

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.

Biology 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 viruses¹, 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 genomes. However, over 90% of plant viruses have ssRNA genomes that are replicated by a virus-encoded RNA-dependent RNA polymerase (RDRP). Plants defend

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 genome⁴. 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 bacteria⁶, it was thought that hybridization of antisense RNA to the target messenger RNA interfered with its transport or translation⁷. 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^{8–10}. It was similarly perplexing when plants transformed with virus-derived transgenes, designed to provide protection through a protein-mediated mechanism¹¹, gave protection against viruses even when little or no transgene protein (transprotein) was produced¹² (Fig. 1).

Further analysis of the co-suppressed and virus-resistant transprotein-free plants revealed that in both cases

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^{12,13} 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 β -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 genes¹⁴.

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^{12,13}. 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^{14–17}. 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 RNA¹⁸. 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^{18–20}. 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 silencing¹⁹, and analysis of similar loci detected the presence of hpRNAs transcribed from them²¹.

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 infection²². 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 β ²³ suggests that the plus and minus strands of a ssRNA virus form full-length dsRNA only as an artefact of extraction²⁴. However, when the mammalian 2',5'-oligoadenylate system (which is activated specifically by dsRNA) was transformed into plants, it was activated by infection with either of the two ssRNA plant viruses tested²⁵. Therefore, replication of a ssRNA plant virus can produce sufficient stretches of dsRNA to be recognized as such within the plant. This is

consistent with the phenomenon of VIGS; here, plant viruses that contain sequences homologous to nuclear-expressed genes act to induce silencing of the targeted genes²⁶.

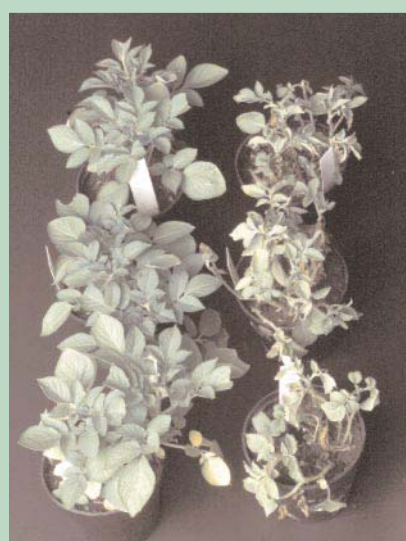
It therefore seems likely that one of the roles of the dsRNA-induced RNA degradation system of plants is to protect them against virus infection. This is a way to generically detect the replication of an invading ssRNA virus and destroy it, by specifically degrading both replicating and translatable 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^{14,27,28}. 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^{29–33}. 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*^{34,35}.

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^{36–40}. 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^{39,40}. 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 termini⁴⁰. 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 virus¹⁸.



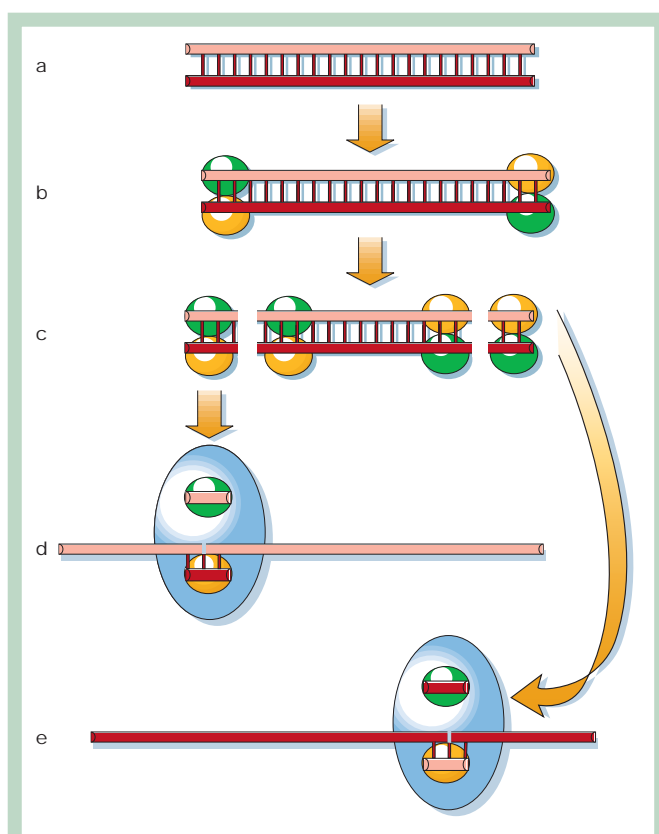


Figure 2 Proposed mechanism, based on RNAi, for dsRNA-directed ssRNA cleavage in PTGS. Introduced dsRNA (a) attracts Dicer-1-like proteins to its termini (b). The heterodimer complex at each end cleaves a 21-nucleotide dsRNA fragment (c), and the exposed ends of the shortened dsRNA each attract a new 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 complex³⁹. 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 turns³⁶) 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 nucleus²⁷. 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^{15,41}. 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 plasmodesmata, scanning the RNAs that pass through. This would allow mRNAs to be efficiently screened and destroyed, if recognized, as they exit the nucleus, thus leading to gene silencing. Viral RNAs

Table 1 Genetic components of post-transcriptional silencing pathways

Gene function	Plants*	Worms*	Flies*	Fungi*	Algae*	TE act.†	PAZ‡
RDRP	SGS2 SDE1§	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 MUT2				Yes	
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.*⁵⁴.

§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 initiation⁴², blocks silencing^{32,43–48}. 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*^{49–53}. 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^{39,54} (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.

The role of methylation and chromatin remodelling in PTGS

DNA methylation and chromatin structure have an integral role in TGS. In this form of silencing, the promoter and sometimes the

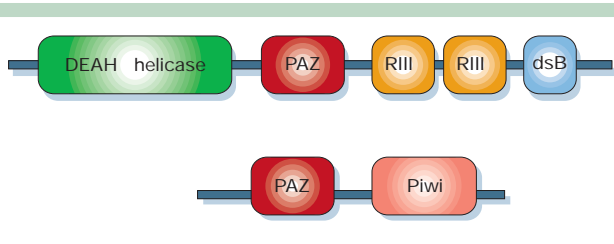


Figure 3 Distribution of domains on DCR1-like and AGO1-like proteins. Top panel illustrates the domains on DCR1-like proteins of ~2,000 amino acids (including DCR1, CAF1 and K12H4.8); bottom panel represents AGO1-like proteins of ~900 amino acids (including AGO1, RDE1 and QDE2). RIII, RNase III domain; 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 methylated⁵⁵. Methylation, or methylation-associated chromatin remodelling, of promoter sequences is thought to prevent binding of factors necessary for transcription⁵⁵. 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 plant⁵⁶. 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 methylation³⁰. Similarly, virus-replicated RNAs direct sequence-specific DNA methylation^{57,58} and are associated with short dsRNAs⁵⁸. 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/altering 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^{58,59} 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^{4,60}. *De novo* DNA methylation was first detected in plants during the inactivation of class II TEs⁶¹ and has been associated with both transcriptional inactivation

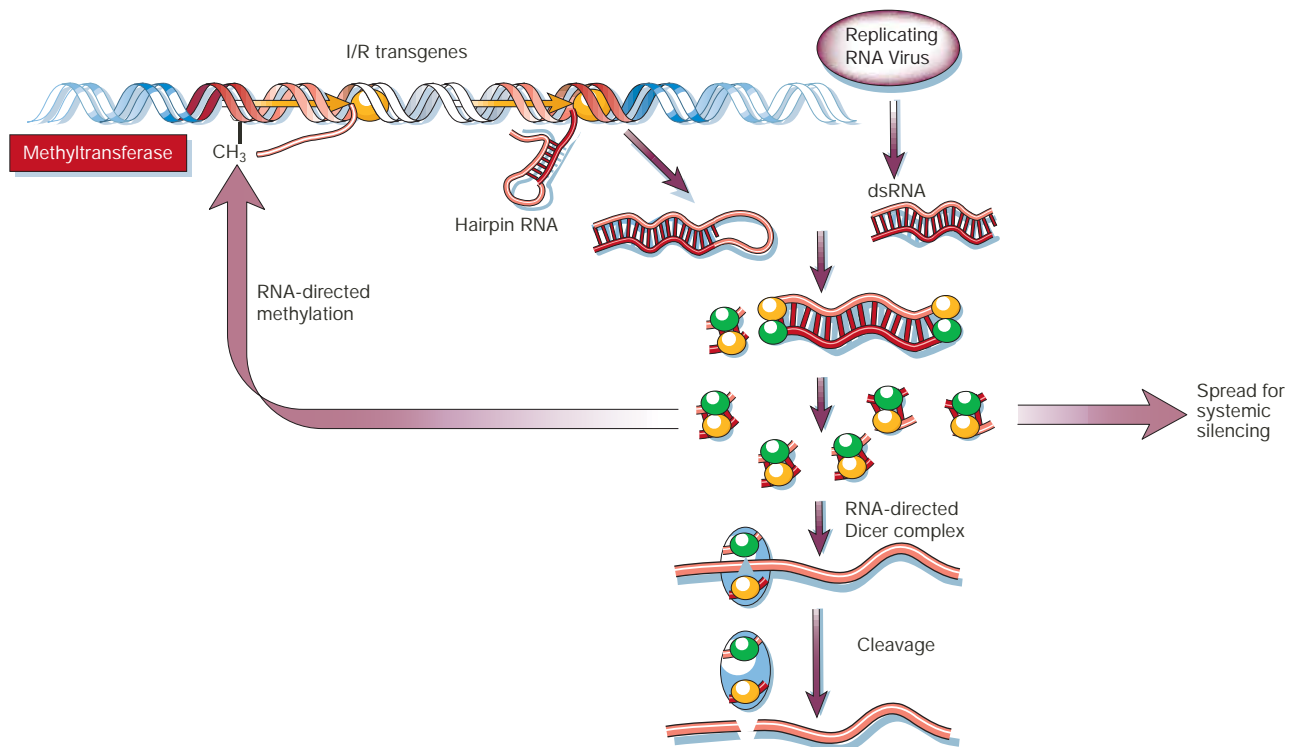


Figure 4 A model for the initiation and operation of PTGS. The hpRNA or dsRNA produced from either an inverted-repeat transgene or a replicating virus is cleaved into ~21-nucleotide fragments by the Dicer-containing complex and used as guides for cleavage of homologous ssRNA (described in more detail in Fig. 2). The ~21-nucleotide dsRNA, in some sort of complex, is transported into the nucleus to direct DNA methylation of homologous sequences or into neighbouring cells to act as a mobile PTGS signal.

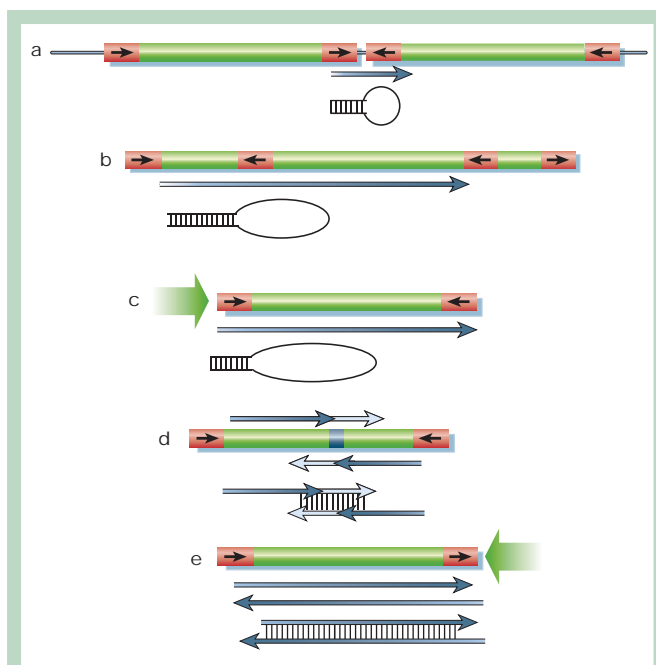


Figure 5 Possible ways in which transposons may generate hpRNA or dsRNA. **a**, LTR transposon integrated as an inverted repeat. **b**, LTR transposon integrated in the opposite polarity into another copy of the same transposon. **c**, TIR transposon adjacent to an endogenous promoter. **d**, TIR transposon (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^{62–64}. Many studies have shown the involvement of methylation with transposon inactivation^{61,65–67} and demethylation with transposon activation^{68,69}. A recent demonstration of this comes from the study of the retrotransposon Tto1 in *Arabidopsis*⁷⁰. 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 elements³. 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 other⁷¹. 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 promoter directing transcription from the reverse end of a TE could produce antisense RNA that might hybridize with the TE RNA to produce dsRNA. It is also possible that the reverse transcription of

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 response⁷². 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.

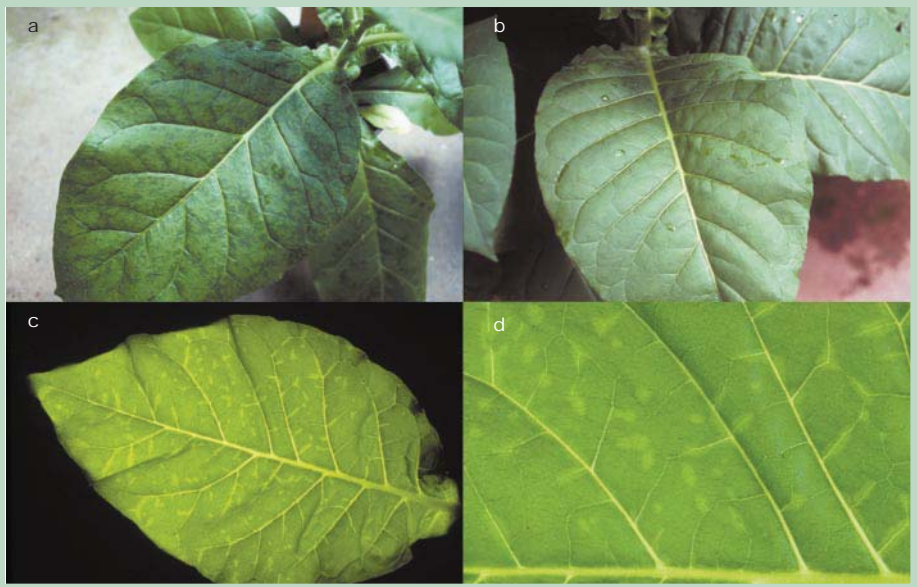
PTGS can spread systemically through a plant

PTGS has three phases: initiation, maintenance and, remarkably, spread^{16,73}. Transgenes and viruses can initiate PTGS, as can exogenous DNA delivered by bombardment or *Agrobacterium* infiltration¹⁶, and grafting of unsilenced scions onto silenced rootstock⁷³. 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^{16,73}. For example, new tissue growing from a GUS-expressing scion, grafted onto a GUS-silenced rootstock, shows progressive silencing of its GUS transgene⁷³. 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 rootstock⁷³.

To account for the specificity of the signal, it must consist (at least in part) of the transgene product, probably in the form of RNA⁷³. 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 plasmodesmata⁷⁴. 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 proteins⁷⁵.

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 mRNA⁷⁶, 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 cell⁷⁷. 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^{78,79}. 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 rootstock⁷⁸. 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 same transgene. This raises three questions — is the signal the ~21-nucleotide dsRNA, how is the signal propagated, and what is the natural (non-transgenic) role of the signal?

Figure 6 Tobacco plants showing potato virus Y (PVY) susceptibility, immunity and resistant/recovery symptoms. **a**, Non-transgenic tobacco plant challenged with PVY, showing a uniform chlorotic mosaic on leaves throughout the plant. **b**, Virus-challenged transgenic plant containing a transgene that expresses a hpRNA derived from PVY sequences, showing immunity to the virus and no symptoms. **c**, A whole leaf, and **d**, close-up, from a PVY-challenged transgenic plant showing resistance/recovery symptoms. As the plant grows the leaves show fewer and fewer yellow patches; only the yellow patches contain detectable levels of virus. The pattern suggests that as the virus attempts to spread from the phloem into the surrounding cells, a signal is emitted that allows more distal cells to be forewarned and resist and restrict the virus.



Defence is a two-step process

Most plant species are immune to most plant viruses, possibly due to compatibility requirements between the RDRP or movement protein of a specific virus and the host factors present in the plant. However, it is also possible that the only virus/host combinations leading to an infection are ones in which the virus can overcome or avoid the plant's PTGS defence mechanism. For example, potyviruses encode a protein, HC-Pro, that potentially disables PTGS^{31,80,81} by directly or indirectly preventing the dsRNA cleaving activity of the Dicer complex. Therefore, a plant challenged with a potyvirus becomes a battleground in the fight between a defence and a counter-defence strategy. It is a race between the cell's recognition and degradation of viral RNA, and the virus expressing HC-Pro to inactivate the degradation machinery. Perhaps the mobile signal, seen in the artificial situation of transgenes and grafting experiments, is a reflection of a plant's fallback strategy. If the first-challenged cells are not quick enough to recognize and destroy the virus, but can send a warning message to uninfected cells of their imminent invasion, these cells can have their degradation machinery prepared. If the signal contains fragments of the virus sequence, the recipient cells can be ready to degrade RNAs containing these sequences before the virus arrives (Fig. 6). This model might explain a number of observations: (1) plants transformed with virus-derived transgenes can show a recovery response (that is, initial virus infection and symptoms followed by new growth that is both without symptoms and resistant to virus infection); (2) infection of non-transgenic plants with nepo-, tobra- and caulimoviruses can induce responses very much like the recovery phenotype^{72,82,83}; and (3) plants showing co-suppression tend to initially show activity of the target gene, but this becomes progressively more silenced in developing tissue. All of these progressive silencing phenotypes could be the result of cells that induce PTGS too slowly or poorly to significantly degrade the virus or transgene RNA, but produce a signal that is amplified in recipient cells such that they can perform effective silencing.

Viruses are capable of rapid evolution to overcome the plant's host defences, even their fallback plans, wherever they can. So it is of little surprise that at least two genera of plant viruses (potex- and cucumoviruses) overcome their host's PTGS mechanism by producing proteins that seem to prevent the spread of the signal^{84–86}.

What is the signal?

Initial impressions might suggest that the ~21-nucleotide dsRNAs produced by Dicer not only direct degradation, but are also the signal (Fig. 4) that moves into the nucleus to direct DNA methylation and

that moves from cell to cell to be amplified and direct RNA degradation and DNA methylation there. But there is strong evidence from grafting experiments, which exploit HC-Pro, that these short dsRNAs are not the signal⁸⁷. As discussed above, the expression of HC-Pro restores activity to transgenes previously showing PTGS and prevents the production of short dsRNAs. In addition, the transgene in a GUS-expressing scion grafted onto a GUS-silenced rootstock becomes silenced. However, when a GUS-expressing scion is grafted onto a rootstock in which a once-silenced GUS transgene has been reactivated by HC-Pro, GUS activity in the scion is silenced. In this grafted plant, short RNAs are detected in the scion, but not in the rootstock. This indicates that short RNAs are not the mobile signal, as they were not detected in the rootstock of the graft, yet the rootstock transmitted a silencing signal to the scion. In addition, once-silenced GUS transgenes, which have been reactivated by HC-Pro (with the concomitant absence of short RNAs), still develop PTGS-like methylation. This casts doubt on the idea that short RNAs act as guides for methylation, although other workers have found that inhibition of silencing and short RNA production by HC-Pro reduced the level of target gene methylation⁸¹.

HC-Pro probably interacts in the PTGS pathway upstream of the production of short RNAs^{31,87}, but downstream of the production of the signal. So what might the signal molecule be, if not ~21-nucleotide dsRNA with or without Dicer-1? It might be a modified product from the methylated target gene⁸⁷ or dsRNA itself. Perhaps dsRNA is recognized in a cell by both the Dicer complex and by another complex containing RNA-movement proteins (Fig. 7). This latter complex facilitates the passage of the dsRNA through nuclear pores and plasmodesmata. In the nucleus, the dsRNA is unwound and used to direct methylation. The dsRNA entering cells, in which the PTGS mechanism is not yet activated (such as in a graft situation), attracts degradation complexes which then produce short RNAs. These act as primers on target mRNAs for a host RDRP, for example, SGS2 (perhaps in association with AGO1, and the helicase SDE3), to produce complementary strands of RNA, thus making dsRNA. The dsRNA is then the substrate for both the movement and PTGS-degradation complexes, thereby re-amplifying the mobile signal and propagating PTGS. This scenario would make the plant's RNA-movement protein and RDRP (and associated factors, including an RNA helicase) essential for amplifying weak signals into effective silencing. Evidence supporting this primer model is that the signal seems to amplify from the target mRNA. GFP-expressing plants silenced by bombardment with DNA encoding one-third of the GFP sequence are resistant to a virus containing the other two-thirds of

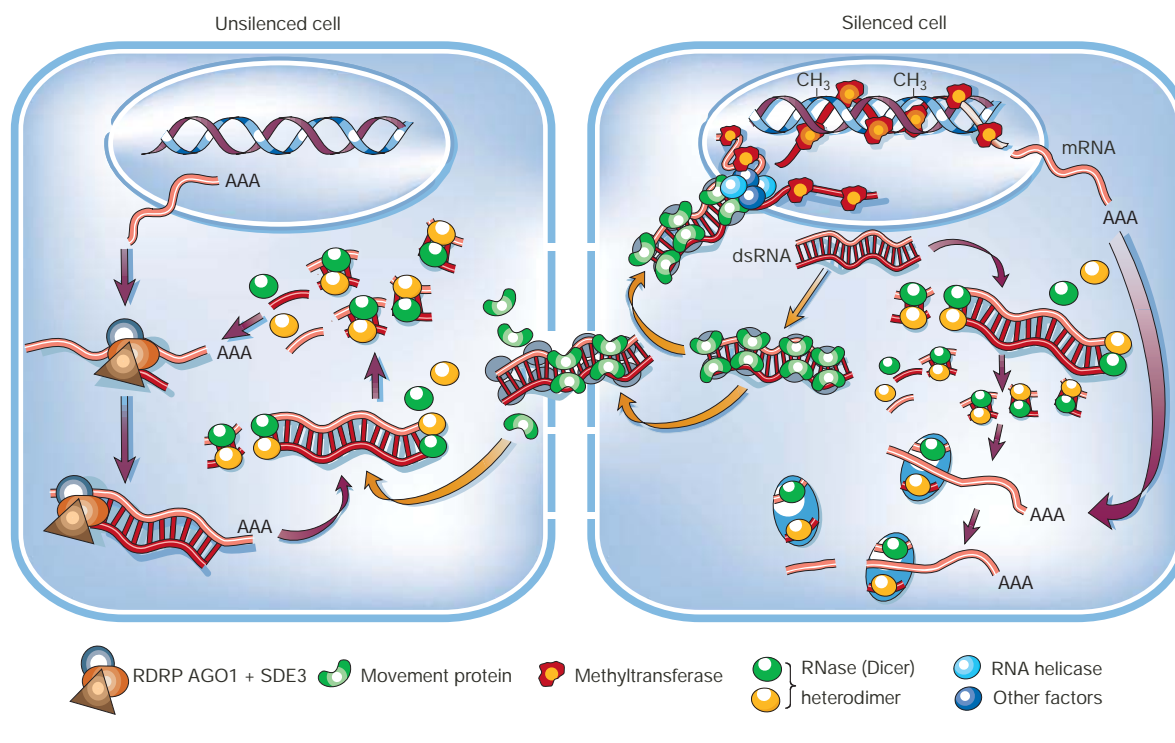


Figure 7 A model for mobile PTGS and methylation signals. The dsRNA-induced cleavage of ssRNA is described in Figs 2 and 4. However, an extra dsRNA-specific movement-protein complex may be required for the spread and amplification of the PTGS signal. This complex transports the dsRNA into the nucleus via the nuclear pore, and into adjacent cells (and ultimately the phloem) by means of the plasmodesmata. In the nucleus, the dsRNA is unwound by a helicase and directs methylation of homologous DNA sequences. In situations such as the grafting of an unsilenced scion onto a silenced rootstock, the dsRNA complex entering the unsilenced cell of the scion is recognized and degraded, releasing some dissociated short RNAs. These RNAs act as primers on a target mRNA for a host RDRP (for example, SGS2) with a helicase (for example, SDE3) and initiating factors (for example, AGO1) to synthesize complementary RNA. This generates dsRNA that is recognized by the components of the movement and degradation complexes, thus initiating degradation of homologous ssRNA and amplifying the mobile signal.

the GFP gene sequence¹⁶. Because there is no overlap between these GFP-derived sequences, it seems that the PTGS targeted against the GFP-containing virus was mediated through the mRNA (or DNA) of the intact GFP transgene in the plant.

The picture so far

As some of the parts of this jigsaw puzzle come together, a picture begins to emerge. A number of the pieces seem to fit together perfectly, some not so perfectly, and many pieces are still missing. The pieces describing the mechanism of dsRNA induction and directed ssRNA degradation fit together well. Those describing the directed methylation and signal amplification fit into the picture but have quite a few adjacent pieces missing. SGS3 and RDE4 are like pieces whose colour and pattern tell us they are part of the puzzle, but as yet they remain unconnected. However, the overall picture is of an ingenious defence mechanism in plants against viruses and TEs. That PTGS is a manifestation of an anti-virus defence mechanism seems beyond doubt from the discovery that viruses encode proteins to specifically disable the system^{81,84–86}. There is considerable evidence that RNAi in nematodes is a defence mechanism against TEs. Out of 30 mutants that activate transposons in nematodes, 22 also cause defects in RNAi⁴⁹. It seems highly likely that homologous genes have the same role in plants.

This plant defence mechanism against viruses and TEs has a number of parallels with the immune system of mammals. The immune system detects foreign bodies and generates agents (antibodies) to specifically recognize them and guide destruction machinery (phagocytes and killer cells) against them. The plant's defence system generates agents (short dsRNAs) with the specificity to recognize sequences in the invading viral RNA genomes and guide destruction

complexes (nucleases) to them. Both systems work systemically, viruses have responded to both systems by evolving ways to circumvent them, and both systems can be enhanced by prior vaccination with virus components.

This plant defence system is an elegant example of how nature can find highly efficient solutions to the problems it faces. Just as translation machinery needs codons of only 3 nucleotides to specify which amino acid goes where in a protein, this defence system requires degradation recognition sequences of only ~21 nucleotides to direct it. With each 21-nucleotide guide sequence, the probability of targeting an inappropriate 1-kilobase mRNA is about 1 in 10¹⁰. The minimum length of dsRNA needed to induce the system is probably ~21 nucleotides, which must place evolutionary constraints on the self-complementarity of mRNAs. Indeed, codon redundancy may be exploited in mRNAs to avoid perfect self-complementarity regions of greater than 20 nucleotides. In addition, plants might use self-complementary motifs to give added instability features to a mRNA. It is possible that chalcone synthase mRNA in plants, which has regions of significant self-complementarity, might exploit this mechanism to generate stochastic pigmented patterns in petals to attract insects (and plant breeders). The PTGS mechanism may be involved in controlling plant development. AGO1, and possibly CAF1, is needed for PTGS; mutants in either of these genes cause developmental abnormalities^{48,88}. It is possible that PTGS controls development by regulating the abundance or transport of endogenous RNAs in plants. Interestingly, a plant host protein, rgs-CaM, has been found which, when overexpressed, disables PTGS in a similar manner to HC-Pro⁸⁹. Perhaps the cell uses this protein to temporarily inactivate PTGS at appropriate times in the cell cycle or in developmental states when PTGS could be detrimental.

We are beginning to understand that phenotypes such as co-suppression and virus-derived transgene-mediated resistance are manifestations of a plant defence mechanism. We also know some design rules²⁰ of how to exploit the mechanism to generate plants with traits for commercial agriculture (for example, virus protection⁹⁰ and modification of quality traits by inhibiting steps of metabolic pathways) and for gene discovery²⁶ (for example, specifically silencing genes of unknown function in the *Arabidopsis* genome database). But many aspects remain to be elucidated and there are some seemingly contradictory results to be reconciled. It is also intriguing that although directed RNA degradation and DNA methylation are aspects of the plant's defence mechanism, these processes may also be important in whole-plant development and homeostasis mediated by RNA-based intercellular communication. □

1. Mayo, M. A. Developments in plant virus taxonomy since the publication of the 6th ICTV report. *Arch. Virol.* **144**, 1659–1666 (1999).
2. Bennetzen, J. L. Transposable element contributions to plant gene and genome evolution. *Plant Mol. Biol.* **42**, 251–269 (2000).
3. Kumar, A. & Bennetzen, J. L. Plant retrotransposons. *Annu. Rev. Genet.* **33**, 479–452 (1999).
4. Martienssen, R. Transposons, DNA methylation and gene control. *Trends Genet.* **14**, 263–264 (1998).
5. Rothstein, S. J., Dimaio, J., Strand, M. & Rice, D. Stable and inheritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proc. Natl Acad. Sci. USA* **84**, 8439–8443 (1987).
6. Simons, R. W. & Kleckner, N. Translational control of IS10 transposition. *Cell* **34**, 683–691 (1983).
7. Hamilton, A. J., Lycett, G. W. & Grierson, D. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* **346**, 284–287 (1990).
8. Napoli, C., Lemieux, C. & Jorgensen, R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **7**, 599–609 (1990).
9. Van der Krol, A. R., Mur, L. A., Beld, K., Mol, J. N. M. & Stuitje, A. R. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to suppression of gene expression. *Plant Cell* **2**, 291–299 (1990).
10. De Carvalho, F. *et al.* Suppression of the β -1,3 glucanase transgene expression in homozygous plants. *EMBO J.* **11**, 2595–2602 (1992).
11. Powell-Abel, P. *et al.* Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738–743 (1986).
12. Lindbo, J. A. & Dougherty, W. G. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology* **189**, 725–733 (1992).
13. Dougherty, W. G. & Parks, T. D. Transgenes and gene suppression: telling us something new? *Curr. Opin. Cell Biol.* **7**, 399–405 (1995).
14. English, J. J., Mueller, E. & Baulcombe, D. C. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 787–797 (1996).
15. Tanzer, M. M., Thompson, W. F., Law, M. D., Wernsman, E. A. & Ukenes, S. Characterization of post-transcriptionally suppressed transgene expression that confers resistance to tobacco etch virus infection in tobacco. *Plant Cell* **9**, 1411–1423 (1997).
16. Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177–187 (1998).
17. Wassenegger, M. & Pelissier, T. A model for RNA-mediated gene silencing in higher plants. *Plant Mol. Biol.* **37**, 349–362 (1998).
18. Waterhouse, P. M., Graham, M. W. & Wang, M.-B. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA* **95**, 13959–13964 (1998).
19. Wang, M.-B. & Waterhouse, P. M. High efficiency silencing of a β -glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. *Plant Mol. Biol.* **43**, 67–82 (2000).
20. Smith, N. A. *et al.* Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319–320 (2000).
21. Stam, M. *et al.* Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat loci. *Plant J.* **21**, 27–42 (2000).
22. Morris, T. J. & Dodds, J. A. Isolation and analysis of double stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* **69**, 854–858 (1979).
23. Blumenthal, T. & Carmichael, G. G. RNA replication: function and structure of Q β replicase. *Annu. Rev. Biochem.* **48**, 525–548 (1979).
24. Buck, K. W. Replication of tobacco mosaic virus RNA. *Phil. Trans. R. Soc. Lond. B* **354**, 613–627 (1999).
25. Ogawa, T., Hori, T. & Ishida, I. Virus-induced death in plants expressing the mammalian 2',5' oligoadenylate system. *Nature Biotechnol.* **14**, 1566–1569 (1996).
26. Baulcombe, D. C. Fast forward genetics based in virus-induced gene silencing. *Curr. Opin. Plant Biol.* **2**, 109–113 (1999).
27. Lee, K. Y., Baden, C., Howie, W. J., Bedbrook, J. & Dunsmuir, P. Post-transcriptional gene silencing of ACC synthase in tomato results from cytoplasmic RNA degradation. *Plant J.* **12**, 1127–1137 (1997).
28. Metzlaiff, M., O'Dell, M., Cluster, P. D. & Flavell, R. B. RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. *Cell* **88**, 845–854 (1997).
29. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
30. Mette, M. F., Aufatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
31. Llave, C., Kasschau, K. D. & Carrington, J. C. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc. Natl Acad. Sci. USA* **97**, 13401–13406 (2000).
32. Dalmay, T., Hamilton, A., Rudd, S., Angell, S. & Baulcombe, D. C. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for post-transcriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**, 543–553 (2000).
33. Hutvagner, G., Mlynarova, L. & Nap, J. P. Detailed characterization of the posttranscriptional gene-silencing-related small RNA in a GUS gene-silenced tobacco. *RNA* **6**, 1445–1454 (2000).
34. Fire, A. *et al.* Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
35. Kennerdel, J. R. & Carthew, R. W. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**, 1017–1026 (1998).
36. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
37. Yang, D., Lu, H. & Erickson, J. W. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.* **10**, 1191–1200 (2000).
38. Hammond, S. C., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
39. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
40. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200 (2001).
41. Holtorf, H., Schob, H., Kunz, C., Waldvogel, R. & Meins, F. Stochastic and nonstochastic post-transcriptional silencing of chitinase and β -1,3-glucanase genes involves increased RNA turnover—possible role for ribosome-independent RNA degradation. *Plant Cell* **11**, 471–483 (1999).
42. Zou, C., Zhang, Z., Wu, S. & Osterman, J. C. Molecular cloning and characterization of a rabbit eIF2C protein. *Gene* **211**, 187–194 (1998).
43. Cogoni, C. & Macino, G. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**, 166–169 (1999).
44. Mourrain, P. *et al.* *Arabidopsis* *SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542 (2000).
45. Smardon, A. *et al.* EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169–178 (2000).
46. Catalanotto, C., Azzalin, G., Macino, G. & Cogoni, C. Gene silencing in worms and fungi. *Nature* **404**, 245 (2000).
47. Tabara, H. *et al.* The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132 (1999).
48. Fagard, M., Boutet, S., Morel, J.-B., Bellini, C. & Vaucheret, H. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi and RNA interference in animals. *Proc. Natl Acad. Sci. USA* **97**, 11650–11654 (2000).
49. Ketting, R. F., Haverkamp, T. H. A., van Luenen, H. G. A. & Plasterk, R. H. A. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner Syndrome helicase and RNase D. *Cell* **99**, 133–141 (1999).
50. Domeier, M. E. *et al.* A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. *Science* **289**, 1928–1930 (2000).
51. Cogoni, C. & Macino, G. Post-transcriptional gene silencing in *Neurospora* by a ReqQ DNA helicase. *Science* **286**, 342–344 (1999).
52. Wu, Scarf, D., Jeong, B.-R., Zhang, C. & Cerutti, H. Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. *Science* **290**, 1159–1162 (2000).
53. Dalmay, T., Horsfield, R., Braunstein, T. H. & Baulcombe, D. C. SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO J.* **20**, 2069–2077 (2001).
54. Cerruti, L., Mian, N. & Bateman, A. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem. Sci.* **25**, 481–482 (2000).
55. Kooter, J. M., Matzke, M. A. & Meyer, P. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* **4**, 340–347 (1999).
56. Morel, J.-B., Mourrain, P., Berlin, C. & Vaucheret, H. DNA methylation and chromatin structure mutants affect both post-transcriptional and transcriptional transgene silencing in *Arabidopsis*. *Curr. Biol.* **10**, 1591–1594 (2001).
57. Jones, A. L. *et al.* *De novo* methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO J.* **17**, 6385–6393 (1998).
58. Wang, M.-B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. Replicating satellite RNA induces sequence specific DNA methylation and truncated transcripts in plants. *RNA* **7**, 16–28 (2001).
59. Van Houdt, H., Ingelbrecht, L., van Montagu, M. & Depicker, A. Post-transcriptional silencing of a neomycin phosphotransferase II transgene correlates with the accumulation of unproductive RNAs and with increased cytosine methylation of 3' flanking regions. *Plant J.* **12**, 379–392 (1997).
60. Yoder, J. A., Walsh, C. P. & Bestor, T. H. Cytosine methylation and ecology of intragenomic parasites. *Trends Genet.* **13**, 335–340 (1997).
61. Brutnell, T. P. & Dellaporta, S. L. Somatic inactivation and reactivation of Ac associated changes in cytosine methylation and transposase expression. *Genetics* **138**, 213–225 (1994).
62. San Miguel, P. *et al.* Nested transposons in the intergenic regions of the maize genome. *Science* **257**, 765–768 (1996).
63. Bennetzen, J. L. The contributions of retroelements to plant genome organisation, function and evolution. *Trends Microbiol.* **4**, 347–353 (1993).
64. Kunze, R., Sædler, H. & Lonnig, W. E. Plant transposable elements. *Adv. Bot. Res.* **27**, 331–470 (1997).
65. Bennetzen, J. L. The Mutator transposable element system of maize. *Curr. Top. Microbiol. Immunol.* **204**, 195–229 (1996).
66. Schlappi, M., Raina, R. & Fedoroff, N. Epigenetic regulation of the maize Spm transposable element: novel activation of a methylated promoter by TnpA. *Cell* **77**, 427–437 (1994).
67. Liu, W. M., Maraia, R. J., Rubin, C. M. & Schmid, C. W. Alu transcripts: cytoplasmic localisation and regulation by DNA methylation. *Nucleic Acids Res.* **22**, 1087–1095 (1994).
68. Singer, T., Yordan, C. & Martienssen, R. A. Robertson's Mutator transposons in *A. thaliana* are regulated by the chromatin-remodeling gene Decrease in DNA Methylation (DDM1). *Genes Dev.* **15**, 591–602 (2001).
69. Miura, A. *et al.* Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. *Nature* **411**, 212–214 (2001).
70. Hirochika, H., Okamoto, H. & Kakutani, T. Silencing of retrotransposons in *Arabidopsis* and reactivation by the ddm1 mutation. *Plant Cell* **12**, 357–368 (2000).
71. Hershberger, R. J. Characterization of the major transcripts encoded by the regulatory MuDR transposable element of maize. *Genetics* **140**, 1087–1098 (1995).

72. Covey, S. N., Al-Kaff, N. S., Langara, A. & Turner, D. S. Plants combat infection by gene silencing. *Nature* **385**, 781–782 (1997).
73. Palauqui, J. C., Elmayan, T., Pollien, J. M. & Vaucheret, H. Systemic acquired silencing: transgene specific post transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* **16**, 4738–4745 (1997).
74. Lucas, W. J. & Gilbertson, R. L. Plasmodesmata in relation to viral movement within leaf tissues. *Annu. Rev. Phytopathol.* **32**, 387–411 (1994).
75. Ding, B., Kwon, M. O., Hammond, R. & Owens, R. Cell to cell movement of potato spindle tuber viroid. *Plant J.* **12**, 931–936 (1997).
76. Lucas, W. J. *et al.* Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* **270**, 1980–1983 (1995).
77. Kuhn, C., Franceschi, V. R., Schulz, A., Lemoine, R. & Frommer, W. B. Macromolecular trafficking indicated by localisation and turnover of sucrose in enucleate sieve elements. *Science* **275**, 1298–1300 (1997).
78. Ruiz-Medrano, R., Xocosnostle-Cazares, B. & Lucas, W. J. Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* **126**, 4405–4419 (1999).
79. Sasaki, T., Chino, M., Hayashi, H. & Fujiwara, T. Detection of mRNA species in phloem sap. *Plant Cell Physiol.* **39**, 895–897 (1998).
80. Kasschau, K. D. & Carrington J. C. A counter-defensive strategy of plant viruses: suppression of post-transcriptional gene silencing. *Cell* **95**, 461–470 (1998).
81. Marathe, M. *et al.* Plant viral suppressors of post-transcriptional silencing do not suppress transcriptional silencing. *Plant J.* **22**, 51–59 (2000).
82. Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. A similarity between viral defense and gene silencing in plants. *Science* **276**, 1558–1560 (1997).
83. Ratcliff, F. G., MacFarlane, S. S. & Baulcombe, D. C. Gene silencing without DNA: RNA mediated cross protection between viruses. *Plant Cell* **11**, 1207–1215 (1999).
84. Brigneti, G. *et al.* Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746 (1998).
85. Li, H. W. *et al.* Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *EMBO J.* **18**, 2683–2691 (1999).
86. Voinnet, O., Lederer, C. & Baulcombe, D. C. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167 (2000).
87. Mallory, A. C. *et al.* HC-Pro suppression of gene silencing eliminates the small RNAs but not the transgene methylation or the mobile signal. *Plant Cell* **13**, 571–583 (2001).
88. Jacobsen, S. E., Running, M. P. & Meyerowitz, E. M. Disruption of an RNA helicase/RNaseIII gene in *Arabidopsis* causes unregulated cell division in floral meristems. *Development* **126**, 5231–5243 (1999).
89. Anandakshmi, R. *et al.* A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* **290**, 142–144 (2000).
90. Wang, M.-B., Abbott, D. & Waterhouse, P. M. A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. *Mol. Plant Pathol.* **1**, 347–356 (2000).

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