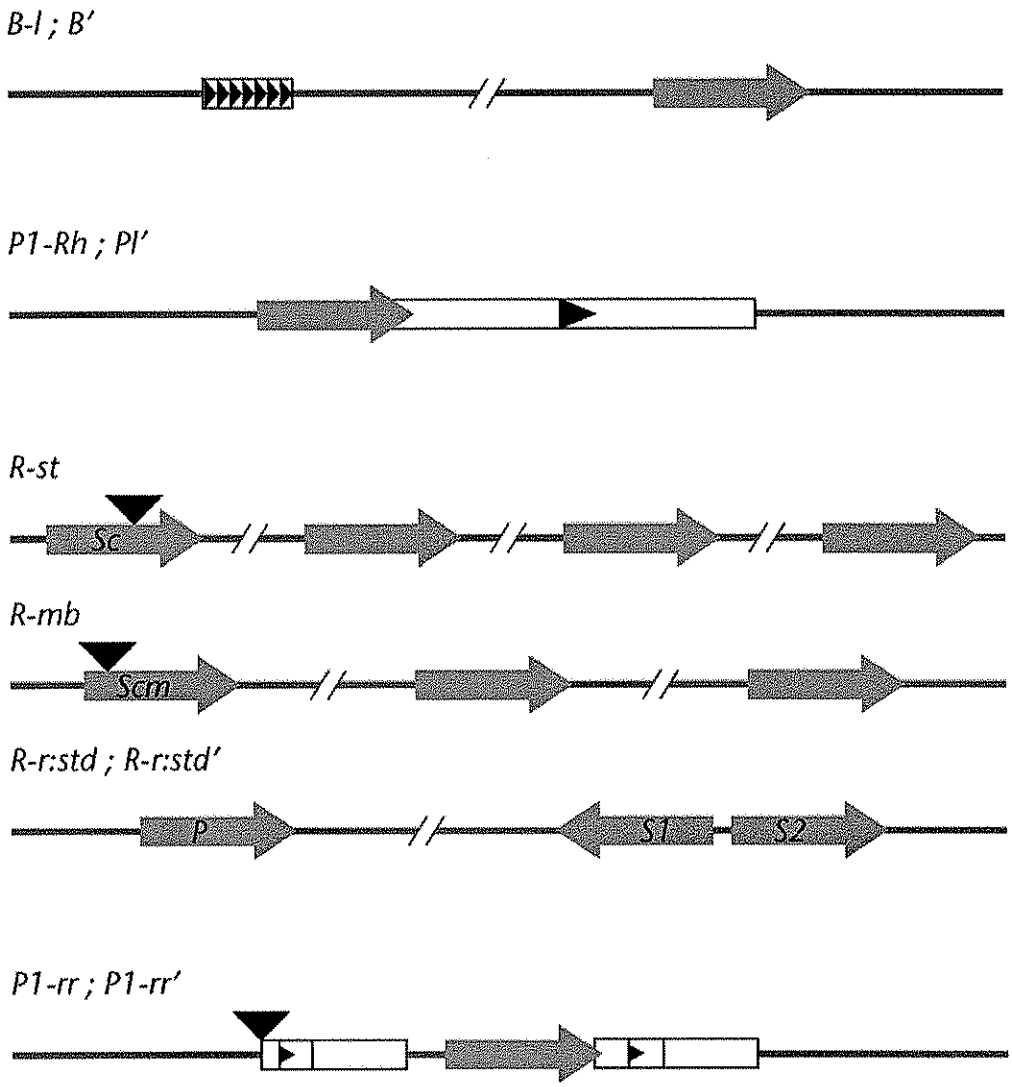

Figure 4#Paramutable versus paramutagenic allele and haplotype structures. Schematics (not to scale) represent genome organizations of the various alleles and haplotypes referred to in the text. Solid black arrows denote transcribed regions. Open boxes represent repetitive regions. Small horizontal triangles represent individual repeats; in the *pl1* and *p1* alleles these represent 3' regions of the respective transcribed regions. Vertical triangles denote the presence of transposable elements. Hash marks indicate large regions of intervening sequences. Several individual *r1* units are labeled; *P* is only expressed in the plant, *S1* and *S2* regions are expressed in the kernel, *Self coloured* (*Sc*) and *Self coloured marbled* (*Scm*) are highly expressed in kernels but are interrupted by the presence of two unrelated transposable elements. Somatic excisions of these elements from *Sc* and *Scm* during kernel development restore gene function and give rise to the pigment patterns displayed in Figure 3. Remaining unlabelled *r1* regions are very weakly expressed in kernels.

Glossary

Epigenetic#Heritable information not encoded by primary DNA sequence.

Gene silencing#Repression of gene expression affecting RNA abundance.

Haplotype#Specific form of a multigenic locus.

Paramutable#Susceptible to paramutation. Undergoes regulatory alterations in response to a paramutagenic allele.

Paramutagenic#Possessing an activity that induces paramutation. Causes regulatory alterations to occur among paramutable alleles.

Paramutation#A directed, meiotically heritable alteration of gene regulation influenced by allelic interactions.

Transgenes#DNA elements introduced into an organism using gene transfer technologies.

