Position effects and epigenetic silencing of plant transgenes

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Nuclear processes that silence plant transgenes are being revealed by analyses of natural triggers of epigenetic modifications, particularly cytosine methylation, and by comparisons of the genomic environments of differentially expressed transgene loci. It is increasingly apparent that plant genomes can sense and respond to the presence of foreign DNA in certain sequence contexts and at multiple dispersed sites. Determining the basis of this sensitivity and how nuclear defense systems are activated poses major challenges for the future.

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Abbreviations

BV	binary vector
MAR	matrix-attachment region
MIP	methylation induced premeiotically
MTase	methyl transferase
PEV	position effect variegation
TE	transposable element

Introduction

The unpredictable silencing or variable expression of transgenes is a ubiquitous phenomenon that has received close scrutiny in plant systems, not least because of the desire to either avoid silencing or harness it for genetic engineering purposes. Transgenes are susceptible to silencing in all plant species studied, including green algae [1••]. There is not yet a reliable way to prevent silencing, although the converse effect — consistent gene silencing — has been reported [2].

Silencing resulting from interactions among multiple copies of transgenes and related endogenous genes involves homology-based mechanisms that act at either the transcriptional or post-transcriptional level [3]. Transcriptional silencing is associated with meiotically heritable epigenetic modifications (often, but not always, cytosine methylation). Cases of *trans*-silencing in which a silencing transgene locus induces a meiotically heritable reduction in the transcription of a target locus resemble paramutation of endogenous genes [4••,5••]. (Paramutation is defined as an interaction between alleles that leads to a heritable alteration in the expression of one of the alleles.) Post-transcriptional silencing (cosuppression) involves enhanced RNA turnover in the cytoplasm and is reset during meiosis [6•,7]. Each type of silencing might reflect a distinct host defense response to either transposable elements (TEs) or viruses, respectively [8].

A separate category of silencing concerns position effects, which refer to repressive influences exerted on transgenes by flanking plant DNA and/or an unfavorable chromosomal location. This type of silencing presumably reflects the epigenetic state of neighboring host sequences or the relative tolerance of particular chromosome regions to invasion by alien DNA. Often assumed in lieu of a better explanation, position effects are being clarified by analyses of the genomic environments of differentially expressed transgenes. Further insight is coming from an improved understanding of the natural targets and signals for epigenetic modifications in eukaryotic genomes. These issues are addressed in this review.

Cytosine methylation

Recent reviews highlight the importance of DNA cytosine methylation for plant development [9–11]. The discussion here, centers on features of DNA structure and sequence that appear to attract *de novo* methylation and to perpetuate methylation once acquired.

Signals for *de novo* methylation

The function of DNA methylation can be considered in view of two hypotheses, one proposing a contribution to the developmental regulation of gene expression, and the second suggesting a primary role as a defense against invasive DNA [12••]. Given the ancient and pervasive presence of TEs in many plant genomes [13] and their common occurrence in the immediate 5′ and 3′ flanking regions of normal plant genes [14], it can be argued that a primordial defensive role has evolved into a means to developmentally regulate gene expression [4••,8]. At present, the only known signals for *de novo* methylation concern its action as a defense mechanism.

Integration intermediates, such as hairpin structures, that form when TEs or transgenes invade a genome, can be targets for *de novo* methylation [15••]. The ability of paired DNA structures to attract methylation has been demonstrated by the MIP (methylation induced premeiotically) phenomenon in the filamentous fungus *Ascobolus immersus*, where sequence duplications are coordinately inactivated and methylated during the sexual phase [16,17••]. These structural targets for methylation do not account fully for patterns of transgene modification in plants because not all inserts become methylated; moreover, multiple copies that could conceivably pair are not invariably methylated.

The disparate abilities of similar sequences to trigger and maintain methylation is illustrated by experiments with the filamentous fungus *Neurospora crassa*, which

neutralizes potentially recombinogenic DNA sequence duplications by the process of RIP (repeat-induced point mutation) [18••]. RIP has both genetic and epigenetic aspects: each copy of a duplication in premeiotic haploid nuclei becomes studded with C to T base mutations; sequences mutated by RIP are then frequently, but not invariably, methylated *de novo* at remaining C residues. Although the mutations possibly result from deamination of 5-methylC to yield T, with pairing of duplicated regions triggering methylation, the signal for *de novo* methylation of single copy sequences mutated by RIP is more mysterious.

A series of glutamate dehydrogenase (*am*) alleles that had undergone RIP were reintroduced into a specific site in the *N. crassa* genome and analyzed for methylation [19]. Those am^{RIP} alleles that contained fewer than 21 mutations in a 2.6 kb region remained unmethylated; those comprising 45 and 56 mutations became methylated only during the sexual cycle; and those harboring 84 to 158 mutations were methylated both premeiotically and when reintroduced into vegetative cells [19]. Thus, deviant DNA sequences or structures created by RIP were able to directly or indirectly influence the activity of methyltransferase (MTase). A comparison of methylated and unmethylated sequences failed to reveal any obvious signals for methylation. In addition to the varying abilities of different am^{RIP} alleles to attract *de novo* methylation, some sequences also maintained methylation better than others [18••,19] for reasons that are unclear. This analysis of sequence requirements for *de novo* and maintenance methylation in *Neurospora* supply a precedent for future similar studies in higher eukaryotes and highlight the complex regulation of methylation.

Patterns and inheritance of methylation

Dense methylation at all Cs is observed in sequences modified by RIP and MIP, where the respective methylation signals appear to be an altered sequence composition [19] or paired DNA segments longer than 400 bp [17••]. Although most methylation in plants is present in symmetrical CG and CNG nucleotide groups (N standing for A, G, C or T), methylation of nonsymmetrical Cs has been observed in both foreign sequences (transgenes and TEs) and endogenous genes (reviewed in [10]). The heavy methylation incurred by nonsymmetrical Cs around the transcription start site and in most of the transcribed region of weak epialleles of the single copy endogenous gene *SUPERMAN* [20•] indicates that this pattern of methylation does not require extensive DNA–DNA pairing or a readily recognizable foreign DNA sequence, such as a TE or a repeat of appreciable length. The *SUPERMAN* gene does, however, contain a 50 bp run of the dinucleotide C[A/T] that comprises the transcription start site. This short microsatellite might attract methylation that decreases expression.

The *Ascobolus* methyl transferase (MTase) gene, *Masc1*, responsible for *de novo* methylation during MIP of all Cs in paired DNA regions, encodes an enzyme unlike known eukaryotic MTases [17••], which preferentially modify hemimethylated DNA and therefore perform primarily a maintenance methylation function. Maintenance methylation is not perturbed in *masc1* mutants, so a second novel MTase that perpetuates methylation of nonsymmetrical Cs must also be present in *Ascobolus* [16]. Further characterization of the three types of MTases discovered so far is necessary to determine whether MTases with similar activities and substrate specificities are present in plants [10].

Position effects on transgene expression

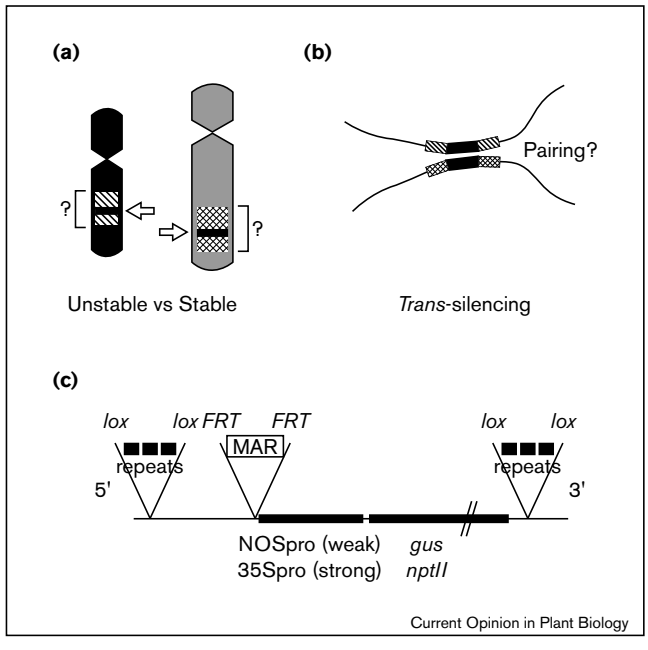
In yeast and *Drosophila*, silencing resulting from position effects can occur at telomeres and centromeres, which are heterochromatic in *Drosophila* [21,22]. These silencing phenomena are not identical: telomeric position effect variegation (PEV) in *Drosophila* is insensitive to suppressors and enhancers of centromeric PEV [23]. In addition to centromeres and telomeres, most higher plant genomes contain substantial amounts of intercalary heterochromatin and repetitive DNA, which can be used as landmarks to evaluate regions associated with transgene silencing. Position effects involving one or more transgene loci can be studied in various ways (Figure 1).

Genomic context of stably and unstably expressed transgenes

Characteristics of flanking plant DNA

Only a few reports provide comprehensive data on the genomic context of transgenes in genetically well characterized lines [24,25,26••]. Recurring sequence motifs have appeared in plant DNA that flanks transgenes introduced using binary vectors and *Agrobacterium*-mediated transfer into diploid and tetraploid *Nicotiana* species. Of twelve transgene loci examined so far ([25,26••]; AJM Matzke, J Jakowitsch, F Mette, C Kunz, H van der Winden, unpublished data), matrix-attachment regions (MARs) have been found in the flanking regions of four; various microsatellites in three; highly repetitive tandem repeats in two; and retroelement remnants in five. Except for the frequent presence of binary vector sequences (see below), the remaining DNA contains no defining characteristics. Prokaryotic sequences, a GA-rich microsatellite, retroelement remnants, and a tandem repeat array are the primary elements correlated so far with silencing. MARs have been associated with some stably expressed inserts [26••]. In work on plant species other than *Nicotiana*, highly repetitive sequences have been found adjacent to an unstable transgene in petunia [24]. Molecular analysis of three integration site structures in rice transformed by direct gene transfer indicated a common AT-rich repetitive sequence and telomeric repeats at all three junctions of the target genome [27]. Additional studies on DNA sequences flanking transgene inserts are required to substantiate the current view that certain repeats or prokaryotic sequences tend to be associated with poorly expressed or silenced inserts.

Figure 1



Analysis of position effects in transgenic plants. Position effects include a variety of phenomena that can be investigated as follows. **(a)** The sequence of flanking plant DNA (diagonally-lined and cross-hatched regions) and chromosomal locations (arrows; two nonhomologous chromosmes are coloured black and grey) of transgene inserts that unstably or stably express the same construct can be compared. **(b)** *trans*-silencing effects possibly involving pairing of homologous sequences (black bars) on separate chromosomes can be considered with respect to the genomic contexts of interacting genes (as in [a]) and their ability to associate in somatic cells. This can be examined directly using three-dimensional fluorescence *in situ* hybridization; alternatively, somatic pairing can be measured using systems for site-specific recombination (not shown; described in [49•]). **(c)** Repetitive sequences are often associated with silencing and a variety of transgene constructs can be designed to study the effects of different types of repeats when they are positioned 5′ or 3′ to transgenes. Possibilities include testing the effect of the same repeat on weak or strong promoters, such as the nopaline synthase (NOSpro) and 35S promoter (35Spro), respectively, and various reporter genes, such as β-*glucuronidase* (*gus*) and *neomycinphosphotransferase* (*nptII*). Other elements that might influence expression, such as MARs, can be engineered upstream or downstream of transgenes. Repeats and MARs can be selectively removed by flanking them with *lox* or *FRT* sites, thus allowing excision by the Cre or FLP recombinases respectively. The coding region is condensed by the slashes.

Transfer of binary vector sequences

Binary vectors, which consist of a bacterial wide-host range plasmid containing the transferred (T-DNA) region defined by short direct repeats at the left and right borders, are frequently used to obtain genetically altered plant cells by *Agrobacterium*-mediated transformation. It has long been assumed that only the T-DNA is incorporated into nuclear DNA. A spate of recent reports, however, has established that binary vector (BV) backbone sequences are transferred frequently into plant genomes (see [28••,29••] and references therein). This transfer is independent of the BV system, transformation method, or plant species used. Although it has long been known that bacterial sequences reduce transgene expression in mammals [30•], BV probes have rarely been used to analyze transgenic plants because of the entrenched notion that T-DNA is strictly delimited by the border direct repeats. BV sequences that join T-DNA and plant DNA appear to be particularly deleterious for transgene expression; BV fragments separated from T-DNA have been found associated with stably expressed inserts ([26••]; C Kunz, AJM Matzke, unpublished data). The apparent tendency of multicopy transgene loci to become silenced (which has given rise to the idea that pairing of these copies triggers *de novo* methylation) might actually reflect a higher probability that BV sequences are present in complex inserts.

Prokaryotic vector sequences introduced by other transformation techniques will probably also contribute to transgene silencing. Although it is difficult to avoid integration of vector DNA with current transformation protocols, screening transgenic lines with vector probes would allow recovery of those containing minimal amounts of these sequences. Note, however, that vector DNA might only contribute to silencing when present in a particular relationship to the transgene (for example, linking transgene and plant DNA) and these cases are most reliably identified by isolating and sequencing junction fragments.

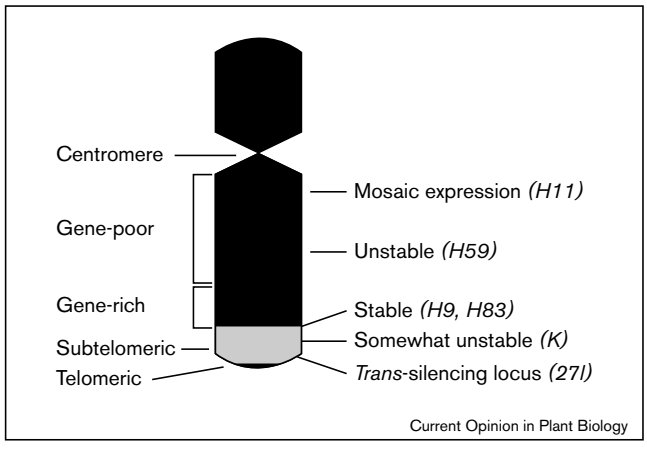
Chromosomal location

Cytogenetic analyses have been performed on transgene loci in a number of plant species, but in only a few cases have correlations been made with stability of expression (Figure 2). Stable inserts, which lack BV sequences, have been located in the vicinity of telomeres [26••]. Transgenes have a tendency to integrate toward the distal end of chromosome arms [31], which are gene-rich regions in wheat and maize [32,33]. In addition to centromeric PEV in *Drosophila*, centromeric locations give rise to mosaic patterns of transgene expression in tobacco [26••], fission yeast [34] and mice [35]. *Trans*-silencing loci, which autonomously acquire methylation, are associated with heterochromatic regions: one has been localized to a telomere [36], and a second to intercalary heterochromatin comprising a tandemly repeated sequence (J Jakowitsch, E Moscone, AJM Matzke, unpublished data).

Engineering repeats into transgene constructs

The numerous possibilities for incorporating various repeats into transgene constructs and testing their effects on expression have yet to be exploited fully (Figure 1). A repetitive sequence from petunia was found to destabilise expression of a *gus* reporter gene in tobacco and petunia [37]. Using the Cre-*lox* system, which allows precise

Figure 2



Influence of chromosomal location on transgene expression. The chromosomal locations of genetically well characterized transgene loci (in italics, right) in tobacco as determined by fluorescence *in situ* hybridization [25,26••,36] are shown relative to landmarks (left) on a model chromosome.

excision by the Cre recombinase of sequences flanked by *lox* sites, we have found that removing a tandem repeat positioned upstream of a nopaline synthase promoter could reduce methylation and alleviate silencing of an adjacent transgene (MA Matzke, AJM Matzke, unpublished data).

Recognition of foreign DNA

The varying propensities of *N. crassa am*^{RIP} alleles to attract methylation [19] and the different sensitivities to methylation of a monocot versus dicot *A1* transgene in petunia [38,39] suggest that methylation can be provoked by particular sequence contexts. The basis of this response is not known but might involve incompatibilities in base composition. Eukaryotic genomes are mosaics of compositionally distinct compartments known as isochores [40]. It is likely that the isochore structure of genomes will play an increasing role in attempts to understand position effects, as evolutionary considerations suggest that DNA segments must conform to compositional or sequence patterns characterizing different domains [41]. In line with this view are several unusual features of PEV in *Drosophila*, including the variegated expression of heterochromatic genes when relocated to euchromatin (reverse position effects) and the discontinuous compaction of chromatin from a heterochromatin–euchromatin breakpoint [42].

This issue is especially relevant for higher plants, many of which are allopolyploids derived by interspecific hybridization between two morphologically and genomically distinct species. Polyploid genomes can accumulate considerable amounts of repetitive DNA; a large fraction consists of TEs, some of which are species-specific and hence enriched in one subgenome [43]. A case in point is tobacco (*Nicotiana tabacum*), which is an allotetraploid originating from two diploid progenitors, *N. sylvestris* (S subgenome) and either *N. tomentosiformis* or *N. otophora* (T subgenome). Tetraploid tobacco has a bimodal sequence composition, indicating that, despite several local S–T exchanges [8,43], extensive intermixing of subgenomes has not yet occurred. The GC contents of parental subgenomes in tobacco range from 37–41% (T) to 42–50% (S) [44]. The distribution of tobacco TEs is not completely random and presumably reflects integration into compatible regions. Retroelement *Tnt*1 is enriched in a compartment characterized by a GC content of 36.6% [45]. The average GC content of four remnants of a different retroelement family [25,26••] is 41%, consistent with enrichment of this family in the T subgenome. Incompatibilities could arise from cross-infiltrations of species-specific elements: a copy of a T subgenome enriched element present on an S chromosome was found in the flanking plant DNA of an unstable transgene [26••].

Prokaryotic DNA might be recognized as foreign because of its generally high GC content and/or because it cannot be packaged properly with eukaryotic proteins. Sharp discontinuities in GC composition are present at the junction of nopaline synthase promoter and BV fragments at a *NOSpro trans*-silencing locus; all of these sequences are heavily methylated (J Jakowitsch, J Narangayavana, AJM Matzke, unpublished data).

TEs and sequence incompatibilities can be targets for DNA methylation. Thus, the types and frequencies of silencing effects observed in different plant species will reflect the repetitive DNA content and the compositional characteristics of their genomes. Position effects might predominate in plants with large, complex genomes containing a substantial TE component.

Gene interactions

Position effects can be considered for *trans*-silencing systems with respect to the ability of two alleles or loci on different chromosomes to interact physically. In *Drosophila*, there are many examples where gene expression is affected by homologous chromosome associations or long-range interactions between genes on separate chromosomes [46••]. These can involve pairing between dispersed DNA repeats or the formation of silencing complexes mediated by specific proteins, such as those of the Polycomb group [22]. It is not yet known whether paramutation or *trans*-silencing of transgenes in plants involves pairing or the participation of specific proteins [4••,5••]. The seeming imposition of methylation from one gene to another in some cases of *trans*-silencing and paramutation at the *r* ('red') locus in maize suggests a mechanism similar to the meiotic transfer of methylation between alleles in *Ascobolus immersus*, which involves heteroduplex formation [47]. *Trans*-silencing phenomena involving methylation might be triggered by foreign sequences (transgenes or TEs) [4••,48••] that can be

coordinately inactivated, possibly by homologous pairing among dispersed copies.

The telomeric location of a *trans*-silencing locus could account for its ability to find and inactivate targets on other chromosomes (Figure 2, [26••]). Transgene loci that are relatively resistant to *trans*-silencing [8] might reside in a protected chromosomal location, as has been postulated for the large ribosomal RNA genes that are immune to RIP in *Neurospora* [18••]. MARs can be somewhat effective in promoting stable transgene expression, but they are unable to protect a locus from *trans*-silencing [26••].

Much additional work combining genetic, cytogenetic and molecular approaches is required to understand interactions that appear to involve physical contact between genes at allelic or nonallelic sites. Because interactions leading to similar types of silencing effects are being found in a wide range of organisms, they point toward a universal means of modifying gene expression that relies on nuclear architecture and physical proximity of interacting loci [46••]. Ideally, one would like to be able to use fluorescence *in situ* hybridisation (FISH) to visualise three-dimensional associations of genes within interphase nuclei, a technique employed successfully with *Drosophila* and human cells [46••]. The potential of this method, however, has not yet been fully realized with plant systems. Moreover, pairing that initiates methylation transfer between genes might be transient and, therefore, difficult to capture by FISH. As an alternative, detection of homologous associations in somatic cells could be facilitated by the use of systems for site-specific recombination because the frequency of recombination between genes reflects the incidence of pairing between them [49•].

Transcriptional silencing and heritable chromatin states

We have emphasized a role for methylation in our discussion of position effects and epigenetic silencing of trangenes because this DNA modification functions to inactivate foreign or invasive DNA [12••]. Moreover, many cases of gene silencing are indeed correlated with increased methylation. There are exceptions, however, including paramutation of endogenous *b* ('booster') and *pl* ('purple plant') alleles in maize [5••] and transgene silencing in *Chlamydomonas* [1••]. It is unclear why some cases of silencing do not seem to involve methylation; an improved understanding of signals that induce methylation will help clarify this issue as will a resolution to the problem of whether methylation is a cause or a consequence of silencing. Methylation is not required for generating mitotically and meiotically heritable epigenetic states as exemplified by chromatin-based modifications in yeast [21,50] and *Drosophila* [51], which lack methylation. While *b* and *pl* paramutation could involve increased chromatin condensation [5••], as has been observed for a transgene subject to repeat-induced gene silencing in *Arabidopsis* [52], silencing in *Chalmydomonas* appears to require neither methylation nor significant alterations in chromatin configuration [1••], suggesting additional ways to achieve heritable gene inactivation in eukayotes. These results emphasize the need for a broad approach in assessing the relative contributions of various types of epigenetic modifications to gene silencing [11]. Whether position effects on transgene expression in multicellular plants can be due solely to changes in chromatin structure in the absence of significant changes in methylation remains to be determined.

The identification of plant proteins involved in methylation and inactive chromatin complexes should follow studies of silencing mutants in yeast and *Drosophila* [21,22]. *Arabidopsis* plants that are deficient in methylation are available [9,10], and mutants in transcriptional or post-transcriptional silencing are being analyzed [10,11]. Interestingly, while several mutants defective in quelling (post-transcriptional silencing in vegetative *N. crassa* cells) have been identified [53], no RIP mutants have yet been recovered, possibly because they are lethal [18••].

Conclusions

The vagaries of transgene expression serve as reminders that genomes are 'highly sensitive organs' able to sense and react to violations of their integrity [54]. Despite having plausible hypotheses invoking sequence incompatibilities, DNA–DNA pairing, proximity to heterochromatin, and adverse chromosomal locations, we still know little about what triggers a response to foreign DNA and provokes epigenetic silencing of transgenes (see also discussion in [55]. As more is learned about the genomic contexts of transgenes, it should become possible to define requirements for stable expression. Plant genomes will probably continue to surprise us, however, as illustrated by the apparent highly variable degree of silencing of single copy transgenes targeted to specific genomic sites [56].

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- • of special interest
- •• of outstanding interest

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The first integrated study combining molecular and cytogenetic approaches to compare genetically well characterized transgene loci in tobacco. Stable expression was correlated with natural matrix-attachment regions and a telomeric location; unstable expression was associated with binary vector sequences that joined T-DNA and plant DNA, and intercalary or centromeric chromosomal sites. The potential wider influence of host genome organization is discussed.

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Using binary vectors containing a *gus* gene positioned outside the T-DNA borders, the authors found that GUS activity could be measured in 20% of tobacco transformants and *gus* sequences could be detected in 75%, demonstrating extremely frequent intergration of non-T-DNA sequences. Vector sequences were present either to the left or the right of T-DNA or integrated separately. Implications for the mechanism of T-DNA integration and governmental regulatory issues concerning the release of transgenic plants are discussed.

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