

# Gene silencing as an adaptive defence against viruses

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Gene silencing was perceived initially as an unpredictable and inconvenient side effect of introducing transgenes into plants. It now seems that it is the consequence of accidentally triggering the plant's adaptive defence mechanism against viruses and transposable elements. This recently discovered mechanism, although mechanistically different, has a number of parallels with the immune system of mammals.

**B**iology students are taught that the concept of vaccination came from Edward Jenner's discovery that milkmaids and dairymen infected with the mild cowpox virus were protected against smallpox. It is less widely appreciated that plants can also be protected from a severe virus by prior infection with a mild strain of a closely related virus. This cross protection in plants was recognized as early as the 1920s, but its mechanism has been a mystery — plants do not possess an antibody-based immune system analogous to that found in animals. This was probably the first observation of a plant's intrinsic defence mechanism against viruses which, 75 years later, is just beginning to be understood. In the past decade, there has been considerable research into transgene-mediated virus resistance, co-suppression, virus-induced gene silencing (VIGS), antisense suppression and transcriptional gene silencing (TGS) in plants. There has also been intense research into RNA interference in *Drosophila* and nematodes, and quelling in fungi. These seemingly disparate endeavours have produced pieces of a jigsaw puzzle which, when put together, begin to reveal the existence and characteristics of a natural defence system in plants against viruses and transposable DNA elements. Many of the details and ramifications have yet to be determined, but the current picture is that of a wonderfully elegant system that can generically recognize invading viruses and transposable elements (TEs) and marshal the plant's defences against them.

## Plant viruses and transposable DNA elements

There are currently 72 different defined genera of plant viruses<sup>1</sup>, containing over 500 species, and there is scarcely a plant species — mono- or dicotyledon — that is not host to at least one virus. Plant viruses have a whole array of different particle morphologies, host ranges, vectors (for example, insects, nematodes, fungi, pollen, seeds or humans), genome organizations and gene expression strategies. They cause symptoms which at their least severe are unnoticeable, but range upwards through ringspots or mosaic leaf patterns, to widespread necrosis. The genomes of some plant viruses are encoded using single-stranded (ss) or double-stranded (ds) DNA; others have dsRNA

themselves by exploiting this requirement of most plant viruses to replicate using a double-stranded replicative intermediate.

TEs are DNA sequences that have the capacity to move from place to place within a genome. They have been divided into two classes. Class I TEs are retroelements that amplify their copy number through reverse transcription of an RNA intermediate. They are particularly abundant in eukaryotes, and in plants comprise the greatest mass of TEs (in maize, this class of TE makes up over 70% of the nuclear DNA). Of the four types of retroelements in plants, the main class contains retrotransposons with direct long terminal repeats (LTRs). Class II TEs occur in all organisms, particularly prokaryotes; they have terminal inverted repeats (TIRs) ranging in size from 11 to several hundred base pairs. Within a class II TE family, one or more elements encode a transposase that has the potential to interact with TIRs to excise the elements and integrate them into other regions of the genome (for recent reviews of plant TEs, see refs 2, 3). Both classes of TEs can move around plant genomes, altering the function and structure of genes, and so accelerating genomic evolution. However, they are also parasitic mutagenic agents that have the potential to lacerate a genome<sup>4</sup>. To ensure survival, a plant needs to keep TE activity in check.

## Targeted RNA degradation

Although not recognized at the time, evidence of a plant's intrinsic defence mechanism to counter viruses and transposons came from the initially mystifying results of co-suppression and transgene-mediated virus resistance. Transformation with antisense gene constructs has been used in plant research since 1987 (ref. 5). From previous work on natural antisense in bacteria<sup>6</sup>, it was thought that hybridization of antisense RNA to the target messenger RNA interfered with its transport or translation<sup>7</sup>. So it was surprising to find subsequently that transformation of plants with transgene constructs encoding sense mRNA homologous to endogenous genes could also suppress the activities of these genes<sup>8-10</sup>. It was similarly perplexing when plants transformed with virus-derived transgenes, designed to provide protection through a protein-mediated mechanism<sup>11</sup>, gave protection against viruses even when little or no transgene protein (transprotein) was

the transgenes were being highly transcribed in the nucleus, but the steady-state levels of their mRNAs in the cytoplasm were very low. This led to the proposal<sup>12,13</sup> that the transgene mRNA was somehow perceived by the cell as unwanted and induced sequence-specific degradation, by a targeted nuclease, of itself and other homologous or complementary RNA sequences in the cytoplasm. Thus, in the co-suppressed and virus-resistant lines, not only the transgene mRNAs but also the mRNA from the homologous endogenous gene and the invading virus RNA (with homology to the transgene) were being degraded. The concept of transgene RNA-directed RNA degradation was supported by the results of an experiment in which plants, with a co-suppressed  $\beta$ -glucuronidase (GUS) reporter gene, were inoculated with a wild-type plant virus or the same virus engineered to contain GUS sequences in its genome. The plants were susceptible to the wild-type virus, but resistant to the virus containing the GUS-encoding sequence. The virus has an RNA genome and replicates exclusively in the cytoplasm, so the simple explanation is that the GUS sequence within the virus genome was specifically degraded in the cytoplasm by the same mechanism that was causing co-suppression of the nuclear-expressed genes<sup>14</sup>.

This conclusion raised a number of questions. How do the nucleases in the cell know which RNAs to degrade and which to leave alone, or more specifically, how do they distinguish transgene RNA from endogenous gene mRNA? Why does this not happen to the mRNAs from all transgenes? And why would a plant want to specifically degrade these RNAs? A critical observation was that in both the co-suppression and virus-transgene transformation experiments, only a proportion of the initial transformants showed co-suppression or virus resistance, and these plants generally contained multiple, methylated copies of the transgenes.

### How is the degradation system triggered?

Several theories have been advanced to explain how this sequence-specific degradation system might be activated. It was proposed initially that the high copy number of the transgenes produced excessively high levels of transgene mRNA and that this level induced the degradation system<sup>12,13</sup>. Other researchers suggested that the methylation of the transgenes made them produce aberrant (for example, prematurely terminated) RNA and that this aberrance induced the system<sup>14-17</sup>. A compelling proposal was that the system is induced and directed by dsRNA and that multiple transgenes favoured the likelihood of their integration as inverted repeats which, by transcriptional readthrough from one transgene into the other, would produce duplex-forming, self-complementary RNA<sup>18</sup>. This was supported by the demonstration that transgenes deliberately designed to produce self-complementary (hairpin or hp) RNA or dsRNA were highly efficient at inducing targeted virus resistance and gene silencing<sup>18-20</sup>. Furthermore, an investigation of simple co-suppression and antisense constructs found a perfect correlation between the integration of these constructs as inverted repeats and the induction of silencing<sup>19</sup>, and analysis of similar loci detected the presence of hpRNAs transcribed from them<sup>21</sup>.

Why would the plant want to degrade dsRNA and ssRNAs of similar sequence? Healthy plants do not contain dsRNA or extensively self-complementary ssRNA. In fact, for many years, plant virologists have used the presence of dsRNA in plant extracts to diagnose viral infection<sup>22</sup>. This seems to be the key. Most plant viruses have ssRNA genomes and replicate in the cytoplasm using their own RDRP to produce both sense and antisense (termed plus-strand and minus-strand) RNA. Evidence from research on the RNA bacteriophage Q $\beta$ <sup>23</sup> suggests that the plus and minus strands of a ssRNA virus form full-length dsRNA only as an artefact of extraction<sup>24</sup>. However, when the mammalian 2',5'-oligoadenylate system (which is activated specifically by dsRNA) was transformed into plants, it was activated

consistent with the phenomenon of VIGS; here, plant viruses that contain sequences homologous to nuclear-expressed genes act to induce silencing of the targeted genes<sup>26</sup>.

It therefore seems likely that one of the roles of the dsRNA-induced RNA degradation system of plants is to protect them against virus infection. This is a way to generically detect the replication of an invading ssRNA virus and destroy it, by specifically degrading both replicating and translatable forms of its genome.

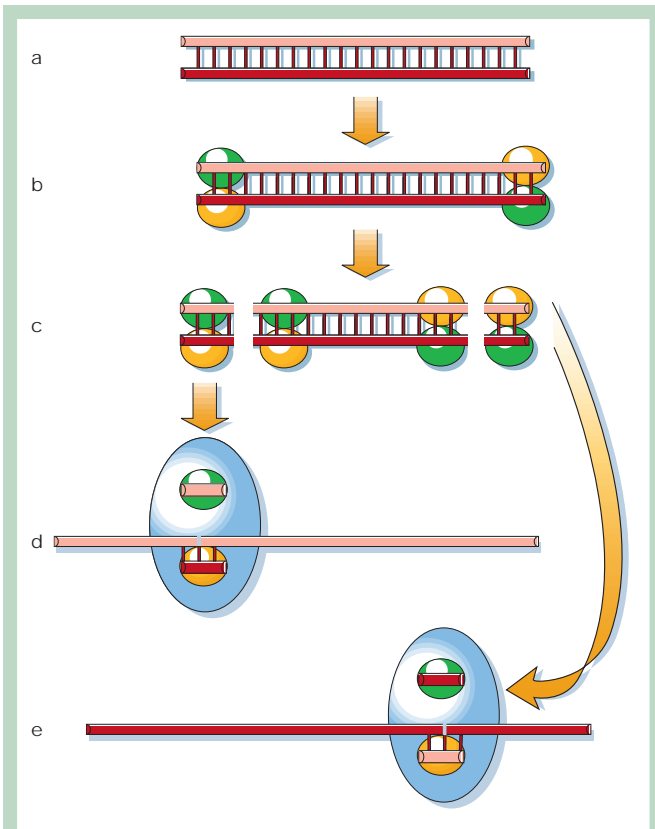
### Nuclease specificity and location

Specific fragments from mRNAs or viral genomes have been identified in gene-silenced or virus-resistant tissues, indicating that the targeted RNA degradation starts with endonucleolytic cleavage at one or more sites and is followed by exonucleolytic degradation<sup>14,27,28</sup>. Further investigation has found that sense and antisense ~25-nucleotide RNAs, with homology to the target RNA, are found consistently in plants showing co-suppression, antisense suppression, VIGS and virus resistance, but not in the appropriate control plants<sup>29-33</sup>. This is a critical finding. It supplies further evidence that these different forms of silencing are all acting by the same mechanism; from here on we refer to them generically as post-transcriptional gene silencing (PTGS). It also provides a strong link between PTGS and a phenomenon called RNA interference (RNAi), which is the targeted inhibition of gene activity by introduction, usually by injection, of dsRNA into a number of lower eukaryotes, including nematodes and *Drosophila*<sup>34,35</sup>.

Looking at the biochemistry of the process of RNAi provides a good indication of what is probably happening in plants (Fig. 2). The processes of RNAi have been examined in *Drosophila* embryos, and embryo extracts, using radiolabelled dsRNA and target ssRNA<sup>36-40</sup>. Target ssRNA is not significantly degraded when sense or antisense RNAs are also introduced. However, the target RNA is degraded within minutes of adding homologous dsRNA. The degradation rapidly produces short sense and antisense ~21-nucleotide RNAs from both the dsRNA and the target ssRNA as a two-step process<sup>39,40</sup>. The dsRNA is degraded in ~21-nucleotide steps from both ends by an enzyme called Dicer-1 (CG64792, *DCR1*). The cleavage process, which is similar to that of *Escherichia coli* RNase III, produces ~21-nucleotide dsRNA fragments with 3' overhangs of 2-3 nucleotides, and 5'-phosphate and 3'-hydroxyl termini<sup>40</sup>. Each fragment is associated with, and cleaved by, a separate Dicer-containing complex. The current model for the second step of the degradation is that the Dicer-containing, small interfering ribonucleoprotein (siRNP) complex alters in such a way that the strands of short dsRNA become unpaired and guide the complex to complementary target RNAs. This probably requires recruitment of

**Figure 1** Potato plants challenged with potato virus Y. The three plants on the right are non-transgenic and are susceptible to the virus. The three plants on the left contain an untranslatable virus-derived transgene yet are immune to the virus<sup>18</sup>.





**Figure 2** Proposed mechanism, based on RNAi, for dsRNA-directed ssRNA cleavage in PTGS. Introduced dsRNA (a) attracts Dicer-1-like proteins to its termini (b). The heterodimer complex at each end cleaves a 21-nucleotide dsRNA fragment (c), and the exposed ends of the shortened dsRNA each attract a new Dicer complex, which cleaves a further 21-nucleotide dsRNA fragment. This progressive exonuclease-like shortening continues until the dsRNA is completely cleaved. Dicers, loaded with dsRNA, acquire further components (blue ellipse), melt their dsRNA fragments and use one strand to hybridize to homologous ssRNA and cleave it in the middle of the 21-nucleotide guide-recognized sequence. One half of the dimer (shown as gold) directs hybridization and endonuclease cleavage giving it sense specificity. Thus Dicer complexes loaded from one end of a dsRNA will cleave sense-strand mRNA (d), whereas complexes loaded from the other end will cleave mRNA of the opposite sense (e).

additional proteins to the complex<sup>39</sup>. Once hybridized to a target RNA, the complex cleaves it at a position approximately in the middle of the recognized 21-nucleotide sequence. The whole two-step process results in dsRNA being cleaved with a ~21-nucleotide (two helical turns<sup>36</sup>) periodicity from their termini, and the appropriate target RNAs being cleaved with the same periodicity but with a frame shift of ~10 nucleotides.

The second step of the degradation probably takes place exclusively in the cytoplasm, as silencing does not reduce the full-length transcript levels in the nucleus<sup>27</sup>. However, the first step could occur in both the cytoplasm and the nucleus. Many mRNA degradation mechanisms involve the association of RNA with ribosomes, so it might be assumed that this would be the site of siRNP-mediated degradation. But several studies using protein-synthesis inhibitors have shown that neither ongoing translation nor association of the target RNAs with the ribosome are required for this degradation<sup>15,41</sup>. Furthermore, for each ribosome to be associated with enough siRNP complexes to ensure effective degradation of target RNA, the siRNPs would have to be expressed at very high levels. Perhaps the degradation complexes act as gatekeepers, located at the nuclear pores and

**Table 1** Genetic components of post-transcriptional silencing pathways

Gene function	Plants*	Worms*	Flies*	Fungi*	Algae*	TE act.†	PAZ‡
RDRP	SGS2	EGO1		ODE1			
Translation initiation factor	AGO1	RDE1		ODE2		No	Yes
RecQ, DEAH or Upf1p helicase	SDE3	MUT7   SMG-2		ODE3	MUT6	Yes	
RNaseIII+ helicase¶	CAF1	K12H4.8	DCR1				Yes
Chromatin remodelling	DDM1					Yes	
Methyl transferase	MET1						
?	SGS3						
?		RDE 4				No	
?		RDE2&3				Yes	
		MUT2					
Methylation	Yes?	No	No	No			

\*Plants, *Arabidopsis thaliana*; worms, *Caenorhabditis elegans*; flies, *Drosophila melanogaster*; fungi, *Neurospora crassa*; algae, *Chlamydomonas reinhardtii*.

†TE act., activation of transposon activity in organisms mutant for this gene function.

‡PAZ, presence of the PAZ domain identified by Cerruti *et al.*<sup>54</sup>.

§SGS2 and SDE1 are different descriptions of the same gene.

||The effect on transposon activation was assessed only for the MUT7 and MUT6 helicases. MUT7 also has an RNase D motif.

¶CAF1 and K12H4.8 have been identified by sequence homology to DCR1 but they have not been shown formally to be involved in PTGS.

would be scanned and destroyed as they attempt to spread from cell to cell through the plasmodesmata.

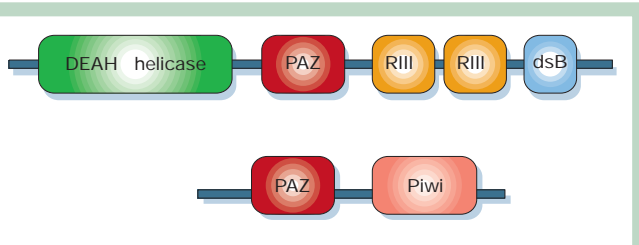
### What genes are involved in PTGS and RNAi?

The similarity of induction, degradation and associated short dsRNAs in RNAi, quelling and PTGS indicates an underlying evolutionarily conserved mechanism. Analysis of mutants defective in these processes in *Caenorhabditis elegans*, *Neurospora* and *Arabidopsis* confirm this closeness, showing that there are a number of common essential enzymes or factors (Table 1). In all three species, mutation of an RDRP or a protein with homology to eIF2C, a rabbit protein thought to be involved in translation initiation<sup>42</sup>, blocks silencing<sup>32,43-48</sup>. Another class of essential silencing proteins, those with homology to one of three types (RecQ, DEAH or Upf1p) of helicase, has been found in *C. elegans*, *Neurospora*, *Chlamydomonas reinhardtii* and *Arabidopsis*<sup>49-53</sup>. The roles of these proteins remain to be elucidated. They are probably not the equivalents (or parts thereof) of Dicer-1 in *Drosophila*; comparisons of the Dicer-1 sequence with genome databases identify K12H4.8 in *C. elegans* and CAF1 in *Arabidopsis* as homologues. These two Dicer-like proteins each have an RNA helicase domain, RNase III motifs and a PAZ domain<sup>39,54</sup> (Fig. 3).

There are two further categories of silencing-deficient mutants in plants and nematodes. One contains mutations of proteins that affect the structure and/or transcriptional status of chromatin, including DDM1, which remodels chromatin structure, MET1, which is a methyltransferase, and RDE2, RDE3 and MUT2, which seem to be involved with repressing the activity of TEs. The other category contains SGS3 in *Arabidopsis* and RDE4 from *C. elegans*, whose functions are a complete mystery. SGS3 has been cloned and sequenced, but has no recognizable motifs or matches with other sequences in available databases.

These mutation studies show that PTGS, RNAi and quelling are not just the result of Dicer complexes waiting to degrade dsRNA and homologous ssRNA that invades a cell. Other associated processes are clearly involved, including a possible link to the translation apparatus, an RDRP, and interactions with chromosomal DNA.





**Figure 3** Distribution of domains on DCR1-like and AGO1-like proteins. Top panel illustrates the domains on DCR1-like proteins of ~2,000 amino acids (including DCR1, CAF1 and K12H4.8); bottom panel represents AGO1-like proteins of ~900 amino acids (including AGO1, RDE1 and QDE2). RIII, RNase III domain; dsB, double-stranded RNA-binding domain(s). For more detailed information on domains, see <http://www.sanger.ac.uk/Software/Pfam>.

coding region of the silenced transgenes are densely methylated<sup>55</sup>. Methylation, or methylation-associated chromatin remodelling, of promoter sequences is thought to prevent binding of factors necessary for transcription<sup>55</sup>. The coding sequences of PTGS-inducing transgenes are also frequently found to be methylated. PTGS can be established in plants with a mutant methyltransferase (*met1*), but during growth, the silencing becomes impaired, reactivating the silenced gene in sectors of the plant<sup>56</sup>. Furthermore, PTGS can fail to establish in mutant plants lacking the chromatin remodelling protein DDM1. These results suggest a role for DNA methylation and/or chromatin structure in both establishment and maintenance of PTGS.

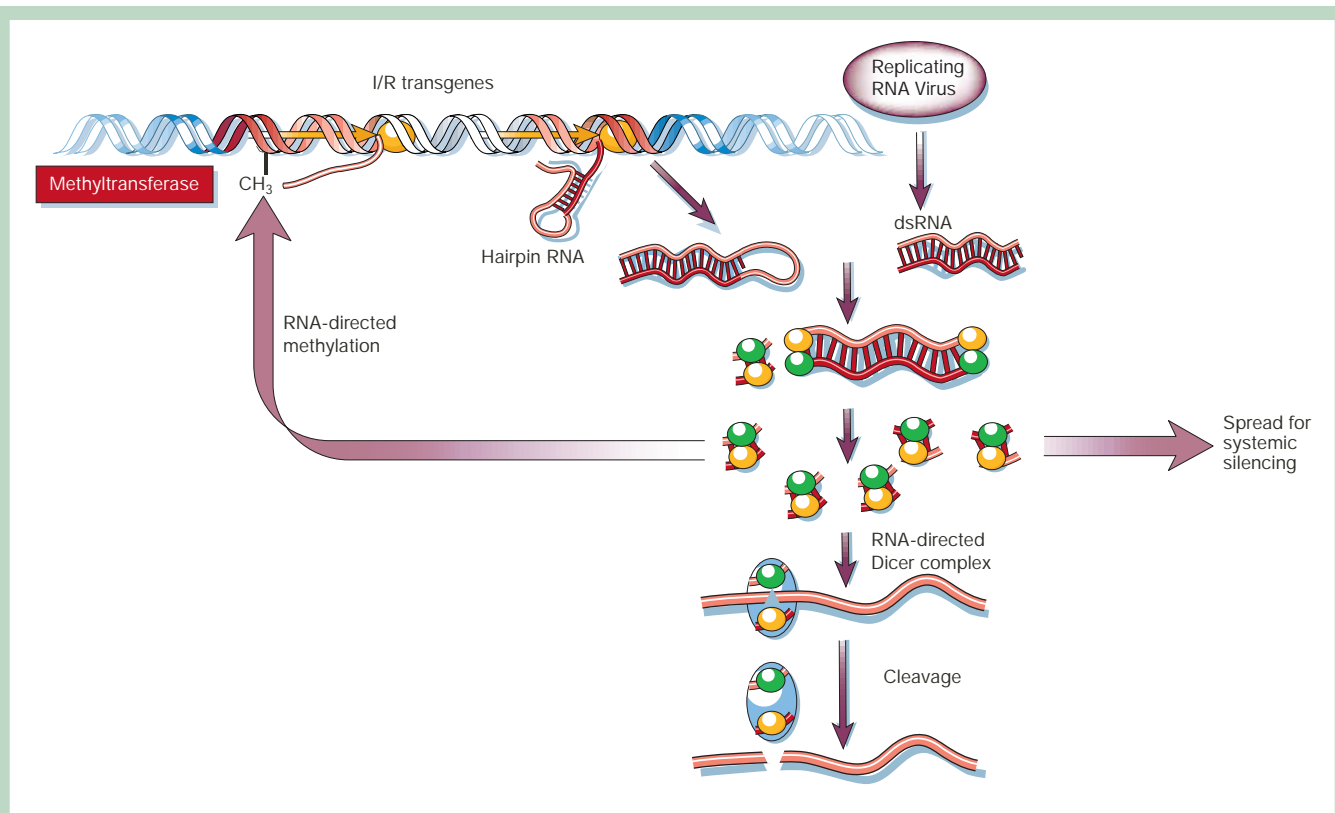
The mechanisms of PTGS and TGS may have more in common than was previously thought. In PTGS, the short RNAs derived from the transcribed region of the transgene act as guides for siRNPs to degrade target ssRNA. In TGS plants, hpRNAs containing promot-

er-region sequences are processed into short dsRNAs, and seem to direct methylation<sup>30</sup>. Similarly, virus-replicated RNAs direct sequence-specific DNA methylation<sup>57,58</sup> and are associated with short dsRNAs<sup>58</sup>. It is possible that the steps of PTGS and TGS are, in fact, the same and differ only in their target sequences: hpRNA or dsRNA is cleaved by the plant homologue of Dicer-1 into ~21-nucleotide dsRNAs to guide specific ssRNA degradation in the cytoplasm, and a similar ribonucleoprotein complex passes into the nucleus to direct chromatin remodelling/methylation of homologous DNA (Fig. 4). Thus, production of hpRNA/dsRNA that contains promoter sequences leads to the methylation/alterd state of the promoter DNA, causing TGS, whereas hpRNA/dsRNA that contains coding-region sequences leads to the degradation of homologous mRNA, causing PTGS. The methylation of coding-region DNA in PTGS and the potential degradation of promoter-sequence transcripts in TGS would be irrelevant by-products, as methylated coding regions are readily transcribed<sup>58,59</sup> and promoter sequences are not usually transcribed.

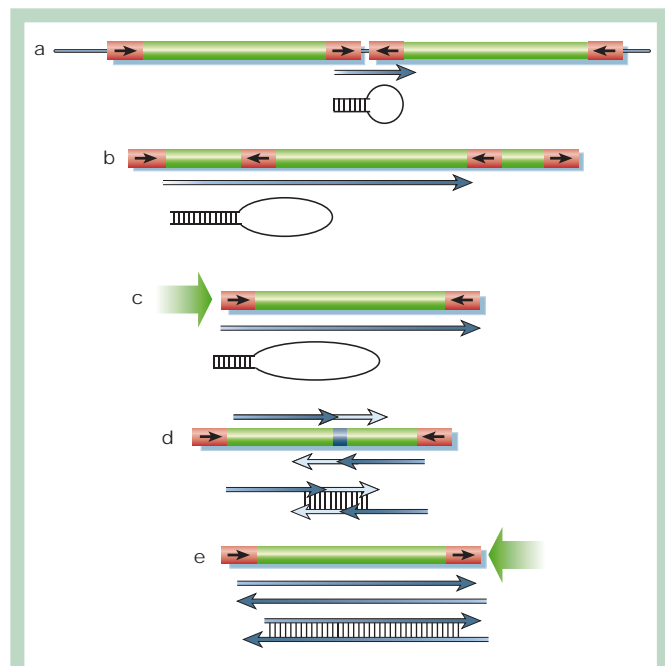
It seems unlikely that the DNA methylation mechanism associated with PTGS and TGS is involved directly in protecting plants against most RNA viruses. The vast majority of these viruses have exclusively cytoplasmic lifecycles and no homologous DNA sequences in plant genomes. It is possible that dsRNA-directed methylation is involved in inhibiting the handful of known plant retroviruses or pararetroviruses during their DNA phases within the nucleus. It is even more likely that the mechanism is primarily for defence against TEs.

### Defence against transposons in plants

DNA methylation may have evolved as an epigenetic means of containing the spread of TEs in host genomes<sup>4,60</sup>. *De novo* DNA methylation was first detected in plants during the inactivation of class II TEs<sup>61</sup> and has been associated with both transcriptional inactivation



**Figure 4** A model for the initiation and operation of PTGS. The hpRNA or dsRNA produced from either an inverted-repeat transgene or a replicating virus is cleaved into ~21-



**Figure 5** Possible ways in which transposons may generate hpRNA or dsRNA. **a.** LTR transposon integrated as an inverted repeat. **b.** LTR transposon integrated in the opposite polarity into another copy of the same transposon. **c.** TIR transposon adjacent to an endogenous promoter. **d.** TIR transposon (Mu). **e.** LTR transposon adjacent to an endogenous promoter. Red blocks represent either the inverted or direct terminal-repeat sequences. Green blocks represent the coding regions of the TEs. Dark blue arrows below represent the transcripts generating dsRNAs or hpRNAs, and the drawing below each transcript represents the structure it may form (that is, either a hairpin or a dsRNA structure). Blue box represents the transcription terminator sequence and light blue arrows represent readthrough transcription. Large green arrows represent endogenous promoters.

and increased transitional mutation<sup>62-64</sup>. Many studies have shown the involvement of methylation with transposon inactivation<sup>61,65-67</sup> and demethylation with transposon activation<sup>68,69</sup>. A recent demonstration of this comes from the study of the retrotransposon Tto1 in *Arabidopsis*<sup>70</sup>. As this TE becomes transcriptionally silenced, it also becomes increasingly methylated, and demethylation of the element, in a hypomethylation mutant *ddm1* background, reactivates its transcription. However, it is still not entirely clear whether the methylation itself inactivates the transposon or whether it is a secondary effect of inactivation caused by a change in chromatin structure.

Epigenetic inactivation of TEs occurs in many, possibly most, cases by its insertion into or near an already heterochromatic block of genomic DNA and the radiation of this repressed state into the elements<sup>3</sup>. But TEs integrating into euchromatic areas may well be the target for dsRNA-induced silencing. There are a number of scenarios (Fig. 5) of how TEs could produce dsRNA or hpRNA to trigger this mechanism. The LTRs of class I TEs contain promoter sequences, so two TE copies integrating as an inverted repeat could produce hpRNA transcripts of these sequences. TEs often integrate within each other, potentially generating transcribable, complex inverted-repeat sequences. Some transposons, such as Robertson's mutator (Mu), have convergently arranged genes which produce transcripts that, by failing to terminate or be polyadenylated at the appropriate sequences, have regions of complementarity with each other<sup>71</sup>. Insertion of a class II TE adjacent to an endogenous promoter directing transcription across the elements could produce RNA with self-complementarity from the TIRs. An adjacent endogenous

retrotransposon RNAs produces intermediates in the cytoplasm similar to replicating RNA viruses which, although RNA/DNA hybrids rather than RNA/RNA hybrids, act as triggers for dsRNA-induced silencing. Indeed, normal infection of plants with cauliflower mosaic pararetrovirus, which will produce a similar RNA/DNA intermediate, triggers a PTGS-like response<sup>72</sup>. But the most convincing evidence that the dsRNA-induced silencing mechanism is suppressing TEs is that such silenced elements are reactivated in a number of PTGS- and RNAi-defective mutants (Table 1), and that some of the ~21-nucleotide dsRNAs from RNAi and PTGS extracts contain sequences of TEs (ref. 40, and A. J. Hamilton and D. C. Baulcombe, personal communication). The TEs are probably controlled by methylation of their DNA and degradation of their transposase mRNA.

### PTGS can spread systemically through a plant

PTGS has three phases: initiation, maintenance and, remarkably, spread<sup>16,73</sup>. Transgenes and viruses can initiate PTGS, as can exogenous DNA delivered by bombardment or *Agrobacterium* infiltration<sup>16</sup>, and grafting of unsilenced scions onto silenced rootstock<sup>73</sup>. These last methods give localized delivery points for PTGS that spreads from these points into other tissues. It seems to spread by a non-metabolic, gene-specific diffusible signal that is capable of travelling both between cells through plasmodesmata, and long distances via the phloem<sup>16,73</sup>. For example, new tissue growing from a GUS-expressing scion, grafted onto a GUS-silenced rootstock, shows progressive silencing of its GUS transgene<sup>73</sup>. The signal seems to be sequence specific, to move uni-directionally from source to sink tissue, and can traverse at least 30 cm of wild-type stem grafted between the GUS-expressing scion and GUS-silenced rootstock<sup>73</sup>.

To account for the specificity of the signal, it must consist (at least in part) of the transgene product, probably in the form of RNA<sup>73</sup>. The concept of cell-to-cell and long-distance spread of endogenous RNAs within plants remains somewhat controversial, but is not unprecedented. For instance, plant viruses have genomes composed of RNA and, when they infect their host, their RNA spreads throughout the plant. Viral-encoded movement proteins facilitate the movement of viral RNA between cells through plasmodesmata in the form of either a ribonucleoprotein complex or intact virions. To fulfil this role, movement proteins have the capacity to move between cells, bind viral RNA and dilate the size exclusion limit of plasmodesmata<sup>74</sup>. Simpler still, viroids — plant pathogens with small (~350 nucleotide) naked RNA genomes encoding no proteins — also infect and spread through plants, presumably associated with host proteins<sup>75</sup>.

There is an emerging picture of RNA mobility in plants that potentially impacts on other plant processes, including transport of the gene-specific silencing signal. Examples of host RNAs moving from cell to cell include the KNOTTED1 transcription factor and its corresponding mRNA<sup>76</sup>, and the transcript that encodes sucrose transporter 1, which has been localized to the enucleate sieve elements, presumably having been transported there from the associated companion cell<sup>77</sup>. Perhaps the most convincing demonstration of intercellular movement of endogenous plant RNA, and potentially signalling, is the demonstration that mRNA is found in the phloem of rice and cucurbits<sup>78,79</sup>. Mobility of pumpkin phloem RNA was demonstrated using grafting experiments. In one instance, a transcript encoding a transcription factor, NACP, was detected in the meristem of cucumber scions that had been heterografted onto a pumpkin rootstock<sup>78</sup>. Thus RNA molecules, derived from the silenced transgene, might move from cells where this gene is silenced, possibly with cellular protein factors fulfilling a role similar to viral movement proteins, to induce silencing in other cells expressing the

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