
Some cases of paramutation of endogenous plant genes and silencing of introduced genes suggest the action of a genomic defence system that inactivates and methylates invasive DNA sequences such as transposable elements and multiple copies of transgenes. Paramutation can occur when promoters of repeated endogenous genes contain transposable elements that are highly homologous to other elements in the genome; the endogenous gene is then mistakenly identified as invasive. Transgenes can be recognized as being invasive either during integration or after integration if multiple copies are present. Because transposable elements are often associated with plant genes, the dividing line between endogenous and 'foreign' genes is not always clear cut. The blurring of this distinction could account for the similar epigenetic behaviour of many transgenes and paramutable endogenous genes, and might have broader implications for the regulation of plant gene expression.

When present as multiple copies, introduced and endogenous sequences in transgenic plants can exhibit a variety of epigenetic phenomena that are collectively termed homology-dependent gene silencing\(^1\) or repeat-induced gene silencing\(^2\) ('epigenetic' refers to the information content of the genome that does not reside in the primary nucleotide sequence). Some examples of these phenomena appear to be similar to paramutation, which is an epigenetic process described for several endogenous plant genes. The hallmark of both homology-dependent gene silencing and paramutation is that one allele or locus is able to induce a heritable change, in the form of weakened expression, in a second allele or locus. The reduced activity is often, but not always, associated with increased cytosine methylation. Despite the similarities, it is not known whether a common mechanism underlies both phenomena. The degree to which this mechanism impinges on normal gene expression is also unclear. Here, we review data suggesting that some cases of endogenous gene paramutation and transgene silencing share a common basis in a response to invasive DNA, which effectively neutralizes proliferating sequences by methylation. In this view, transgenes that become silenced are recognized as being invasive because they are present in multiple copies; endogenous genes are

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Fig. 1. Trans-silencing. A target locus or allele (T), which is usually expressed and not methylated, can become methylated and exhibit reduced activity either during (transgene silencing, as shown here) or following (r paramutation, as shown in Fig. 3) an association with a methylated silencing locus or allele (S). The methylation and silencing can persist for a few generations but is gradually reversible in the absence of the silencer, which is unchanged by the interaction. More completely methylated, partially methylated and sparsely methylated states are indicated by filled circles, half-filled circles and open circles, respectively.

incorrectly identified as invasive because they are repetitive, and because their promoter regions contain transposable elements that are highly homologous to other elements in the genome.

DNA methylation as a defence response to invasive sequences

Evolutionary considerations suggest that DNA methylation in eukaryotes derives from a prokaryotic genomic defence system that disables invasive or foreign DNA sequences. The advantages of such a system appear obvious: proliferating sequences introduce the potentially lethal problems of insertional mutagenesis and ectopic recombination that could disrupt chromosome structure. Multiple copies of a particular DNA sequence can arise through replicative transposition of transposable elements or genomic turnover processes (e.g. unequal crossing over). Filamentous fungi possess two alternative ways for dealing with these potentially deleterious sequence duplications.

Fate of duplicated sequences in filamentous fungi: repeat-induced point mutation and methylation induced premeiotically

Transformation experiments have shown that filamentous ascomycete fungi frequently eliminate one copy of a tandem duplication (i.e. where two copies of a gene occur in series) by recombination. Neurospora crassa and Ascobolus immersus have also taken a 'bolder step'4, which involves modification of both copies of a duplicated sequence. In Neurospora this occurs by C-to-T transitions (RIP, 'repeat-induced point mutation') accompanied by frequent methylation, and in Ascobolus by methylation (MIP, 'methylation induced premeiotically')5. Although RIP involves point mutations, these might actually be the result of aborted methylation attempts under conditions in which the methyl donor, S-adenosylmethionine, is limiting5. Therefore, it is possible that RIP and MIP represent the same response to DNA duplications. Both RIP and MIP neutralize repeats by sequence diversification (rapidly in RIP, more slowly via spontaneous deamination of 5-methyl-C to T in MIP) and by methylation, which inhibits transcription and probably also prevents recombination5. Recently, an attenuated MIP
response has been found in *Coprinus cinereus*, a basidio-
mycete fungus that is the most ‘evolutionarily advanced’
organism yet shown to exhibit true MIP activity.

Invasive DNA sequences and cues for de novo methylation

Both RIP and MIP demonstrate the central role of methyl-
lation in alleviating the potentially harmful effects of inva-
sive DNA. Intrusive sequences could trigger de novo methyla-
tion during or subsequent to integration if multiple copies of
a sequence are present. The DNA methyltransferase enzyme
may be able to recognize integration intermediates,
such as hairpins, which form when transposable elements
or transgenes insert into a genome. Pairing between multi-
ple homologous DNA sequences can also provoke de novo
methylation. The MIP process in *Ascochulus* appears to
involve a pairing signal, because the pattern of methylation
is exactly coextensive with a duplicated DNA region and
always occurs in both copies (i.e. it never happens that just
one copy is methylated).

Plant transposable elements and methylation

Transposable elements are acted upon by the methyl-
atation machinery in the plant nucleus. DNA elements [e.g.
Ac ('activator'), Spm ('suppressor-mutator') and Mu ('mu-
tator')! undergo reversible inactivation associated with
methylation, and communication of methylated states can
occur between homologous transposable elements in non-
allelic (ectopic) positions. Retroelements (DNA sequences
that transpose through reverse transcription of an RNA
intermediate) are usually highly methylated. An element
that is transpositionally active could become methylated
when integrating into the genome. The coordinate methyl-
atation of unlinked copies of Mu elements in maize possibly
occurs by means of ectopic pairing, as suggested by methyl-
atation being restricted largely to the elements and not flank-
ing plant DNA (Ref. 9). Some infiltration of methylation
from transposable elements into flanking plant sequences
has also been observed.

Trans-silencing of transgenes and endogenous genes in
plants

Homology-dependent gene silencing has been reported
for a number of transgenes in a variety of plant species.
Paramutation has been observed for several endogenous
genes in maize [r ('red'), b ('booster') and pl ('purple plant')],
*Antirrhinum majus* and tomato. Here, attention is focused
on two systems that show strikingly similar epigenetic
behaviour: promoter homology-dependent silencing of trans-
genes in tobacco and paramutation at the r locus in maize.
In both of these cases, a ‘silencing’ allele or locus induces
increased methylation and a heritable reduction in the
activity of a ‘target’ allele or locus; the silencer remains
unchanged by the interaction (Fig. 1).

Promoter homology-dependent silencing of transgenes

Two classes of homology-dependent gene silencing have
been identified in transgenic plants. One class involves a
post-transcriptional process (presumably cytoplasmic RNA
turnover) and does not induce substantial methylation of
promoter regions or a heritable reduction in gene activity.
A second class is associated with transcriptional inacti-
vation, increased promoter methylation and meiotically
heritable reductions in gene activity that persist in the
absence of the original silencing stimulus. Because this
latter process induces heritable alterations in gene ex-
pression, it most closely resembles the paramutation of
endogenous genes.

Two promoter homology-dependent silencing loci that are
able to trans-inactivate and methylate genes at unlinked
target loci in tobacco have been identified and analyzed in
more detail. One of these loci, H2 ('hygromycin resistance'),
silences genes under the control of the nopaline synthase
promoter. The second locus, 271, is a potent silencer of
genes that are under the control of the 35S promoter of
cauliflower mosaic virus. Both of these loci contain
multiple copies of the respective transgene construct (Fig. 2);
single copies of the same construct do not have silencing
activity. Although the exact arrangement and complete-
ness of the multiple copies are not yet known, silencing
activity appears to be associated with methylation in the
promoter regions that are the main region of homology to
the target locus. Spontaneous methylation of the silene-
locus possibly occurs when multiple copies of the trans-
gene construct at the locus pair in a process related to MIP.
The cis-methylated silencing locus would then be capable of
imposing methylation on the target locus in trans via pair-
ing of homologous promoter regions, a process termed ‘epi-
gene conversion’. cis-Inactivation is used to refer to the
silencing of closely linked genes on the same DNA molecule,
while trans-inactivation involves homologous genes on dif-
ferent DNA molecules. These genes can be at either allelic
or ectopic locations. The methylated, silenced target locus
does not revert to full activity or completely lose methyla-
tion when a silencing locus is crossed out by breeding,
which can result in significant epigenetic variability within
the target locus in backcross progeny.

Paramutation at the maize r locus

Paramutation is defined as an interaction between alleles that
leads to directed, heritable change at the locus with high
frequency, and sometimes invariably, within the
time span of a generation. It was first identified and
characterized at the maize r locus. Members of the r gene
family, including the displaced homologous b and lc ('leaf
color') loci, regulate anthocyanin expression in various plant
and seed parts via production of a transcriptional acti-
ator of the myc family of helix-loop-helix proteins.

Paramutation at the r locus involves a heritable reduction
in the activity of a sensitive (paramutable) allele, R-r, after
it has been associated with an inducing (paramutagenic)
allele, R-st (st: 'stippled'), in the heterozygote (Fig. 3). Recent
work on the structure and composition of epigenetic elements
in maize has shed new light on the features of the
paramutation process (20, 21).

To date, all r alleles that participate in paramutation
have been found to be complex, comprising multiple copies
of the r transcription unit (Fig. 4). The paramutable R-r
allele contains multiple complete and incomplete r genes
in both direct and inverse orientation. A complete r gene, P
('plant color'), is expressed in plant tissues. The S1 and S2
('seed color') genes form an inverted duplication flanking a
truncated and rearranged copy of a *doppia* mobile element.
This *doppia* element contains sequences normally found in
the P promoter and confers seed (aleurone)-specific expression
on the S1 and S2 genes. The P gene lacks a *doppia* element.

The paramutagenic R-st allele contains four complete
copies of the r gene in direct orientation (Fig. 3). The Sc ('self-
colored') gene is capable of conferring strong anthocyanin
expression in the aleurone, but is irregularly expressed because of the presence of an $I-R$ ($I$: inhibitor of aleurone color) mobile element near its 3’ end. The three additional r genes, Nc1, Nc2 and Nc3 (‘near-colorless’), together express a lightly mottled aleurone phenotype. With respect to paramutation, an intriguing observation is that a doppia element is present in the promoter of each Nc gene; doppia thus represents the only common element in the promoter of the S1 and S2 genes and r gene copies in R-st (Ref. 21) (W. Eggleston, unpublished). Reducing the number of Nc genes within R-st not only leads to increased pigmentation by the remaining copies of Nc but also to a reduced ability to induce paramutation$. Thus, copy number-dependent silencing at r (cis-inactivation) and paramutagenicity (trans-inactivation) appear related.

The Sc promoter region in R-st is moderately methylated, and the promoter regions of the three Nc genes are highly methylated$. As the number of Nc genes is reduced, the methylation level of the Sc promoter decreases such that little or no methylation is detected in simplex derivatives retaining only the Sc promoter (W. Eggleston and J. Kermicle, unpublished). Such simplex alleles have also lost all paramutagenicity$\text{23}$. Paramutation of R-r results in increased methylation in the 5’-ends of the S1 and S2 genes (M. Alleman and J. Kermicle, unpublished; cited in Ref. 20). A paramutated R-r allele (paramutated alleles in maize are designated by addition of a ‘prime’ symbol after the allele name), which is weakly paramutagenic, gradually recovers activity and becomes less paramutagenic over a few generations in the absence of the R-st allele.

Comparisons between promoter homology-dependent silencing of transgenes and r paramutation

The similarities between the promoter homology-dependent silencing of unlinked transgene loci and r paramutation are inescapable. Both involve:

1. Silencing and target loci or alleles that share homology in promoter regions; these regions of homology consist of sequences that could be considered invasive DNA (i.e. either multiple copies of transgene promoters or doppia elements). Paramutation of R-r results in increased methylation in the 5’-ends of the S1 and S2 genes (M. Alleman and J. Kermicle, unpublished; cited in Ref. 20). A paramutated R-r allele (paramutated alleles in maize are designated by addition of a ‘prime’ symbol after the allele name), which is weakly paramutagenic, gradually recovers activity and becomes less paramutagenic over a few generations in the absence of the R-st allele.

2. A multicopy silencing locus or allele that autonomously cis-inactivates.

3. A target locus or allele that is weakened in a heritable (but gradually reversible) way during or following an interaction with the silencer.

4. An association between silencing and methylation. This similar epigenetic behaviour and involvement of methylation implies a common mechanism based on a methylation/defense system that incapacitates invading DNA.

Recognizing endogenous genes as invasive

It is easy to see how transgenes — even when present as single copies — might be identified as invasive, since they must integrate into a plant genome (Box 1). Nevertheless, multiple copies of a transgene are usually required to incite silencing associated with methylation$\text{2}$ (although there are exceptions$\text{14,24}$), and both loci with strong silencing ability, $H2$ and $271$, contain multiple copies of the transgene construct. A high transgene copy number might be considered equivalent to a proliferating sequence by the methylation machinery in the nucleus.

Endogenous genes may also be recognized as being invasive if they resemble a proliferating entity. Two features could be responsible for this in the case of R-r and R-st alleles. First, both alleles contain multiple copies of the r gene. Second, at least some of these r genes are associated with doppia transposable elements.

r paramutation, complex alleles and doppia elements

In principle, the coordinate methylation and inactivation of homologous transposable elements could bring under their control any gene that contains a copy of that element. Although it is tempting to consider the doppia elements as prime players in r paramutation, the role of multiple copies of the Nc genes at the R-st allele cannot be overlooked, because one copy of Nc together with Sc is only weakly paramutagenic$\text{23}$. Moreover, substituting Lc, which lacks a doppia element, for Nc has little effect on paramutagenic strength$\text{23}$. Therefore, an essential feature for generating paramutagenicity of R-st appears to be multiple copies of r sequences, at least one of which should contain a doppia element in its promoter. The doppia elements in the Nc genes would be required for the trans-inactivation of the R-r allele because they provide the only region of homology between the Nc promoters and the two r genes that are most sensitive to paramutation (S1 and S2). Paramutation could occur when the highly methylated state of the doppia elements in

![Fig. 4. Structures of paramutable (target) R-r and paramutagenic (silencing) R-st alleles. Both complex alleles contain multiple copies of the r gene. The four tandemly arranged copies at R-st (Sc and Nc1–3) are separated by several kilobases. The Sc gene contains an $i$-R transposable element, which plays no role in paramutagenicity. The R-r allele comprises three functional r genes (P, S1 and S2), and q, which is homologous to the 5’ region and promoter fragments of P (hatched region) but does not contain an r coding sequence (solid lines). The distance between P and q is approximately 190 kb (Ref. 21). The doppia transposable elements (filled boxes), which are proposed to mediate the paramutagenic interaction between R-r and R-st, are present in q and in the promoter regions of S1, S2 and Nc1–3. The S1 and S2 genes form an inverted repeat centred around a rearranged doppia element containing sequences homologous to the P promoter. The promoter regions of the Sc and Nc1–3 genes at R-st are moderately and highly methylated, respectively. The 5’ ends of the r genes at R-r, including q, are increasingly methylated following an association with R-st. One-way horizontal arrows indicate the orientation of the genes. The open box is the Sc promoter.](image-url)
Although transgenes qualify as invasive DNA without needing to invoke the presence of transposable elements, multi-copy inserts are generally more susceptible to silencing than are single copies of transgenes. However, the great variability observed with transgene silencing and the occasional inactivation and modification of single copies, suggest that factors other than copy number may be involved. One possibility is that silenced transgenes are associated with transposable elements. Because of the ubiquity of transposable elements and their remnants (particularly retroelements) in plant genomes, it is likely that transgenes frequently integrate near to transposable elements. However, data obtained to date on the sequence of DNA flanking stably and unstably expressed inserts of the same transgene construct in tobacco have not revealed a correlation between proximity to known transposable elements and silenced inserts. Nonetheless, much of the flanking plant DNA appears on DNA (Southern) blots to be moderately repetitive (V. Iglesias and A. Matzke, unpublished) and it is possible that it is derived from transposable elements. Another interesting observation requiring further investigation is that stably expressed transgenes are often in the vicinity of nuclear matrix attachment regions (MARs) (Ref. 47) (V. Iglesias, A. Matzke and S. Michalowski, unpublished). In maize, single copy genes can be embedded within extensive regions of moderately to highly repeated sequences that are highly methylated, with MARs present at repeat/single copyjunctions. It may be that MARs are able to modulate the influence of repeated DNA on the expression of both endogenous genes and transgenes.

Paramutation at other maize loci

Other loci in maize that undergo paramutation do not appear to contain complex alleles like those found at the r locus. Moreover, there is no obvious correlation either with a specific transposable element or with methylation. In the case of pl, another anthocyanin-regulating gene, three different simplex alleles – Pl-Rh (Rh: ‘Rhoades’) (a strongly expressed allele), and two weakly expressed derivatives, Pl-bl (bl: ‘blotted’) and Pl-mah (mah: ‘mabogany’) – have a doppia element in their promoters. The Pl-mah allele can paramutate Pl-Rh (Ref. 26), although this is not associated with increased methylation; however, Pl-bl does not participate in paramutation (V. Chandler and J. Hollick, pers. commun.).

Transposable elements are present in the promoter regions of paramutable and paramutagenic b alleles but their relationship to paramutation is unclear because a paramutation-insensitive allele, B-Peru, contains many of the same sequences (V. Chandler, pers. commun.). The paramutagenic activity of the B allele has been mapped to the 5'-flanking region and transcription of the paramutated gene is reduced 20-fold. Both of these features are reminiscent of promoter homology-dependent silencing of transgenes, which also involves increased promoter methylation. Following extensive testing, however, no methylation changes have yet been correlated with b paramutation (Ref. 27). Heritable silencing of both b and pl may result from an altered chromatin structure, or methylation at cytosines that have not yet been examined.

Paramutation at r may represent a special type of interaction that involves a paramutagenic allele comprising multiple, closely linked copies of a gene, one or more of which contains in its promoter a particular transposable element that is also present in the paramutable allele. In the case of the R-st allele, this configuration might potentiate cis-inactivation associated with methylation, and increase the likelihood of pairing with the copies of doppia in the R-r allele. Consistent with this proposal is the finding that two r genes present on each of two chromosomes are less paramutagenic than four r genes on a single chromosome. These considerations may also apply to the transgene silencing loci, H2 and 271, which likewise have probably acquired methylation and strong silencing ability because of multiple tandemly arranged copies of the transgene construct. In those cases of paramutation that do not involve complex alleles and methylation, it is conceivable that transposable elements are involved in other ways. For example, the elements could generate certain rearrangements of plant DNA, such as inverted repeats, which might in turn promote paramutagenic interactions.

Paramutation-like behaviour has been observed for other transposable element-associated genes in maize: the phenotypes of mutations resulting from insertions of defective Mu and Spm elements can be sensitive to the presence or absence of autonomous members of these two families at other locations in the genome. These effects can also involve coordinate changes in methylation of unlinked transposable elements. The possible involvement of Tam (transposable element from Antirrhinum majus) transposable elements in paramutation of two simplex niv (‘niva’) alleles encoding chalcone synthase in Antirrhinum majus has also been proposed; however, the basis of this interaction is still unclear, as loss of the Tam element from one allele does not seem to interfere with paramutation. Apparent cases of paramutation involving nonallelic endogenous genes in maize and Arabidopsis have also recently been reported, with discussion of the possible role that transposable elements play in these processes.

Transposable elements and requirements for differential gene silencing

The silencing of transgenes and some transposable element-associated endogenous genes have been discussed as pathological cases that reveal the action of a genomic ‘immune’ response to invasive DNA. Because of the ubiquity of transposable elements and transposable element-derived repeats in eukaryotic genomes, it is worth considering whether some of these elements might play a more general role in regulating plant gene expression.

Gene silencing is an essential part of eukaryotic gene regulation, as up to 50% of the structural genes must be silenced in differentiated cell types. Conceivably, the spread and accumulation of transposable elements and their insertion adjacent to and/or into promoters of structural genes could contribute to the extensive silencing that is required in differentiated cells. Depending on
their distribution, abundance and degree of homology, these sequences could participate in the differential regulation of a 'generic' protein-coding genome by directing differential gene silencing through networks of homologous pairing.

Recent work has uncovered transposable elements (primarily retrotransposons and miniature inverted-repeat transposable elements) or their remnants in the 5'- or 3'-flanking regions of many wild-type plant genes. Most of the retrotransposon sequences appear to have diverged sufficiently such that pairing between multiple copies might be prohibited. Miniature inverted repeat transposable elements (it is not yet known whether these transpose via a DNA or RNA intermediate) are short entities, of about 100–350 bp, that might be unable to pair effectively even if they were highly homologous. Based on these considerations, homology-dependent interactions between these transposable elements might be expected to occur relatively infrequently. However, the length (about 250–300 bp) and homology (>90%) estimates derived from conventional homologous recombination models might not be applicable for DNA homology-based silencing, and alternative possibilities for pairing could also be considered.

Pairing between ectopic DNA elements might be mediated by transposases binding to single ends of two elements at unlinked sites. Genomes of higher eukaryotes might also have a special mechanism requiring a smaller window for recognizing homology than that associated with the standard homologous recombination machinery. The potential for short windows of homology to induce silencing is supported by the observation that only 90 bp of sequence identity in the promoter of the target gene is required for sensitivity to trans-inactivation by the 271 silencing locus. Large and complex genomes seem to possess a remarkably efficient homology-searching mechanism, which may even exceed the capabilities found in fungi and yeasts. Finally, repeats too short to promote recombination could provoke silencing if different proteins are involved in pairing and/or if less-intensive pairing is required.

Pairing interactions leading to silencing and methylation of homologous sequences could be under developmental control. DNA elements in maize (e.g. Spm and Mu) can exhibit developmentally regulated methylation and expression. Genes into which Spm elements are inserted can be coupled to the developmental expression pattern of Spm. A group of genes coordinately expressed in maize pollen contains the same retroelement. Other retroelements have been found to be transcribed in specific cell types, implying that these sequences are inactive in all other cells, possibly silencing structural genes in their vicinity.

**Mammalian imprinted genes recognized as 'foreign'?**

Although a phenomenon analogous to paramutation has not yet been observed in mammals, methylation and silencing of DNA recognized as being invasive does occur in these organisms. The progressive silencing of tandem arrays of transgenes under the control of tissue-specific promoters happens frequently in transgenic mice. Retroviruses integrated into promoters of mammalian genes can also cause epigenetic effects not observed with the wild-type gene, such as mosaic patterns of methylation and expression. Silencing and de novo methylation of retroviral vectors used to transduce genes into animal cells have also been reported.

**Gametically imprinted genes associated with repeats and pairing interactions**

Gametically imprinted genes are expressed differently depending on the sex of the parent from which they are inherited. Imprinted genes might be recognized as 'foreign' and thereby be subject to methylation. All known imprinted genes in mammals contain directly repeated sequences, which might form secondary structures comprising the 'foreign' recognition signal. The possible role of these repeats has been discussed in relation to a study from *Drosophila* in which a transgene insert adopted a more condensed chromatin conformation as the number of tandemly arranged copies increased, presumably in a pairing-dependent process. A recent study using three-dimensional fluorescent *in situ* hybridization has revealed transient association of imprinted regions on homologous chromosomes. In maize, *r* alleles subject to paramutation also undergo gametic imprinting, suggesting a mechanistic link between the two phenomena.

**Concluding remarks**

A role for transposable elements in paramutation was first suggested by Barbara McClintock. Molecular analyses of *r* alleles involved in paramutation have provided support for this view. However, further work is required to determine whether transposable elements play a role in other cases of paramutation where association with a specific element has yet to be demonstrated. Transgenic plants have offered defined systems with clear parallels to paramutation of endogenous genes and an impetus for examining cases of paramutation involving methylation as a response to invasive DNA.

Two distinct roles for methylation have been proposed to explain the pattern of genomic methylation observed: first, operation as a defence mechanism against foreign or invasive DNA; and second, as a means of controlling gene expression during development. It is becoming apparent that this distinction may not always be clear cut in plants. The extraordinarily similar epigenetic behaviour of some transgene silencing systems and of endogenous genes subject to transposable element-associated paramutation suggests that a defence response to foreign or invasive DNA can also be used against a plant's own genes. A beneficial consequence of the pervasive presence of transposable elements and their remnants in plant genomes might be differential transposon-induced silencing of genes. This implies that the function of methylation as a part of the defence response has merged to a significant degree with its role as a developmental regulator of gene expression in plants.

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