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RNA silencing and antiviral defense in plants

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Much progress has been made recently in identifying the molecular components of RNA silencing in plants, and in understanding their roles in the biogenesis of small interfering RNAs and microRNAs, in RNA-directed DNA methylation, and in RNA-mediated antiviral defense. However, many crucial questions remain unanswered. What are the molecular bases of sense and antisense transgene-mediated silencing? Why does silencing only appear to spread through transgenes? Plant viruses encode silencing suppressors to counteract host RNA silencing, and some of these suppressors affect microRNA accumulation and function and hence normal plant development. Is viral pathogenicity determined, partly or entirely, by their silencing suppressor activity?

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Introduction

RNA silencing is a general term used to describe post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals [1•,2•]. To those interested in its practical applications, the basic process of RNA silencing might look simple and straight-forward: double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) is cleaved by Dicer, an RNaseIII-type enzyme, into small interfering RNAs (siRNAs) of 21–26 nucleotides (nt), which then guide an RNA-induced silencing complex (RISC) to destroy single-stranded cognate RNA. To those trying to understand the underlying biology, however, RNA silencing is a complex process that involves RNA–RNA, RNA–DNA, RNA–protein and protein–protein interactions [2•].

RNA silencing might have arisen as an ancient RNA surveillance system that is conserved among eukaryotes, and that acts as a natural defense mechanism against invasive nucleic acids, including viruses, transposons and perhaps other highly repetitive genomic sequences. RNA silencing also plays a pivotal role in plant and animal development by providing an elegant system of gene control that can occur through RNA degradation, translational inhibition or chromatin modification. There are two overlapping but distinct RNA silencing pathways in plants and animals, the siRNA pathway and the micro-RNA (miRNA) pathway [1•,2•]. The siRNA pathway is induced by the presence of perfect dsRNAs, and is believed to play a defensive role against viruses and transposons [1•]. miRNAs are small ∼22-nt RNAs that are generated by Dicer cleavage of short hairpin structures in primary miRNA transcripts [3]. Many of these miRNAs control the spatial and temporal expression of key regulatory genes in plants and animals by binding to mRNA, either targeting its destruction by cleavage or preventing its translation into protein [3,4].

This review focuses on recent advances in understanding RNA silencing in plants and its defensive role against viruses. We also discuss some important questions concerning the molecular details of the siRNA pathway in plants.

RNA silencing in plants and the associated protein factors

Some aspects of RNA silencing (e.g. the requirement for Dicer and Argonaute proteins) are common to all eukaryotic organisms, whereas others occur in some but not all eukaryotes. Plants appear to have more diverse aspects of RNA silencing than do other organisms. Silencing in plants is systemically transmissible (systemic silencing), and can spread from the initial target region to adjacent 5′ and 3′ non-target sequences (transitive silencing) [5•,6]. Similar systemic and transitive silencing occurs in the nematode *Caenorhabditis elegans*, but appears to be absent from mammals and insects [1•]. The presence of dsRNA can induce sequence-specific DNA methylation, and this RNA-directed DNA methylation (RdDM) has been demonstrated in various plant systems and in response to various dsRNA inducers [7]. Recent studies suggest that RdDM also occurs in mammals [8] but does not exist in fungi [9]. Silencing in plants is associated with two distinct size classes of siRNAs, 21-nt and 24-nt siRNAs [10,11•], which appear to have different functions. The 21-nt siRNAs guide the cleavage of target mRNA by a RISC, and the 24-nt siRNAs direct systemic silencing and RdDM [10]. Silencing of transgenes in the fungus *Mucor circinelloides* is also associated with two size classes of siRNAs, 21-nt and 25-nt siRNAs [12], but animals only appear to produce the ∼21-nt species of small RNAs.

Several silencing-associated protein factors have been identified in plants. These include Dicer-like (DCL) proteins, RNA-dependent RNA polymerases (RdRP), proteins of the Argonaute family, RNA helicases and a few other proteins such as HEN1 and HYL1 [1[•],2[•]]. Unlike animals and fungi, which encode one or two Dicer proteins, *Arabidopsis* and rice have four DCL proteins, which appear to have distinct functions. DCL1 is structurally and functionally similar to human Dicer and *Drosophila* Dicer-1, having two RNaseIII domains plus dsRNA-binding, RNA helicase and PAZ domains [13]. DCL1 is required, together with HEN1 and HYL1 [14[••],15,16], for plant miRNA biogenesis. It also has similarities with the animal miRNA biogenesis enzyme Drosha [17[•]], and appears to function in the nucleus to process both primary transcripts and precursors of miRNAs [18[•],19]. DCL1 is unlikely to be involved in the processing of long dsRNAs: a loss-of-function mutation has no effect on viral RNA accumulation [14[••]] and does not relieve silencing induced by long hpRNA transgenes [13]. Although the function of DCL4 has yet to be determined, it could be responsible for the processing of long dsRNA (e.g. long hpRNA) because it is the only *Arabidopsis* DCL that lacks a PAZ domain [13]. The PAZ domain binds to the 2-nt 3′-overhang of dsRNA termini [20], and the absence of this domain seems to be a typical feature of long-dsRNA-processing enzymes including *Drosophila* Dicer-2, the fission yeast Dicer, and the *Escherichia coli* RNaseIII [17[•]]. The exact function of DCL2 is unclear but it might play a role in antiviral defense: an *Arabidopsis dcl2* mutant showed delayed viral siRNA accumulation and increased susceptibility to one of three viruses tested [14[••]]. DCL3 is required for RdDM in *Arabidopsis* [14[••],21] and, in conjunction with HEN1 [16], is also required for the production of ∼24-nt endogenous (transposon) siRNAs [14[••]]. Hence, it is likely to be involved in systemic silencing.

Another class of proteins that is particularly important for silencing in plants is RdRP, which is thought to contribute to silencing by copying target RNA to generate secondary dsRNA. Indeed, in *Arabidopsis*, RdRPs are required for systemic and transitive silencing [5[•],6] and for RdDM [14[••],21]. The exact nature of the RISC in plants has not been determined, but it is likely to include a member(s) of the Argonaute family [22].

Sense and antisense transgene-mediated silencing: how are they induced?
Most of the known silencing factors, including HEN1, HYL1, AGO4 and the four *Arabidopsis* DCLs, appear to localize in the nucleus [14[••],15]. One or more DCLs might, however, also function in the cytoplasm, as suggested by the capacity of wheat-germ extract (mostly of cytoplasmic content) to process long dsRNA [11[•]]. This might provide clues to some of the important processes involved in co-suppression (sense-transgene-induced

silencing) and antisense-mediated silencing in plants. It has been postulated that co-suppression is induced by read-through hpRNA transcripts from inverted-repeat (IR) transgene copies (Figure 1; [23]). This is consistent with reports showing a direct correlation between transgene silencing and IR transgene integration [24,25]. However, single-copy transgenes or multiple transgenes that are not arranged as IRs also induce effective gene silencing [26]. Another model proposes that transgene-derived 'aberrant RNA' is used as a template for RdRP to produce dsRNA, thereby triggering silencing, but the nature of the 'aberrant RNA' remains a mystery [27[•]]. It has been proposed that nuclear-accumulated transcripts might be the 'aberrant RNA' template for RdRP (Figure 1; [25]).

An alternative scenario for co-suppression is illustrated in Figure 1a. In this scenario, nuclear-accumulated sense transcripts form imperfect hairpin structures that resemble miRNA precursors (pre-miRNA). These precursors are processed by a Drosha-like protein, or by one or more of the DCL enzymes (e.g. DCL1 or DCL3), into miRNA-like small RNAs. These small RNAs have partial complementarity with the target mRNA, which allows them to hybridize with the cytoplasmic mRNA and to initiate RdRP-catalyzed synthesis of secondary dsRNAs, resulting in silencing. The nuclear RNA model is consistent with the frequent observation that transgene or endogenous gene (e.g. retroelement) silencing is accompanied by RNA accumulation in the nucleus [28,29[•]]. This model could also account for the observations that transgenes of bacterial origin appear to be more susceptible to co-suppression than are endogenous sequences. Bacterial genes have not co-evolved with RNA silencing and are more likely than endogenous sequences to contain pre-miRNA-like secondary structures in their RNA transcripts.

A seeming paradox regarding antisense transgenes is that they rarely induce efficient silencing in plants despite their transcripts having the potential to form duplex RNA with the target mRNA. It is possible that antisense transcripts do not usually hybridize with the target sense mRNA *in vivo* to form dsRNA and trigger silencing. We postulate that a similar nuclear pathway (as shown in Figure 1b) accounts for antisense-mediated silencing; the antisense-derived small RNAs have perfect complementarity with the target mRNA and hence can direct cleavage of the mRNA as well as acting as primers for RdRP.

Spread of silencing in plants: why does it only appear to act on transgenes?
One puzzling observation regarding systemic and transitive silencing in plants is that they seem to occur only with transgenes and not with endogenous genes. For instance, a green fluorescent protein (GFP) transgene was found to be susceptible to both systemic and transitive silencing,

Figure 1
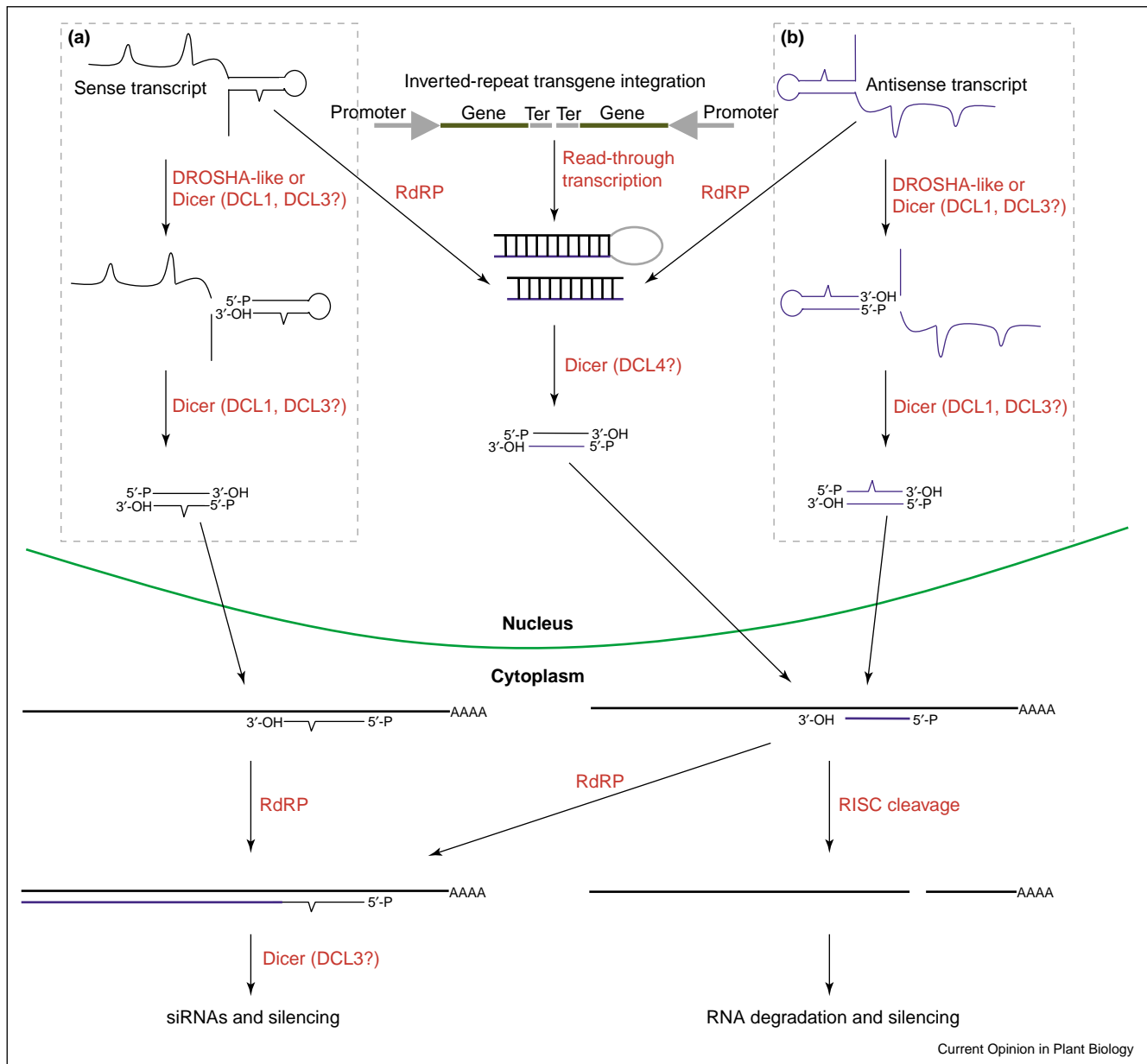
A nuclear model for sense and antisense transgene-mediated silencing. **(a)** Nuclear-accumulated sense transcript forms a pre-miRNA-like structure and, upon cleavage by Dicer or Drosha-like proteins, gives rise to miRNA-like small RNAs. These small RNAs are then used as primers by RdRP to synthesize secondary dsRNA, resulting in gene silencing (co-suppression). **(b)** Similarly, nuclear-localized antisense transcript can also form pre-miRNA-like structures and hence miRNA-like small RNAs. These small RNAs have perfect complementarity with the target mRNA. They guide RISC to cleave target mRNA or are used as primers for RdRP or both. Alternatively, nuclear sense or antisense transcript is the preferred template for RdRP to synthesize secondary dsRNA in either a primer-dependent or a primer-independent manner [11•]. Another possibility is that read-through transcription occurs in a tail-to-tail inverted transgene repeat, generating long hpRNA and triggering silencing. Ter, transcriptional terminator.

whereas the endogenous Rubisco small subunit (RbcS) gene is recalcitrant to both types of silencing [5•,6]. Target mRNA levels do not appear to be responsible for this difference because GFP and RbcS transcripts accumulate to similar levels [5•]. Sequence composition is also unlikely to be responsible because endogenous gene sequences, when expressed as transgenes, become susceptible to systemic silencing [30]. A possible explanation is that certain transgene loci might have features (e.g. tandem repeats or lack of introns) that make them highly

susceptible to RdDM-mediated heterochromatic modification that is triggered by primary siRNAs or systemic siRNA signals. The heterochromatic marks (e.g. DNA and histone methylation) and associated factors might recruit enzymes such as RdRP and DCLs to the target locus and hence to the nascent transcripts originating from it [14••,27•] Alternatively, these marks might result in the production of the nuclear-localized transcript. Either of these processes can trigger further silencing. This model is supported by the finding that AGO4 and SDE4, proteins that are required for cytosine (DNA) methylation, are also required for the accumulation of endogenous retroelement siRNAs in *Arabidopsis* [14••,31]. Furthermore, silencing that is induced by exogenous hpRNA, and the associated accumulation of siRNA, requires both an RdRP and a histone methyltransferase in fission yeast [32]. A possible nuclear action by RdRP is suggested to occur in both plants and fission yeast. The *Arabidopsis* RDR2 appears to interact both physically and functionally with DCL3 that is presumably localized in the nucleus [14••]; in fission yeast, RdRP physically associates with silent heterochromatin [33].

RNA silencing and antiviral defense in plants

The infection of plants by both RNA and DNA viruses results in the accumulation of viral siRNAs. Viruses are therefore inducers of RNA silencing that is directed against their own replication. The siRNA pathway of RNA silencing is generally believed to be a natural antiviral defense mechanism in plants. The exact pathway for the biogenesis of viral siRNAs is unclear. It is thought that dsRNA replication intermediates are the source of viral siRNAs. However, direct processing by Dicer of duplex structures formed within single-stranded viral RNAs could also contribute to the siRNA pool. Furthermore, the probable involvement of RdRP in antiviral defense [34] and in DNA virus-induced gene silencing [35] in plants suggests that RdRP-mediated synthesis of secondary viral dsRNA might also play a role in viral siRNA accumulation. The long-dsRNA-mediated siRNA pathway does not seem to operate in most mammalian cells [36•], and is therefore less likely to play a major role in antiviral defense in mammals.

Transgene-mediated virus resistance is a classical example of RNA silencing and its role in antiviral defense in plants. However, viruses are different from plant genes with respect to their response to transgene-induced silencing. This is demonstrated by the different susceptibilities of potyviruses and plant genes to sense and antisense transgene-mediated silencing. For instance, co-expression of a sense and an antisense transgene from two separate transcriptional units gives high levels of resistance to potato virus Y [37], but the same strategy does not cause the silencing of plant genes [38]. Also, a viral sense transgene that expresses high steady-state levels of RNA confers good resistance to tobacco etch virus, although the transgene is clearly unsilenced before virus infection [39]. A probable explanation of this difference is that viruses are themselves the source of siRNAs, and these siRNAs can initiate the RdRP-mediated synthesis of secondary dsRNAs using the sense and antisense viral transgene RNA as templates, leading to the amplification of silencing and, hence, to high levels of virus resistance.

## Suppressors of RNA silencing and viral counter defense

The discovery that almost all plant viruses encode silencing suppressors [40] provides further evidence for the involvement of RNA silencing in plant antiviral defense. These suppressor proteins operate through a variety of mechanisms. For instance, the P1/HC-Pro suppressor from the potyviruses inhibits silencing at a step downstream of dsRNA processing, possibly by preventing the unwinding of duplex siRNAs or the incorporation of siRNA into RISC, or both [41••]. The tombusvirus p19 protein also functions downstream of dsRNA processing, but it physically binds to duplex siRNAs and hence prevents their incorporation into RISC [41••,42,43••]. The cucumisvirus 2b protein and the p25 protein of potexviruses, on the other hand, inhibit the systemic transmission of silencing signals [40]. Thus, plant viruses appear to have evolved diverse counter-defense strategies against RNA silencing.

None of these silencing suppressors appear to block dsRNA processing, but tend to operate by sequestering siRNAs, preventing siRNA unwinding, or blocking the cell-to-cell movement of siRNAs. This might have significant implications for viral self-defense strategies. It is possible that silencing suppressors only function in those cells in which viruses are actively replicating, and might lose their suppressor activity once the viruses have completed their life cycle and moved into neighboring cells. The siRNA-charged suppressor proteins in the pre-infected cells would then release their siRNAs, making them available for silencing against secondary infection by the same or a related virus. Thus, viruses might have evolved a survival mechanism by protecting their hosts from secondary viral infection. This possibility is consistent with the phenomenon of classical viral cross-protection, where a plant that is pre-inoculated with a mild virus strain becomes resistant to subsequent infection by a related severe strain. A recent study suggests that viral cross-protection is mediated by RNA silencing [44]. Cross-protection is only effective when the severe viral strain is inoculated after infection with the mild strain. Simultaneous co-inoculation of the two viral strains does not result in cross-protection.

RNA silencing and viral pathogenicity

Overexpression of viral silencing suppressors can affect miRNA accumulation and function, and can result in

Figure 2
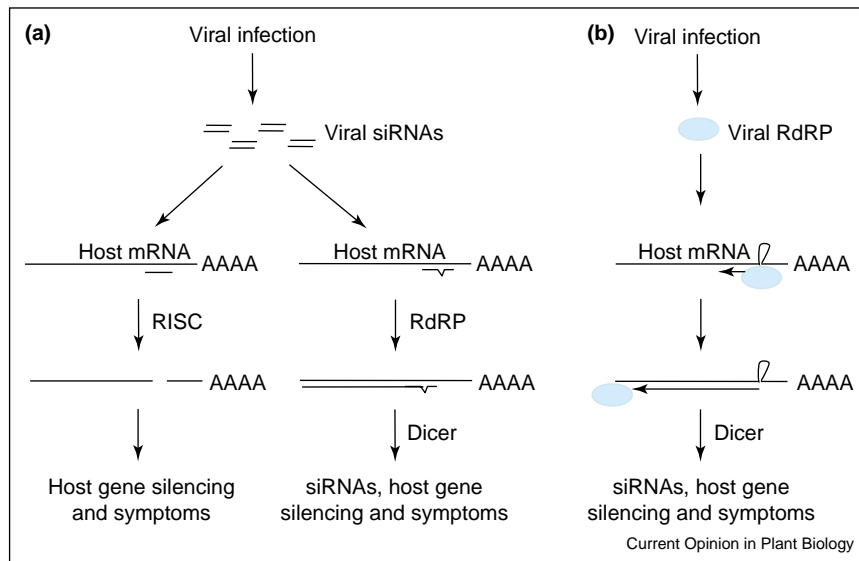
A model for viral pathogenicity mediated by RNA silencing. **(a)** Viral siRNAs share sequence identity with host mRNA and therefore direct the cleavage of host mRNA or initiate the RdRP-catalyzed synthesis of host gene dsRNA, resulting in silencing of the host genes and viral symptoms. **(b)** Viral-encoded RdRP binds to host mRNA sequences that resemble a viral origin of replication and initiates viral-like replication of the host mRNA. This replication generates dsRNAs of the host mRNA, leading to host gene silencing and symptoms.

developmental abnormalities in plants [41[••],43[••],45,46]. This has led to the suggestion that viral pathogenicity is largely determined by the effect of viral silencing suppressors on the host miRNA pathway [45,46]. Evidence against the universality of this pathogenicity model comes from the observation that not all viral suppressors appear to affect the miRNA pathway in plants [43[••]].

An alternative RNA-silencing-mediated pathogenicity model (illustrated in Figure 2) is suggested by the finding that plant subviral RNAs appear to induce symptoms by inducing silencing against host genes [47], and that a human DNA virus expresses miRNAs that have the potential to suppress host gene expression [48[•]]. This model envisages three possible scenarios: first, viral siRNAs induce cleavage of host mRNA because of their sequence identity; second, viral siRNAs are partially complementary to the host mRNA and serve as primers to initiate RdRP-mediated synthesis of secondary dsRNA against the host mRNA; or third, certain host mRNAs contain sequence motifs that resemble viral origins of replication, and consequently, viral-encoded RNA-dependent RNA polymerase recognizes the sequences and initiates the synthesis of antisense RNA against the host mRNA. Each of these scenarios would result in the silencing of host genes, leading to disease symptoms. This pathogenicity model, if proven, would have implications for an additional role for the silencing suppressors; namely, that they might function to minimize virus-induced symptoms by moderating host gene silencing,

thus minimizing the impact on their hosts and, hence, on the viruses themselves.

Conclusions

There is still much to learn about the molecular processes and biological roles of RNA silencing in plants. Our current understanding of this RNA-mediated mechanism of gene control has already opened up new horizons for molecular biology and virology research. It is clear that RNA silencing plays a defensive role in plants, but its fundamental role in gene regulation is only beginning to be recognized. The recent finding that miRNAs mainly target transcription factor and other regulatory genes [3,4] indicates that they constitute the primary control elements in gene regulatory cascades. The discovery that the accumulation of certain endogenous small RNAs is responsive to environmental stresses [49] suggests that at least some epigenetic traits in plants might also be determined by this small-RNA-mediated control mechanism. It will be exciting to see if all epigenetic modifications in plants are directed by small RNAs or other non-coding RNAs. Recent evidence seems to indicate that RdDM is the main, or only, source of *de novo* DNA methylation in plants [7,21]. The ability of viruses to modulate the normal functioning of RNA silencing pathways in plants has led us to ponder whether we should continue to see them solely as pathogens. Like some other environmental stimuli [50], viral infections might disturb the RNA-silencing-mediated control of transposons through their silencing suppressor activity,

and might thereby enhance the transposon-mediated evolution of the host plant genome.

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