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Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses

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Abstract. Linked and unlinked copies of transgenes and related endogenous genes in plants can be epigenetically silenced by homology-based mechanisms that operate at either the transcriptional or post-transcriptional level. Transcriptional inactivation is associated with promoter homology and meiotically heritable methylation. Post-transcriptional silencing requires homology in protein-coding regions and is fully reversed during meiosis. Recently, the notion that both of these processes reflect the action of different host defence systems has been strengthened: (i) Obvious parallels have emerged between promoter homology-dependent silencing/methylation of transgenes and paramutation of endogenous genes that contain transposable elements in their promoters; (ii) remarkable similarities have been observed between post-transcriptional silencing involving transgenes and natural forms of virus resistance in nontransgenic plants. These results and others implicate two distinct cellular defence responses in transgene silencing. One is active in the nucleus and is manifested by transgene methylation, a reaction that might have originated as a means to oppose the spread of transposable elements. A second line of defence resides in the cytoplasm and operates through enhanced RNA turnover, a process that might help plants overcome viral infection.

Key words. Cosuppression; DNA methylation; epigenetics; gene silencing; isochores; paramutation; *trans*-inactivation; transposable elements.

Introduction

Plants have been consistently amenable to genetic and cytological analyses. It should therefore come as no surprise that plants offer some of the best systems for investigating epigenetic effects on gene expression. McClintock's demonstration of the heritable, but potentially reversible, inactivation of maize transposable elements represents the first case of epigenetic silencing in plants [1]. A second long-standing example is paramutation, which involves an allelic interaction that causes meiotically heritable alterations in gene expression [2]. Recent discoveries of various types of gene silencing in transgenic plants have expanded the repertoire of epigenetic effects in this kingdom. Because several examples of transgene silencing closely resemble paramutation, they have reignited interest in this phenomenon, which has long been considered exceptional and obscure. Transgene silencing and paramutation have also fueled interest in the possibility that *trans*-interactions between related genes, even when present on different chromosomes, might frequently provoke epigenetic modifications in higher eukaroytes.

In this short review, we describe the different epigenetic silencing effects that have been observed in transgenic plants. Although these silencing variants have characteristic features and take place in separate cellular compartments, an emerging and unifying view is that transgenes become inactivated as a result of diverse host defence systems that are designed to neutralize invasive DNA sequences and viruses.

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Transgene silencing in plants

Transgenes often become silenced in plants. Determining how and why this occurs has been the focus of a major research effort during the last 8 years, not least because transgene inactivation can be a hindrance to the successful commercialization of genetically engineered crops [3]. Conversely, because certain applications of plant gene technology will require disabled endogenous genes, finding ways to predictably elicit or optimize silencing is also a desirable goal [4]. Basic researchers are primarily interested in transgene silencing because it is revealing novel and unanticipated features of gene regulation in plants.

Types of silencing

Silencing can affect single transgene loci, or two or more transgenes at unlinked loci (fig. 1). Single loci can become silenced and methylated because of ill-defined features of flanking plant DNA and/or chromosomal location (so-called position effects) or because of interactions between multiple, closely linked copies. The latter effect, which has been termed 'homology-dependent' [5] or 'repeat-induced' [6] gene silencing, can also involve interactions between two unlinked loci in which one transgene locus is able to *trans*-inactivate a second, homologous transgene or homologous endogenous gene.

Homology-dependent *trans*-inactivation phenomena can be subdivided on the basis of whether silencing occurs at the transcriptional or post-transcriptional level (fig. 2). Transcriptional inactivation depends on homology in promoter regions and has been associated with increased promoter methylation and meiotically heritable silencing that persists for several generations in the absence of the inducing (silencing) locus. Current mechanistic models for this type of *trans*-silencing invoke DNA-DNA interactions in the nucleus. In contrast, post-transcriptional silencing – termed 'cosuppression' or 'sense suppression' [7] – requires homology in protein-coding regions, which often become methylated at their 3′ ends, and is reset during meiosis. This type of silencing has been associated with enhanced RNA turnover in the cytoplasm.

Mechanisms of transgene silencing. It is unlikely that silencing mechanisms developed in plants only after the advent of transgene technology. Therefore, transgene silencing must manifest natural plant processes [8]. The possible involvement of host-defence responses was first suggested by correlations between transgene silencing and methylation. Cytosine methylation serves primarily as a genome defence against the proliferation of transposable elements, which are inactive when methylated [9]. To extend the concept of cellular defence responses to include all forms of transgene silencing, even those that are not associated with methylation, either transgenes or their expression products must be recognized by a plant as potentially harmful. In this context, we now describe transgene silencing effects in more detail and discuss how they reveal two distinct defence systems that act, respectively, in the nucleus by methylating DNA and in the cytoplasm by triggering RNA turnover.

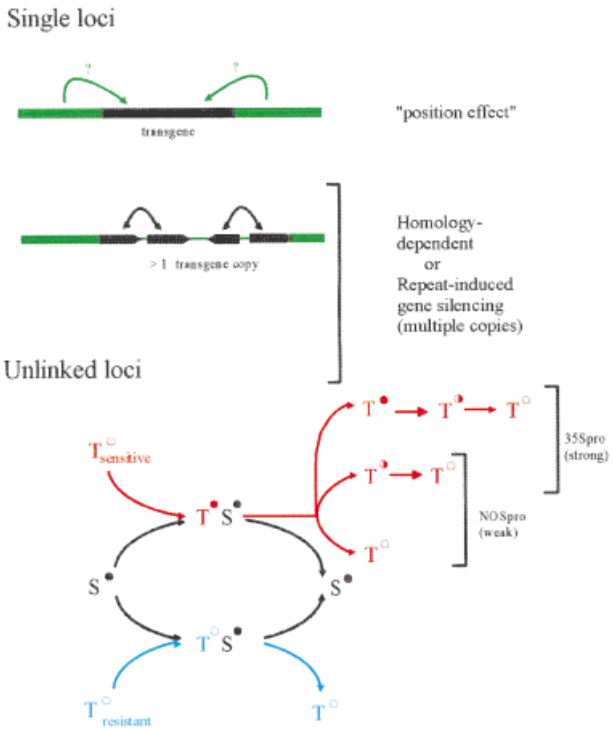

Figure 1. Types of transgene silencing in plants. A single transgene locus can become silenced because of repressive influences exerted by flanking plant DNA (green bars) in so-called position effects and/or because of interactions among multiple copies of closely linked transgenes (homology-dependent or repeat-induced gene silencing). Homology-dependent silencing can also affect genes at unlinked sites; in these cases a silencing locus (S) *trans*-inactivates and methylates a target locus (T) with which it shares DNA sequence homology. The scheme shown here depicts the consequences of interactions between silencing and target loci that share homology in promoter regions. Two constitutive plant promoters that we have used in transgene constructs are the strong 35S promoter derived from cauliflower mosaic virus and the weaker NOS promoter originating from the *A. tumefaciens* tumor-inducing plasmid. A silencing locus independently acquires stable methylation, which it imposes on a normally unmethylated target locus in a process that might require DNA-DNA pairing. A target locus does not completely lose methylation or reactivate fully after segregating away from a silencing locus; the rate of recovery depends on promoter strength. The silencing locus remains unchanged by the interaction. Although most homologous target loci are sensitive to silencing by a given silencing locus, we have identified several potential targets that are largely resistant to silencing, suggesting that not all homologous genes are able to associate physically in the nucleus. Symbols: open, half-closed and completely closed circles represent no methylation, partial methylation and complete methylation, respectively.

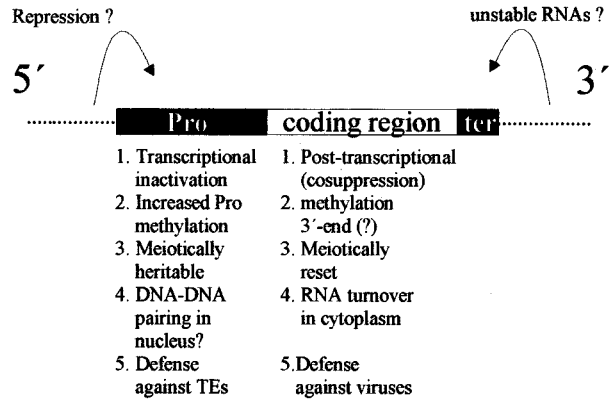

Figure 2. Homology dependence and features of different *trans*-silencing effects. *Trans*-silencing occurring at either the transcriptional or post-transcriptional level involves homology in promoters (Pro) or coding regions, respectively. Additional distinctive characteristics of each type of silencing and the cellular defence system they possibly reflect are listed. Noncoding, homologous sequences neighbouring a gene might also influence expression. Repeated DNA sequences (dotted regions) upstream of promoters could induce promoter methylation and repress gene expression. De novo methylation of repeats downstream of transcriptional terminators (ter) might spread into the 3′ ends of genes, leading to the production of unstable transcripts. TEs = transposable elements.

Transcriptional silencing and methylation: outcome of a defence response that inactivates foreign or invasive DNA

A number of examples of transgene silencing have been associated with increased methylation in promoter regions and reduced transcription (reviewed in ref. 8). Multiple copies of closely linked transgenes have a strong tendency to become methylated (*cis*-inactivation). Moreover, a multicopy transgene locus that has become methylated spontaneously in *cis* can act as a *trans*-silencing locus by transcriptionally inactivating and methylating a second unlinked target locus with which it shares DNA sequence homology.

The two *trans*-silencing systems that we have examined in detail involve silencing and target loci that share homology in promoter regions. These systems are based on two constitutive plant promoters that are commonly used in plant transgene constructs: the strong 35S promoter (ca. 500 bp) originating from cauliflower mosaic virus and the weak nopaline synthase (NOS) promoter (ca. 300 bp) derived from the *Agrobacterium tumefaciens* tumor-inducing plasmid. Silencing loci containing multiple, methylated copies of these promoters are able to transcriptionally *trans*-inactivate and methylate most genes under the control of the respective promoter [10–13].

Both *cis*- and *trans*-inactivation/methylation have been postulated to involve pairing of homologous DNA sequences. Pairing has been an attractive hypothesis because it serves as a signal for de novo methylation of sequence duplications in the filamentous fungus *Ascobolus immersus* [14] and, in the same organism, promotes the transmission of methylation from one allele to another during a meiotic process that is mechanistically related to gene conversion [15]. In transgenic plants, pairing of homologous sequences could account for both the autonomous *cis*-inactivation of multiple transgene copies at a silencing locus, and for the imposition of methylation from a silencing locus onto a target locus, a process that has been termed 'epigene conversion' [12, 16]. These *trans*-silencing effects suggest an extraordinary ability of relatively short homologous promoters on different chromosomes to interact physically in somatic cells to trigger methylation and reduce gene expression. The telomeric location of at least one *trans*-silencing locus [13] might facilitate the homology search that is necessary for this interaction to occur.

Various target loci differ in their susceptibility to inactivation by a given silencing locus. Although most homologous targets are sensitive to silencing, we have recovered a few potential target loci that are largely resistant to inactivation and emerge relatively unscathed from an interaction with a silencing locus (fig. 1). The existence of resistant targets eliminates the possibility that a silencing locus acts by sequestering diffusible transcription factors, and suggests instead that *trans*-silencing depends on the ability of a silencing locus and a target locus to associate physically in the nucleus.

A distinctive aspect of promoter homology-dependent *trans*-silencing is that a target locus does not always fully reactivate or lose methylation after segregating from a silencing locus. This residual imprinting of the target locus transgresses Mendel's first law, which states that alleles (or in this case, unlinked loci) segregate unchanged from a heterozygote. This feature is also seen with paramutation of endogenous genes (see below). Because the target locus usually regains activity to different extents in outcross progeny, significant epigenetic variability is introduced into the population. The rate of recovery of different target loci depends on promoter strength (fig. 1): silenced NOS promoters regain activity one or two generations after segregating away from a silencing locus, whereas silencing of 35S promoters can persist into the fourth backcross generation [13; M. Matzke, unpublished results]. These differences probably reflect the different degrees of methylation required to silence either a weak or strong promoter, respectively, and to more facile loss of sparse methylation from the former.

The epigenetic variability created by the immediate and lingering effects of homologous interactions might have relevance for classical genetic phenomena such as in-

complete penetrance and variable expressivity. Continuous quantitative variation in gene expression, which has long been a major conundrum of population genetics [17, 18], could have a substantial epigenetic basis in a continuum of gene methylation at the population level [19].

Paramutation of endogenous genes. Promoter-homology-dependent *trans*-silencing is remarkably similar to the phenomenon of paramutation, defined as an allelic interaction in which a silencing (paramutagenic) allele induces a meiotically heritable reduction in the activity of a sensitive (paramutable) allele following their association in the heterozygote [2]. As with *trans*-silencing of transgenes, paramutation violates Mendel's first law because the paramutable allele does not emerge unaltered from an interaction with the paramutagenic allele. Initially identified several decades ago for a handful of endogenous genes in maize and tomato [20], paramutation has long been considered a rare and unusual phenomenon [21]. The discovery of new examples in maize, however, and *trans*-silencing interactions in transgenic plants suggest that paramutation might be more common than previously realized. Moreover, the transgene examples demonstrate that not only alleles [22] but also homologous sequences on different chromosomes [10–13] can participate in paramutation-like interactions.

The paramutation system that most closely resembles the promoter homology-dependent *trans*-silencing that we have studied is paramutation at the *r* locus in maize. Similar to the multicopy *trans*-silencing loci, *r* alleles involved in paramutation contain multiple copies of the *r* gene. This configuration probably potentiates the spontaneous methylation of the paramutagenic allele, which imposes methylation on the paramutable allele when they are together in the same genome. The description of *r* paramutation as 'a transference of *cis*-inactivation among DNA sequences in one molecule to homologous sequences on another molecule' [23] applies perfectly to *trans*-silencing of transgene loci [12]. This striking resemblance has been used to argue that *trans*-silencing is not restricted to transgenes, that is 'foreign' DNA. Recent molecular analyses of *r* alleles participating in paramutation, however, have revealed that they contain a built-in foreign component in the form of *doppia* transposable elements in their promoter regions (reviewed in ref. 24). The presence of invasive DNA sequences in crucial regulatory regions of paramutable and paramutagenic *r* alleles probably contributes to inducing methylation and promoting interallelic *trans*-inactivation [24, 25]. Therefore, *r* paramutation, which closely parallels *trans*-silencing of transgenes but takes place in a nontransgenic context, can be considered a defence response against foreign or invasive sequences.

How transgenes and transposable elements trigger de novo methylation. There are at least two ways that transposable elements and transgenes might be recognized as invasive and acted upon by the DNA methylation machinery [26]. Integration intermediates, which form when transgenes or transposable elements insert into a genome, can provide targets for de novo methylation. A second likely signal is pairing of multiple copies of transposable elements or homologous transgenes. Transposable elements at ectopic locations can become methylated coordinately, possibly via pairing interactions [27], which is comparable to the *trans*-silencing effects observed with unlinked homologous transgenes. Whether a transgene becomes modified and inactivated is not completely determined by the above processes, however, because not all integrated transgenes are methylated. Moreover, methylation is not always associated with multiple copies of transgenes [28, 29]. A third feature that might identify transgenes and transposable elements as foreign and hence provoke de novo methylation is abrupt transitions in the base composition at junctions between plant DNA and newly integrated sequences. A consideration of this point brings us to a discussion of position effects and transgene expression.

Position effects

Although it is commonly assumed that the activity of a given transgene can be affected by the genomic context ('position effects') (fig. 1), surprisingly little is known about the features of flanking plant DNA or chromosomal location that permit optimal transgene expression. We are using two approaches to analyse position effects on plant transgene expression. First, transgene loci that either stably or unstably express the same construct are being compared with respect to transgene copy number and arrangement, nucleotide sequence of flanking plant DNA, and chromosomal location. Second, because repeated sequences are often associated with silencing and methylation, different repeats are being introduced upstream or downstream of transgenes and their effects on expression assessed.

In a molecular and cytogenetic analysis of four independent transgene loci [29], stably expressed transgenes were found to be associated with AT-rich plant DNA that bound to nuclear matrices in vitro, and were localized by fluorescence in situ hybridization to the distal ends of chromosome arms, known to be gene-rich regions in wheat, maize and humans [30, 31]. Unstably expressed, methylated loci were adjacent to prokaryotic (binary vector) sequences that led directly into plant DNA, and were found to occupy intercalary or centromeric chromosomal locations that are distant from the gene-rich terminal domains.

Only one published report has described the influence of a repeat that was deliberately engineered next to a transgene. In this study, a repetitive sequence from petunia was found to enhance variegated expression of a reporter transgene in petunia and tobacco [32]. Using the Cre-*lox* system for site-specific recombination to specifically remove a repeated sequence from a transgene locus, we have found recently that a tandem repeat could induce methylation and inactivation of a neighbouring NOSpro-driven gene (fig. 2) (M. A. Matzke and A. J. M. Matzke, unpublished results). Numerous variations on these studies are being performed, including testing (i) repeats from plant and nonplant sources; (ii) repeats derived from tandem and dispersed families; (iii) species-specific repeats; (iv) repeats positioned 5′ or 3′ to a transgene; and (v) the effect of the same repeat on promoters of different strengths.

Discontinuities in base composition at transgene-plant DNA junctions. Single copies of transgenes can become methylated and silenced even if the flanking plant DNA remains unmethylated, suggesting a specific response to foreign DNA [28]. The basis of this response is not known, but has been postulated to involve differences in the base compositions of transgenes and flanking plant DNA, which generally has a higher AT content than many commonly used transgenes. Discontinuities in GC content or deviant base compositions might serve as a signal for de novo methylation [8], as illustrated by the presence of GC-rich binary vector sequences adjacent to transgene inserts that are methylated and unstably expressed [29; J. Jakowitsch and A. Matzke, unpublished results]. In a unique study, a GC-rich *A1* gene from the monocot maize was shown to be more susceptible to methylation and silencing in petunia compared with its homologue from the dicot, *Gerbera*. The *Gerbera* gene, which has a much lower GC content than the maize gene, was probably more compatible with the genome of petunia, which is also a dicot [33]. The genomes of grasses, such as maize, have higher overall GC contents (45–48%) than the genomes of dicots, such as tobacco, sunflower and pea (41% GC), and the coding sequences of grasses can be considerably more GC-rich (60–70%) than comparable sequences in dicots (average 46%) [34]. Whether these variations in base compositions are responsible for different types or frequencies of gene silencing in each class of plants is not yet known. Understanding and controlling gene silencing will require coming to grips with the genomes of agriculturally important plants, which are usually substantially more complex than the genome of the current model plant, *Arabidopsis thaliana* [35].

Distinct base compositions of subgenomes in allopolyploids. Bernardi has documented the compartmentalized structure of eukaryotic genomes, which consist of mosaics of compositionally similar sequences that comprise families with different GC contents (isochores) [36]. Through our work on tobacco, we have become interested in how the distinct base compositions of subgenomes in polyploid plants might influence transgene expression.

Together with the accumulation of repetitive DNA, polyploidy has been one of the major forces in plant genome evolution. Up to 70% of flowering plants have polyploidy in their history [37]. A number of crop plants, including wheat, oats, sugarcane, coffee and cotton, are natural allopolyploids produced by interspecific hybridization between morphologically and genomically distinct diploid species followed by chromosome doubling [38]. Polyploidy is not restricted to the plant kingdom: two genome duplications are believed to have occurred during vertebrate evolution [39]. Despite the importance of polyploidy in the evolution of plants and vertebrates, little is known about the evolutionary processes taking place in newly formed polyploid species. Because many common plant varieties are relatively recent polyploids derived from extant diploid progenitors, they provide a window onto the early stages of polyploid genome evolution, before the distinctive features of parental subgenomes have been erased by intermixing and sequence divergence [40].

Tobacco (*Nicotiana tabacum*; $2n = 4x = 48$) is a natural allotetraploid created approximately 6 million years ago by hybridization between two diploid ($2n = 24$) progenitors, *N. sylvestris* (S subgenome) and *N. tomentosiformis* (T subgenome), which have been diverging from each other for about 75 million years [41]. Several translocations between the S and T subgenomes have occurred subsequent to polyploidization [42 and references therein], and there is some molecular evidence suggesting local exchanges [43, 44]. A bimodal sequence composition that reflects the distinct base compositions of the subgenomes is still discernable in tetraploid tobacco, however, indicating that extensive intermingling between subgenomes has not yet occurred [45]. This is strikingly revealed by the technique of genomic in situ hybridization. When total genomic DNAs from the two diploid parents are labelled with different fluorochromes and used as probes on tobacco metaphase spreads, chromosomes from the S and T subgenomes are easily recognized (fig. 3). The isochore structure observed in the genomes of vertebrates [36], which are probably ancient allooctoploids with a quadruplicated genome [39], could actually be a vestige of diverged parental genomes that have become highly intermixed since the original polyploidization events.

In our studies on the genomic context of stably and unstably expressed transgenes, we noticed in several

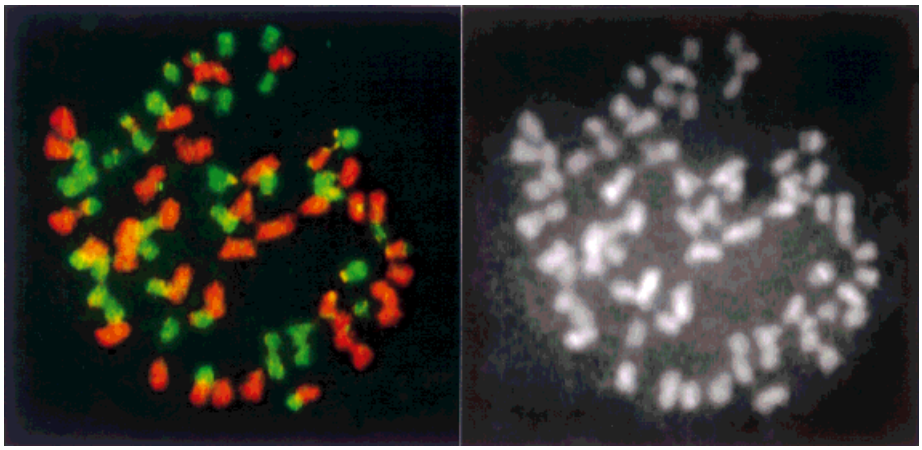

Figure 3. Genomic in situ hybridization (GISH) to somatic metaphases of the allotetraploid *N. tabacum* cv. Petit Havana SR1 (tobacco) (2n = 4x = 48). Total genomic DNA from the two diploid (2n = 24) progenitors of tobacco were labelled with different fluorochromes and used as probes in a GISH analysis of tobacco metaphase chromosomes. Left: Ancestral *N. sylvestris* chromosomes (S subgenome) fluoresce red; the chromosome set derived from *N. tomentosiformis* (T subgenome) fluoresces green. Areas where the red and green signals overlap at homologous nucleolar organizing regions appear yellow. Right: DAPI counterstaining of the same metaphase. These chromosomes were prepared from a spontaneously occurring hyperploid line (2n = 6x = 72). For details of the GISH procedure, see ref. 42. Photographs kindly supplied by E. A. Moscone.

cases a discordance between the molecular and cytogenetic data with respect to the subgenomic allocation of particular transgene loci: two unstably expressed transgene inserts were adjacent to a retroelement remnant that was enriched in the T subgenome, but the transgenes were actually present on chromosome from the S subgenome [29, 44]. The enrichment or species specificity of different transposable element families in either the S or T subgenome suggests that the amplification of these elements has been repressed in one subgenome. Differences in the base composition of the S and T subgenomes, and variations in the GC content and insertion site preference of different retrotransposon families [46; M. Matzke and A. Matzke, unpublished results], might limit the spread of specific elements to the genome with which they are most compatible [36, 46, 47]. The infiltration of species-specific transposable elements into the partner subgenome in recently created allopolyploids could produce interruptions in sequence homogeneity if an element is incompatible with the new host subgenome. The outcome might be a 'foreign DNA response', manifested at the insertion site by increases in methylation that could encroach on neighbouring sequences including transgenes [40]. Testing of these ideas will require information about additional transgene loci, including the analysis of extensive flanking plant DNA sequences, chromosomal location and subgenomic allocation.

Unique features of methylation in plants. Although DNA methylation is found in many higher eukaryotes (familiar exceptions include *Drosophila* and *Caenorhabditis*), this epigenetic modification has unique features in plants, including the preservation of methylatable cytosines in CpG and CpNpG nucleotide groups. Plants and vertebrates methylate cytosines in CpG dinucleotides; in addition, plants also methylate cytosines in CpNpG trinucleotides and other nonsymmetrical cytosine residues [48]. Vertebrate genomes are deficient in CpGs due to the deamination of 5-methylcytosines to thymines that are not recognized by repair mechanisms [49]. In contrast, CpG depletion is not as acute in plants, and there is virtually no CpNpG suppression. This has been interpreted as indicating either that plants have an extremely efficient mismatch repair system or that genes entering the germ line are not methylated [50].

Transposable elements are frequent targets for cytosine methylation [9]; however, it is not known at what point decaying transposable elements cease being modified by methylation. *Copia*-like retrotransposons are ancient, heterogeneous and extremely abundant components of many higher plant genomes [51]. Most copies are defective and in various stages of sequence degeneration. Because of the absence of CpG and CpNpG suppression in plants, these eroding retroelements can contain many potentially methylatable cytosines (I. Papp, C. Kunz and A. Matzke, unpub-

lished results). Consequently, they could still attract methylation and contribute substantially to genomic methylation patterns. Transgenes integrated adjacent to these elements might have a tendency to become progressively silenced [29].

Transposable elements in wild-type plant genes: links between a defence response and regulation of gene expression by methylation? Intimate connections between plant genes and mobile elements are revealed by the surprising finding that transposons, primarily miniature inverted repeat transposable elements (MITES) and retroelement remnants, are present in the flanking regions of many wild-type plant genes, often less than 1 kb from the transcription start or stop sites [52, 53]. In addition to providing novel, tissue-specific *cis*-acting regulatory sequences [54–57], these retroelement relics and MITES might be differentially methylated and contribute to the large-scale gene silencing that is required in different cell types [24]. The relative tolerance of polyploid genomes to transposition has probably played a prominant role in genome evolution by allowing the fixation of mobile elements in the regulatory regions of individual copies of duplicated genes [40].

Post-transcriptional transgene silencing: a reflection of a viral defence response that instigates cytoplasmic RNA turnover

Several recent reviews provide detailed accounts of post-transcriptional silencing and suggest possible mechanisms [58–60]. Post-transcriptional silencing was first discovered for the pigmentation gene chalcone synthase (*chs*) in transgenic petunia plants [61, 62]. Although the original goal of these experiments had been to obtain overproduction of CHS protein and more deeply pigmented petunia petals, the opposite outcome, i.e. completely white or patterned flowers, was frequently observed. This phenomenon was termed 'cosuppression' because both the *chs* transgene and *chs* endogenous gene were found to be inactivated coordinately in the white portions of petals [61]. Transcription run-on experiments with isolated nuclei established that the type of silencing displayed by *chs* genes (and others) occurred post-transcriptionally and involved enhanced RNA turnover [63–65]. The trigger for turnover is not yet known, but possibilities include an overproduction of RNA, such that a sharp threshold concentration is surpassed [7, 66, 67], and the synthesis of aberrant RNAs. The hypothetical aberrant RNA might be produced by the transgene [60] or the homologous endogenous gene [58]. The latter possibility is suggested by the observation that nontranscribed, promoterless transgenes can elicit cosuppression, particularly if two transgene copies are arranged as an inverted repeat (IR) [68]. The IR transgenes are thought to act by pairing with the homologous endogene, which responds by undergoing some sort of epigenetic modification, such as methylation, leading to the production of aberrant transcripts [58–60]. Some cases of post-transcriptional silencing have been shown to involve methylation, which is usually concentrated at the 3′ end of protein-coding regions. This methylation might be responsible for the production of transgene RNAs that are truncated or improperly processed [69, 70].

Similarities between post-transcriptional silencing and natural viral defence mechanisms. An influential study suggested a relationship between genetically engineered virus resistance and post-transcriptional silencing involving RNA turnover in transgenic plants [71]. This connection has been subsequently strengthened, and the turnover process has been localized to the cytoplasm because the viruses involved only replicate their RNA genome in this cellular compartment [69, 70, 72]. Most interesting in the context of this review are recent reports of enhanced RNA turnover in virus-infected nontransgenic plants. In both cases, normal plants inoculated with viruses were able to overcome infection by initiating cytoplasmic turnover of replicating RNA viral genomes [73] or RNA replication intermediates of cauliflower mosaic virus, which has a DNA genome [74]. The authors of both of these studies suggested that post-transcriptional gene silencing in transgenic plants might reflect a natural resistance to virus infection.

How are transgene-derived RNAs incorrectly perceived by a plant as being of viral origin? Transgenes subject to post-transcriptional silencing might produce RNAs having features resembling replicating viruses, such as double-strandedness [73]. Another association between viruses and post-transcriptional silencing arises from the frequent use of the 35S promoter in transgene constructs. This promoter, which has been involved in virtually all cases of post-transcriptional silencing, is derived from cauliflower mosaic virus, which is acted on by a natural host defence in kohlrabi plants [74]. Because it originates from a virus and is a strong promoter that produces abundant RNA (required for the 'threshold hypothesis'), the 35S promoter might produce transcripts that are frequently drawn into a turnover mechanism intended to combat viral infection.

There are several known instances of post-transcriptional silencing of nonviral genes in nontransgenic plants. These all concern natural duplications or rearrangements of the *chs* gene, which is apparently highly susceptible to this type of silencing (reviewed in ref. 60). In one petunia variety created by plant breeders, not only is post-transcriptional silencing of *chs* genes responsible for the petal pigmentation pattern, but the same RNA breakdown products are generated as in cosuppressed transgenic petunias [75]. Endogenous genes can thus undergo structural alterations that give rise to transcripts susceptible to degradation in a manner identical to that occurring in transgenic plants.

Concluding remarks

'Transgene inactivation: plants fight back!' is not only the clever title of an interesting review [3], it is also encapsulates perfectly the major premise of this article, viz. that transgenes are silenced in plants because they fall victim to cellular defence systems directed against harmful genomic or cytoplasmic invaders. This conclusion derives from recent findings on natural epigenetic effects that provide clear parallels to various types of gene silencing in transgenic plants. These results include the discovery of transposable elements in the promoters of *r* alleles subject to paramutation in maize, and the initiation of cytoplasmic RNA turnover in nontransgenic plants recovering from viral infection. The extent to which plants have adapted these defence mechanisms to control the expression of endogenous genes is unknown. The presence of fossil retrotransposons or MITES in the immediate flanking regions of many wild-type plant genes, as well as the endogenous (albeit rearranged or duplicated) *chs* genes that produce transcripts subject to turnover, provide hints that defensive strategies have been co-opted for the regulation of normal plant genes.

The discoveries of *trans*-silencing effects in plants have contributed to the surge of interest in epigenetic modifications induced by long-range interactions and homologous DNA pairing in diverse organisms, including *Drosophila*, fungi and mammals [76]. Determining the molecular basis of these associations, their possible developmental control and their dependence on interphase nuclear architecture poses major challenges for the future.

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