Role of short RNAs in gene silencing

Peter M. Waterhouse, Ming-Bo Wang and E. Jean Finnegan

Recent research has revealed the existence of an elegant defence mechanism in plants and lower eukaryotes. The mechanism, known in plants as post-transcriptional gene silencing, works through sequence-specific degradation of RNA. It appears to be directed by double-stranded RNA, associated with the production of short 21–25 nt RNAs, and spread through the plant by a diffusible signal. The short RNAs are implicated as the guides for both a nuclease complex that degrades the mRNA and a methyltransferase complex that methylates the DNA of silenced genes. It has also been suggested that these short RNAs might be the mobile silencing signal, a suggestion that has been challenged recently.

Peter M. Waterhouse*
Ming-Bo Wang
E. Jean Finnegan
CSIRO Plant Industry, PO Box 1600 Canberra, ACT 2601, Australia.
*e-mail: peterw@pi.csiro.au

Post-transcriptional gene silencing (PTGS) in plants results from the action of a mechanism that surveys the RNAs in a cell and specifically degrades those it perceives as alien. The alien RNAs can be double-stranded (ds) or single-stranded (ss) molecules that have homology to dsRNA present in the cell. Most plant viruses have RNA genomes that replicate to produce plus and minus sense RNAs, with the potential to form duplexes in the cell, and are therefore both inducers and targets of this defence mechanism. Transgenes encoding ds or self-complementary (hairpin) RNAs of endogenous gene sequences are highly effective at directing the cell's degradation mechanism against endogenous (ss) mRNAs, thus giving efficient, targeted gene suppression[1]. This discovery has enabled the transgenic enhancement of a plant's defence mechanism against viruses that it is unable to combat unaided. It has also shed light on how antisense and co-suppression might operate: by the inadvertent integration of two copies of the transgenes in an inverted repeat orientation, such that read-through transcription from one gene into the adjacent copy produces RNA with self-complementarity[2].

The involvement of short RNAs in PTGS was uncovered when ~25 nt RNAs with sequence homology to a transgene were detected only in plants where the corresponding transgene was silenced[3]. These RNAs were present in the same and complementary senses to the transgene. Further studies have shown that these short RNAs are consistently associated with PTGS in plants[4–6].

The relevance of these short sense and antisense RNAs became apparent when similarly sized dsRNAs were found to be an integral part of RNA interference in *Drosophila*[7], a mechanism with many similarities to PTGS in plants. Target ssRNA is specifically degraded when dsRNA of the same sequence is delivered to the cellular machinery. It appears that the dsRNA is first cleaved by an RNAseIII-like enzyme, termed Dicer[8], into ~22 nt dsRNAs, which then act as guides to nuclease complexes that cleave ssRNA with homologous sequences[9] (Fig. 1a).

Post-transcriptional gene silencing can spread systemically through a plant

A remarkable feature of PTGS is that it is non-cell-autonomous; it can be induced in tissue actively expressing a transgene by a mobile, graft-transmissible signal originating from tissue where the same transgene is silenced[10–12]. For example, new tissue growing from a GUS-expressing scion, grafted onto a rootstock with GUS–PTGS, shows progressive silencing of its GUS transgene[13]. The signal appears to be sequence specific and to move uni-directionally from source to sink tissues. It can traverse at least 30 cm of wild-type stem, grafted between a GUS-expressing scion and a GUS–PTGS rootstock. A signal with similar properties can also spread PTGS from leaves transiently expressing a silencing construct, introduced by *Agrobacterium* infiltration, into other parts of the plant not infiltrated with bacteria[14]. Signals for systemic silencing can be produced by silenced transgenes derived from reporter, endogenous or viral genes[13,15].

To account for the specificity of the signal, it has been suggested that it contains at least a part of the transgene product, probably in the form of RNA[13]. The notion that an endogenous RNA can spread from cell to cell for long distances within a plant is controversial, yet such spread of RNAs within plants is not unprecedented. Most plant viruses have genomes composed of RNA and, when they infect their host, their RNA spreads throughout the plant. This is mediated by virus-encoded movement proteins, but viroids – plant pathogens with small (~350 nt), naked RNA genomes encoding no proteins – also infect and spread though plants, presumably associated with host proteins.

There are examples of host RNAs moving from cell to cell. The KNOTTED transcription factor and its corresponding mRNA have been detected moving from cells where they are synthesized to cells not transcribing the gene[16]. The mRNA of the sucrose transporter, SUT1, is found in enucleate sieve elements presumably having been transported there from the associated companion cells[17]. Perhaps the most convincing demonstration of intracellular movement of endogenous plant RNA is the demonstration that pumpkin NACP mRNA can be detected in the meristems of cucumber scions grafted onto a pumpkin rootstock[18].

Thus, RNA molecules derived from the silenced transgene, might move from cells where this gene is silenced to induce silencing in other cells expressing the same transgene. It seems unlikely that all

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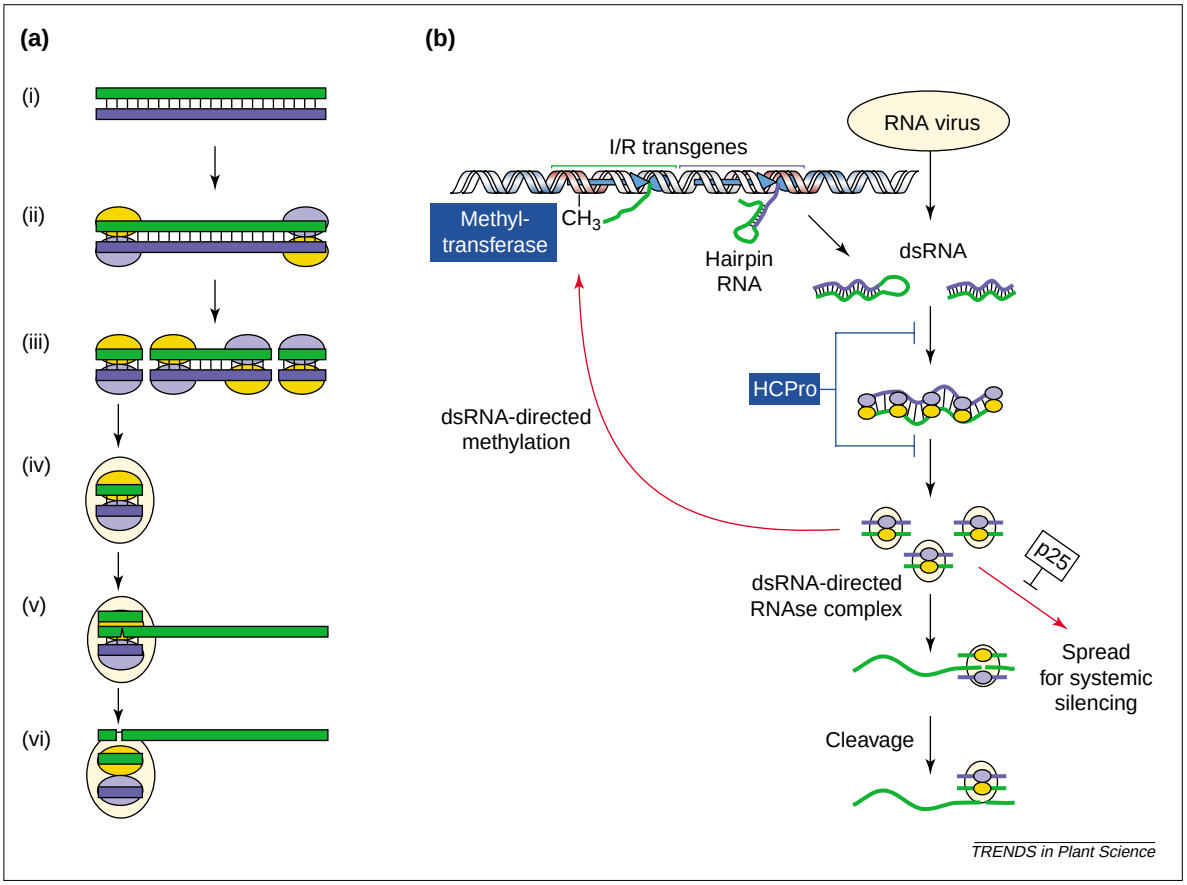

Fig. 1. Proposed mechanism of RNA interference (RNAi) in *Drosophila* (a) and post-translational gene silencing (PTGS) in plants (b). In RNAi, introduced double-stranded RNA (dsRNA) (i) is recognized by proteins (ii) which, starting from the dsRNA termini, cleave it (iii) into ~22 nt fragments to produce nucleoprotein complexes (iv). The strands of the short dsRNA are separated and one strand is used as a guide to recognize single-stranded RNA (ssRNA) with complementary sequences (v). Each complex cleaves the ssRNA at a position approximately in the middle of the guide sequence (v, vi). In PTGS in plants (b), an RNAi-like cleavage mechanism operates. The dsRNA directing the mechanism is introduced by a replicating virus or an inverted repeat transgene that produces self-complementary (hairpin) RNA. The short RNAs from the cleavage (or alternatively un-cleaved dsRNA) enter the nucleus to guide a methyltransferase complex to sequences for methylation and also spread into other cells to direct the cleavage of homologous ssRNAs. The possible points of action of viral silencing suppressors, p25 and HC-Pro, are shown. p25 prevents the spread of the mobile PTGS signal but does not inhibit existing PTGS. HC-Pro is a viral silencing suppressor.

mRNAs are free to roam from cell to cell around the plant, therefore the silencing RNAs must have at least two features: one that enables them to move from cell to cell and one that enables them to mediate the propagation of silencing.

Short RNA molecules direct DNA methylation

The coding region of genes showing PTGS and the promoter regions of genes showing transcriptional gene silencing (TGS) are often methylated. This methylation can be directed by dsRNA from a virus-replicated (satellite) sequence or by hairpin RNA transcribed from a transgene. In both situations, the methylation was associated with the presence of short RNAs (Refs 4,19). It is tempting to speculate that these RNAs are the signals that pass from the cytoplasm into the nucleus to direct methylation of the homologous DNA. Furthermore, the satellite sequence was replicated by a virus that only infects vascular tissue and yet the target DNA sequences became methylated in the majority of plant cells[19]. One explanation for this spread of methylation is that the short RNAs move from the vascular cells into other cells where they pass into the nucleus to direct methylation of homologous sequences.

PTGS and TGS used to be considered as different pathways. However, the discoveries that both PTGS and TGS are induced by dsRNA, are associated with the presence of short RNAs and might be able to spread in a non-cell-autonomous way, suggest that the two mechanisms are inter-woven and that the short RNAs play a central role (Fig. 1b.) Thus the short RNAs in plants showing PTGS, could be not only the guides for degradation in PTGS but also the mobile silencing signal for both DNA methylation and systemic spread of PTGS.

Short RNAs might guide sequence-specific degradation but might not be the systemic signal

The PTGS mechanism probably evolved to protect plants against viruses; indeed viral RNA is an effective trigger for PTGS. However, in almost every plant species, some viruses can breach this defence. They do this by expressing proteins that interfere with one or more steps in the PTGS pathway. The multi-functional HC-Pro protein, encoded by

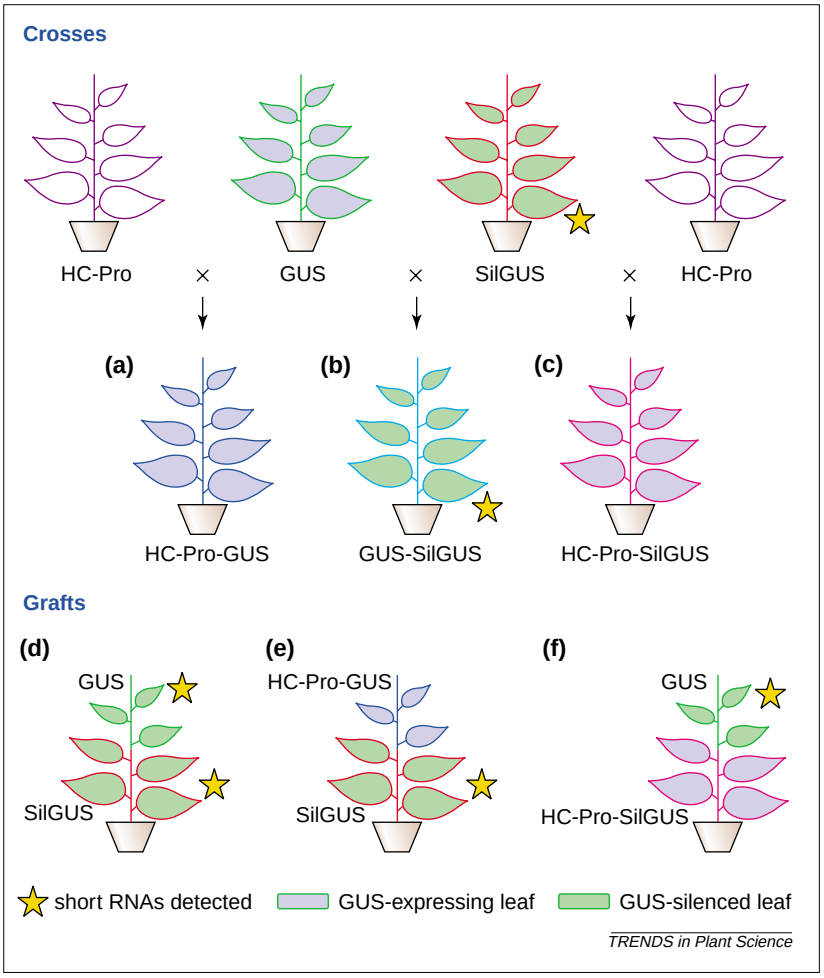

Fig. 2. The effect of HC-Pro on systemic silencing. Transgenic plants expressing HC-Pro (a viral silencing suppressor), expressing GUS, or with a spontaneous GUS-silencing locus (SilGUS) were intercrossed. Progeny of the crosses showed that plants containing both GUS and HC-Pro transgenes expressed GUS (a), the functional GUS gene was silenced in plants also containing the silGUS locus (b), and the HC-Pro transgene reactivated expression of the silGUS locus (c). A GUS scion grafted onto a silGUS rootstock became silenced (d), demonstrating a mobile silencing signal. If the GUS scion also contained HC-Pro, it retained its GUS expression even on a silGUS rootstock (e). GUS expression was silenced when a GUS scion was grafted onto an HC-Pro-reactivated SilGUS rootstock (f). The presence of GUS-derived short RNAs was detected in the plants and tissues indicated by yellow stars. The results of graft (e) show that HC-Pro prevents the signal from the silGUS rootstock from acting in the scion. The results of graft (f) show that the rootstock was producing a mobile silencing signal but not detectable amounts of short RNAs, suggesting that these RNAs are not the signal.

Potyviruses, suppresses the plant host's capacity to carry out PTGS, thus facilitating virus infection (reviewed in Ref. 20). When expressed from a transgene, HC-Pro restores activity to transgenes previously showing PTGS and prevents the production of the short RNAs normally associated with silencing[5,21]. This property of HC-Pro has been exploited elegantly in grafting experiments to investigate the role of short RNAs in the systemic signalling of PTGS (Ref. 21; Fig. 2).

As before, the transgene in a GUS-expressing scion grafted onto a GUS-silenced rootstock becomes silenced; short RNAs are detected in both the scion and rootstock. However, when a scion expressing both HC-Pro and GUS transgenes is grafted onto the same GUS-silenced rootstock, no short RNAs are detected in the scion and the GUS gene remains active, suggesting that HC-Pro blocks the perception or action of the mobile signal (from the rootstock) in the scion. Furthermore, a GUS-expressing scion, grafted onto a rootstock where a once-silenced GUS transgene has been reactivated by HC-Pro, shows GUS silencing. In this grafted plant, short RNAs are detected in the scion but not in the rootstock (Fig. 2), which strongly suggests that the short RNAs are not the mobile signal because they were not detected in the rootstock of the graft and yet the rootstock transmitted a silencing signal to the scion.

In addition, once-silenced GUS transgenes, which have been reactivated by HC-Pro (and devoid of short RNAs), still develop PTGS-like methylation, casting doubt on the idea that short RNAs act as guides for methylation. However, this result conflicts with another report[5] in which HC-Pro-released silencing was accompanied by the inhibition of short RNA production and a reduction of PTGS-associated methylation.

Where does HC-Pro interact in the PTGS pathway? Probably upstream of the production of short RNAs, perhaps preventing either the recognition or the cleavage of dsRNA (Ref. 21), but downstream of the production of the signal. So what is the signal? One of the simplest explanations might be that dsRNA in a cell is recognized by two complexes: one that cleaves it into short molecules to use as guides in degrading homologous ssRNAs, and another containing RNA-movement proteins. This RNA-movement protein complex ushers the dsRNA through nuclear pores and plasmodesmata. The dsRNA entering the nucleus is unwound and used to direct methylation, whereas the dsRNA entering cells, in which the PTGS mechanism is not yet activated (such as in a graft situation), is used as a substrate for degradation complexes to produce short RNAs. These short RNAs act as primers for a host RNA-dependent RNA polymerase to produce cRNA on target mRNAs. This newly formed dsRNA then becomes a substrate for both the movement and PTGS degradation complexes, thus re-amplifying the mobile signal and propagating PTGS.

An alternative explanation is that the short RNAs are indeed the mobile signal, but are only required at a low, and by present technology undetectable, level. Thus, in cells where HC-Pro is expressed, it can reduce the degradation of target RNAs to a level that appears to relieve silencing (e.g. the HC-Pro-reactivated–GUS-silenced rootstock) but that is still able to spread and be amplified in tissues where the PTGS machinery is not inhibited by HC-Pro.

Future perspectives

It is becoming clear that short, non-protein encoding RNAs play important roles in regulating development, intron splicing and RNA editing. For example, several short RNAs in the spliceosome can base pair with short sequences in pre-mRNAs to

Box 1. Intriguing questions and possible routes to some answers

Questions

- Is the same signal molecule responsible for post-translational gene silencing (PTGS) and DNA methylation.
- Is it RNA?
- Is the signal composed of short (22 nt) double-stranded RNAs (dsRNAs), full-length dsRNAs or intermediate RNAs?

Possible approach 1

Compare the transgene or target-derived nucleic acids in phloem sap from a wild-type stem, grafted onto a signal-producing rootstock, with nucleic acids in the phloem of a wild-type stem on wild-type rootstock. The nucleic acids present in a wild-type stem, grafted onto a signal-producing rootstock but not a wild-type stem on wild-type rootstock would be excellent candidates for the signal molecule.

Possible approach 2

Analyse the methylation of a virus-derived transgene in plants also expressing an HC-Pro transgene when challenged with the corresponding virus. Mallory *et al.*[a] have shown that short dsRNAs should not be produced. If the DNA still becomes methylated, this would support the suggestion that the short dsRNAs are not the signal.

Question

What genes are involved in the RNA-directed methylation pathway?

Possible approach

Several genes involved in the RNA degradation pathway have been identified from studies of *Arabidopsis* mutants[b,c]. A similar mutational approach, rescuing hairpin RNA-directed methylation silenced genes with restored activity, might identify the key genes involved in the RNA-directed methylation pathway.

Question

Why does RNAi (RNA interference) pass through to the progeny of silenced Nematodes and *Drosophila* but PTGS in plants does not?

Possible answer

Considerably fewer RNA viruses attack Nematodes and *Drosophila* than attack plants, and only a few plant viruses are seed transmitted. Perhaps the plant's mechanism that stops seed transmission of viruses also prevents the PTGS signal from being passed on to the next generation. Nematodes and *Drosophila* have not needed to evolve such a mechanism.

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delineate regions for splicing[22]; a group of short nucleolar RNAs has been found to direct pseudouridylation and 2′-*O*-ribose methylation in rRNA (Ref. 23). Recently it was shown that a 21 nucleotide RNA regulates the timing of developmental transitions in *Caenorhabditis elegans* through hybridization with the 3′ region of its target RNA, blocking translation. This short RNA is conserved across a wide range of species, from flies to mammals[24]. The recent progress in understanding the mechanisms of PTGS has identified yet another role for short RNA molecules: as guides for sequence-specific RNA degradation and possibly DNA methylation (Box 1). Studies on *Potexviruses*, which lack an HC-Pro equivalent but which employ a different strategy to overcome the plant's defence, might shed light on the role of short RNAs in systemic transmission of this defence system. These viruses express a protein, p25 (also called TGBp1), that prevents the spread of the mobile PTGS signal but does not inhibit existing PTGS (Ref. 25). Identification and characterization of host proteins that interact with p25 might well provide further insights into the identity and mechanism of the mobile signal. If p25 also blocks DNA methylation then this would suggest that the signal for transmission of PTGS and for DNA methylation in TGS and PTGS are the same.

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Balance of power: a view of the mechanism of photosynthetic state transitions

Anna Haldrup, Poul Erik Jensen, Christina Lunde and Henrik Vibe Scheller

Photosynthesis in plants involves photosystem I and photosystem II, both of which use light energy to drive redox processes. Plants can balance the distribution of absorbed light energy between the two photosystems. When photosystem II is favoured, a mobile pool of light harvesting complex II moves from photosystem II to photosystem I. This short-term and reversible redistribution is known as a state transition. It is associated with changes in the phosphorylation of light harvesting complex II but the regulation is complex. Redistribution of energy during state transitions depends on an altered binding equilibrium between the light harvesting complex II–photosystem II and light harvesting complex II–photosystem I complexes.

Anna Haldrup*
Poul Erik Jensen
Christina Lunde
Henrik Vibe Scheller
Plant Biochemistry Laboratory, Dept Plant Biology, The Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark.
*e-mail: anna@kvl.dk

Although they work in series, photosystems I and II (PSI and PSII) are spatially separated in the thylakoids. PSI is mainly located in the non-appressed stroma lamellae and PSII is mainly located in the appressed grana stacks. To optimize the photosynthetic performance and to avoid damage when exposed to excess light, plants must balance the excitation of the two photosystems[1,2]. When plants are exposed to illumination favouring either PSI or PSII, they can redistribute the excitation, giving states 1 and 2, respectively (Box 1). In this way, the light-limited photosystem receives more energy and the light-saturated photosystem receives less.

The accepted view is that the mechanism for this short-term regulation (state transition) is based on the reversible phosphorylation of light-harvesting complex II (LHCII) by a thylakoid-bound kinase[3–6]. According to this view, an imbalance between PSI and PSII is detected at the level of the plastoquinone pool. When PSII is favoured (state 2), the plastoquinone pool becomes more reduced and this activates an LHCII kinase in the thylakoid membranes[7,8]. The kinase phosphorylates the subunits of LHCII (Lhcb1 and Lhcb2) and the phosphorylated LHCII dissociates from PSII because of either electrostatic repulsion or a conformational change that prevents the binding of phospho-LHCII to PSII (Ref. 9). Thus, a mobile pool of LHCII is associated with PSII in state 1 but, in state 2, it is phosphorylated and dissociated from PSII (Fig. 1). Whether the dissociated LHCII is associated with PSI has not been clear.

In addition to state transitions, which are adjustments of the relative activities of PSI and PSII, plants can also regulate the relative amounts of PSI and PSII complexes in the thylakoid membranes. This regulation involves both nucleus- and chloroplast-encoded photosynthetic proteins. Thus, the expression of both *lhcb* genes (encoding LHCII) and *psaAB* (encoding the central core proteins of PSI) has been shown to respond to changes in light composition[10–12]. The adjustment of photosystem stoichiometry is a long-term regulation operating over a period of days. By contrast, state transitions take place within 5–20 min. Several results have appeared over the past few years that have required a modification of the simplest view of state transitions. It is our opinion that there has been an accumulation of evidence that calls for a change in paradigm concerning the mechanism of state transitions.

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