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The complex interplay between plant viruses and host RNA-silencing pathways

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RNA silencing was originally identified as an immune system targeted against transposons and viruses, but is now also recognized as a major regulatory process that affects all layers of host gene expression through the activities of various small RNA species. Recent work in plants and animals indicates that viruses not only suppress, but can also exploit, endogenous RNA silencing pathways to redirect host gene expression. There are also indications that cellular, as opposed to virus-derived small RNAs, might well constitute an unsuspected defense layer against foreign nucleic acids. This complex interplay has implications in the context of disease resistance and evolution of viral genomes.

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Introduction

In most eukaryotes, the perception of RNA that has double-stranded (ds) features triggers its conversion, by homologs of the RNaseIII enzyme Dicer, into 21–24 nt-long RNAs [1,2]. These small RNAs guide RNA-induced silencing complexes (RISCs) that act at the level of RNA to inhibit transcript stability or translation, or at the level of DNA to promote epigenetic modifications [3,4]. These various processes are all manifestations of 'RNA silencing'. In *Arabidopsis*, four Dicer-like enzymes (DCL1–4) account for the production of endogenous small RNA species that mediate RNA silencing [5••]. These small RNA species can be sub-divided into at least two major classes. Small interfering RNAs (siRNAs) are produced as populations from long dsRNAs that result from read-through or bi-directional transcription of DNA repeats or transposon loci, and from the action of host-encoded RNA-dependent RNA polymerases that synthesize complementary strands from cellular RNAs [6,7]. siRNAs are

also generated from transgenic pan-handled transcripts that are used to provide experimental RNA interference (RNAi) [8]. Endogenous siRNAs either direct the endonucleolytic cleavage of homologous transcripts (*trans*-acting siRNAs [9,10]) or promote DNA methylation and heterochromatin formation at the genetic loci from which they originate (*cis*-acting siRNAs [5••]), often resulting in transcriptional gene silencing (TGS). *Cis*-acting siRNAs are produced in the nucleus by DCL3, whereas *trans*-acting siRNAs require DCL1 for their biogenesis. The DCL(s) that are involved in the execution of experimental RNAi remain unknown [5••].

MicroRNAs (miRNAs) constitute the second class of endogenous small RNAs. Those molecules are excised by DCL1 from nuclear and non-coding precursor transcripts, of approximately 70–200 nt in length, which acquire a partial stem-loop structure. Mature miRNAs are cytoplasmic and direct the cleavage or translational inhibition of mRNAs that carry discrete complementary target sites [11,12]. Work with animal miRNAs indicates that the degree of complementarity between small RNAs and their target sequences largely influences the outcome of their interaction [13]. Inhibition of translation is favored by incomplete pairing (prevalent in animals), whereas cleavage is instigated by a perfect or near-perfect complement (prevalent in plants). The first plant miRNA targets to be identified were a series of evolutionarily conserved transcription factors that control important developmental fates [14], but recent work indicates that miRNAs regulate many other biological processes [15,16]. Moreover, gene inversion or duplication events can generate species-specific miRNAs that probably contribute to the ability of plants to adapt to their environment [17•,18].

The dsRNA features of plant viruses are also recognized by the host RNA-silencing machinery, such that the presence of virus-derived small RNAs and the consequent silencing of viral genes dampens the accumulation of the pathogens in a process referred to as virus-induced gene silencing (VIGS) [2,19,20]. Accordingly, viruses have evolved strategies to avoid or suppress this defense, one of which is the production of highly diverse suppressor proteins that target many steps of the silencing machinery (reviewed in [21]). In the most simplistic view, antiviral RNA-silencing can be perceived as yet another illustration of the continuing arms race between hosts and pathogens. In this review, we provide insights into the complex interactions between plant viruses and cellular RNA silencing pathways, and discuss how viruses not

only interfere with but also exploit silencing-based regulatory networks. We also highlight recent work in plants and animals that suggest that the interplay between viruses, host RNA-silencing pathways and classical disease resistance networks might be extremely sophisticated and might have consequences that are beyond the simple scope of defense, including effects on the evolution of pathogen and, perhaps, host genomes.

Triggering and executing VIGS: things are not always what they seem

The small RNAs that are produced by plant viruses are usually thought to promote the endonucleolytic cleavage of pathogen RNAs upon their incorporation into a RISC complex. However, this assumption has never been strictly experimentally validated, because steady-state transcript levels rather than quantitative RNA-cleavage assays [22] are used to measure the impact of viral small RNAs. It is in fact possible that translational repression, as opposed to RNA turnover, also contributes to dampening virus accumulation in infected plant cells. Hence, recent findings indicate that the processing of imperfect hairpins, which are produced by the intra-molecular folding of plus-stranded RNAs, account for a large fraction of the small RNAs produced by tombusviruses and probably by many other types of plant virus [23].

In most cases, small RNAs that are derived from such hairpins would be only partially complementary to the other arm of the stem and could, therefore, favor the translational inhibition of the targeted RNA rather than its cleavage. The demonstrated prevalence of those imperfect RNA hairpins in the VIGS process [23,24] also prompts a re-evaluation of the biochemical nature of virus-derived small RNAs in plants, which are generally considered to be siRNAs. In fact, many of those molecules might be akin to miRNAs, because their hairpins have greater similarity to miRNA precursors than to the perfect dsRNAs that produce the siRNAs that direct experimental RNAi. Supporting this hypothesis, viral-derived miRNAs have been shown to accumulate in human cells that are infected by members of the herpesviridae family [25••–27••]. Thus, plant VIGS probably involves a combination of siRNAs, miRNAs and perhaps other types of small RNAs; therefore, we refer to these molecules collectively as 'virus-derived small RNAs (vsRNAs)' in this review. This more elaborate picture of VIGS raises questions about the identity of the Dicer that would process the imperfect hairpins that are found in phytovirus genomes, because DCL1, the plant miRNA-processing enzyme, is nuclear [28,29], whereas most plant viruses replicate in the cytoplasm. Furthermore, these observations suggest that our perception of antiviral RNA silencing in plants should be more complex than is usually anticipated. For example, vsRNAs that direct the translational inhibition of viral replicases would promote a drop in the steady-state levels of viral RNAs

that could be misinterpreted as a result of RISC endonucleolytic activity.

Viruses not only interfere with but also exploit plant RNA-silencing pathways

A common response of plant viruses to RNA silencing is the production of suppressor proteins [30]; but by inhibiting RNA silencing of their own genomes, viruses often (although not always) also interfere with endogenous silencing pathways that regulate host gene expression. In *Arabidopsis*, the transgenic expression of several viral-encoded silencing suppressors causes a set of recurrent developmental abnormalities. These abnormalities have variable penetrance correlating to the degree of inhibition of the miRNA-directed cleavage of endogenous transcripts [31••,32••]. However, the defects in these transgenic plants are more complex than those observed in *Arabidopsis* mutants in which miRNA-directed functions are compromised, suggesting that other silencing pathways could also be affected. For instance, we have found that several *Arabidopsis* loci that correspond to cloned *cis*-acting siRNAs [5••], which are thought to direct the TGS of those loci, are ectopically transcribed in plants that express silencing suppressors (L Navarro, P Dunoyer, O Voinnet, unpublished). Therefore, viral suppression of RNA silencing might have much wider effects on the expression of host genes than originally anticipated. In an applied context, this large spectrum of inhibition could be exploited for the discovery of novel small RNA targets (Figure 1a) and to assess the impact of RNA-silencing pathways in a variety of biological processes (Figure 1b).

In addition to producing suppressor proteins, viruses can also use RNAs to interfere with the host silencing mechanisms. In human cells, the adenovirus VA1 (for VIRUS-ASSOCIATED 1) non-coding RNA acquires a stable secondary structure that efficiently competes with both Exportin 5 and Dicer, which are involved in export of miRNA precursors and in miRNA maturation, respectively [33•]. Secondary structures that are common in plant viral genomes could have a comparable effect. Moreover, abundant vsRNAs in plant- and insect-infected cells [2,23,34] might out-compete endogenous small RNAs for RISC, which might account for some of the symptoms of virus infections. Viruses can also exploit RNA silencing to modify host gene expression directly because of homologies between vsRNAs and host transcripts. For instance, herpesviridae-encoded miRNAs are predicted to suppress the translation of several classes of human mRNAs, and this probably favors the infection process [25••–27••]. Likewise, in plants, the expression of siRNAs from inverted-repeat transgenes that correspond to pathogenic regions of viroid genomes recapitulates most of the symptoms elicited during viroid infections [35••]. In fact, we can readily anticipate that this phenomenon has widespread implications, and it seems almost paradoxical that, to date, virus- or viroid-induced

Figure 1
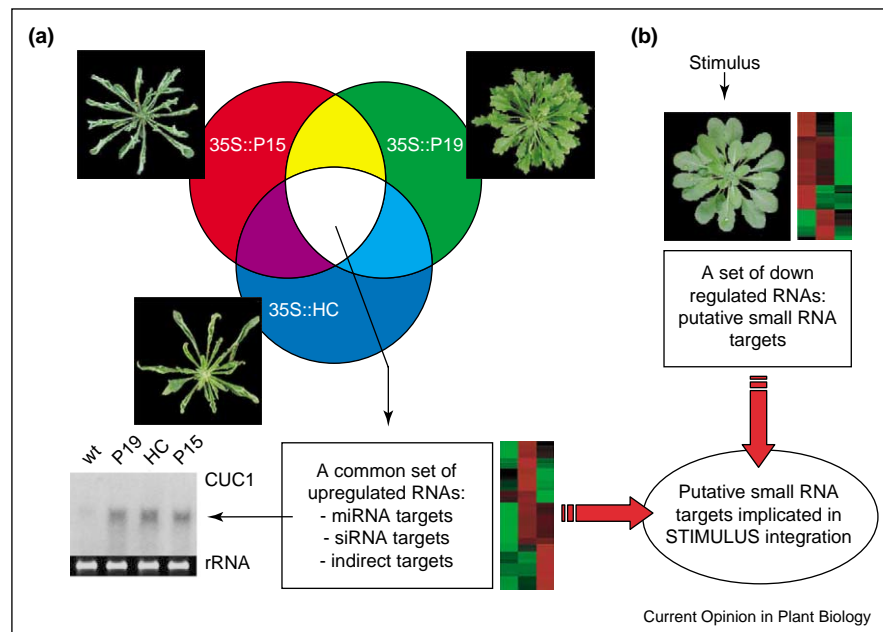
Using viral-encoded silencing suppressors to identify novel small RNA targets. **(a)** Comparative transcript profiling in *Arabidopsis* identifies a common subset of mRNAs that are altered similarly in plants expressing three distinct silencing suppressors (P15, P19 and HcPro). These silencing suppressors cause similar developmental phenotypes with variable penetrance. The common subset of transcripts is largely enriched in endogenous RNA-silencing targets, from which the corresponding small RNA sequences can be bio-informatically retrieved and further experimentally confirmed. For instance, the northern blot on the left shows ectopic expression of the CUP-SHAPED COTYLEDON1 (CUC1) mRNA (targeted by miR164) in the three silencing-suppressor lines. An advantage of this approach is that, unlike cloning and sequencing strategies, it relies on the activity rather than the mere abundance of small RNAs and, therefore, can help to identify rare molecules that would be otherwise underrepresented or absent in cloning libraries. **(b)** The dataset generated in (a) can be compared with the transcript profiles of plants subjected to various stimuli such as biotic and abiotic stresses. Some of the genes that are downregulated upon stimulus perception might be putative small RNA targets, in which case they should be upregulated in the silencing-suppressor lines.

RNA silencing of host genes has escaped the attention of plant virologists. Simple alignments between *Arabidopsis* transcripts and RNA viral genomes reveal dozens of 21–24 nt-long matches that might be significant for symptom expression or even successful infection if they indeed correspond to vsRNAs. Depending on the extent of pairing between vsRNA and host transcripts, such interactions might result either in the cleavage of host mRNA (high complementarity), translational repression (low complementarity) or a combination of both.

Cellular small RNAs have direct and indirect antiviral functions

Several cloned small RNAs, including some with features of miRNAs, do not show sequence homology to cellular protein-encoding genes in *Arabidopsis* [36]. Although it is possible that these small RNAs have protein-encoding targets that have evaded computer prediction because of imperfect base-pairing, this observation has prompted the idea that some of the orphan small RNAs could constitute a reservoir of defensive molecules owing to their complementarity to invading viral genomes [37]. Recent findings in human cells directly support the concept that endogenous small RNAs can confer antiviral defense. For

example, the genome of the primate foamy retrovirus (PFV) contains a sequence that is partially complementary to the cellular miRNA miR-32, potentially resulting in translational inhibition of all PFV transcripts. This target sequence was indeed sufficient to suppress the translation of a reporter gene transcript in a miR-32-specific manner. Moreover, a modified virus carrying synonymous mutations that prevent the annealing of miR-32 accumulated to higher levels than the wildtype virus, and the levels of accumulation were observed upon specific neutralization of miR-32 activity. The significance of this miRNA-directed antiviral defense was further underscored by the finding that PFV encodes a suppressor protein that has broad inhibitory effects on miRNA activities in human cells, including inhibitory effects on the activity of miR-32 [38••].

One key aspect of this finding is the mere fortuitous nature of the recognition process involved: the chances of a match between cellular small RNAs and foreign RNAs increases proportionally with the size of sampled sequences (viral genomes in this case) and will be further improved if the required degree of pairing between small RNAs and targets is moderate, as is the case for most

animal miRNAs [39]. Therefore, virtually any endogenous small RNA could hold an intrinsic, albeit fortuitous, antiviral potential that is independent of its cellular function. This concept applies to all organisms that

exploit silencing-related small RNAs for regulatory purposes, including plants (Figure 2a,b). In some instances, a small RNA might evolve to a point at which antiviral control could constitute its sole cellular function. This

Figure 2

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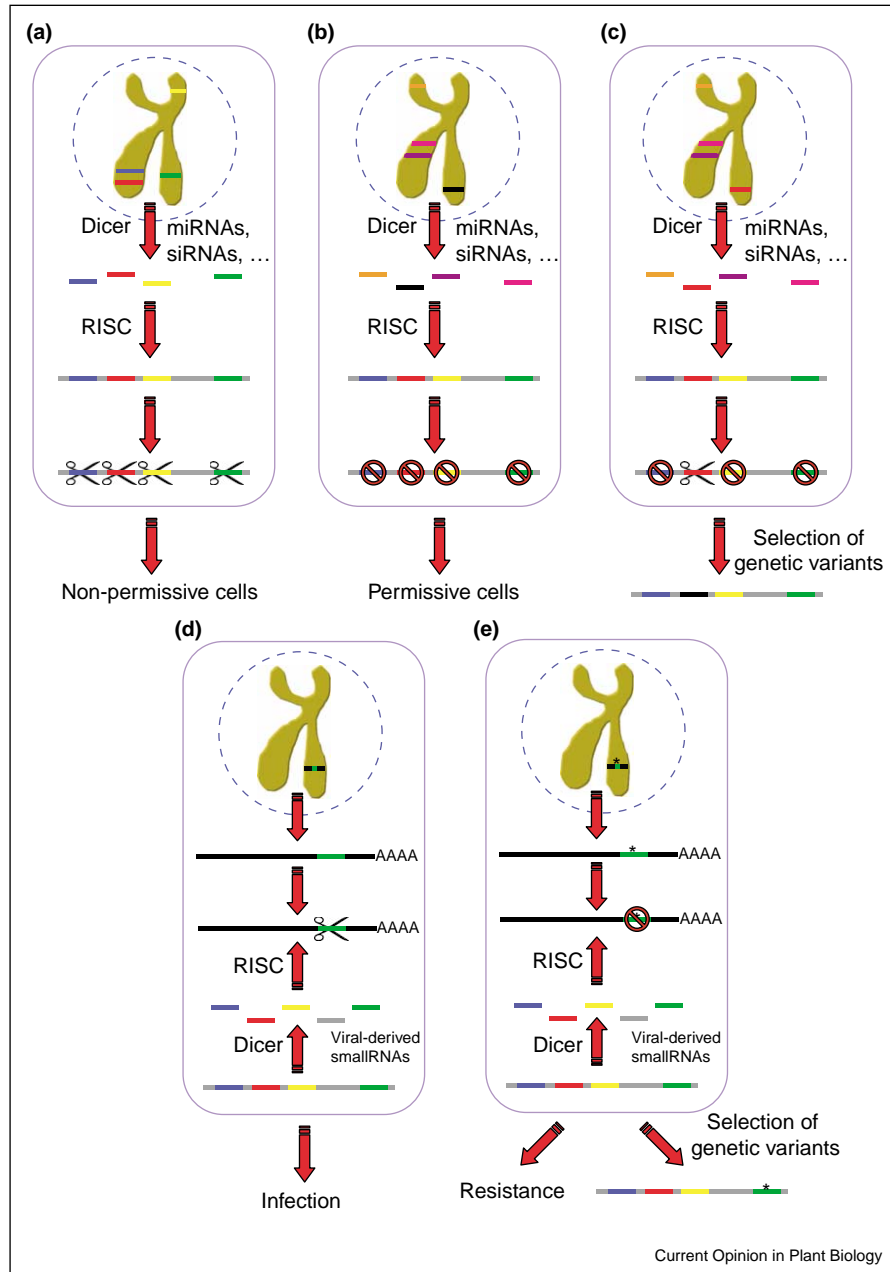
Antiviral RNA silencing mediated by cellular small RNAs. **(a)** In a given cell type, the repertoire of expressed small RNAs is such that several matches are found in the viral genome, potentially leading to complete resistance. These cells are scored as non-permissive. **(b)** In a second cell-type, cellular small RNAs have no homology with the pathogen's genome and will, therefore, be fully susceptible. **(c)** The situation probably encountered in most infections: a profile of small RNAs that have limited complementarity to the intruding genome will only confer a minimal layer of protection, thus allowing the virus to mutate the small RNA target sites, and thereby evading recognition by the small RNA. This process will favor the emergence of genetic variants with a higher fitness for this cell type. **(d,e)** The impact of vsRNAs on host mRNA expression and its consequences on viral genomes. (d) A species of vsRNA shares sequence homology with a host mRNA that encodes a defense factor and directs its cleavage by RISC, thereby allowing infection to proceed. Micro-polymorphism in the host mRNA might preclude such events, leading either to enhanced resistance and/or to the selection of viral genetic variants that have accommodated the polymorphism by modification of the vsRNA sequence.

might be the case for miRNAs that are targeted against retro-viruses or retro-transposons that are stably integrated into host genomes [40,41]. In addition, miRNA target sites within viral genomes might be, in some circumstances, under positive selection if the corresponding cellular miRNAs exert regulatory functions to optimize or coordinate expression of the pathogen's genomes.

The results described above illustrate how cellular small RNAs can exert direct defensive roles. However, these molecules might also exert indirect effects in restricting the accumulation of viruses and other pathogens. For instance, tobacco plants that express the potyviral helper component proteinase (HcPro) show enhanced resistance to a broad range of pathogens [42]. Although not experimentally tested by the authors, one explanation is that HcPro acts as a silencing suppressor that compromises the effects of endogenous small RNAs that normally target positive regulators of plant defense pathways. But it is also possible that this control operates directly on effectors of the plant innate immune response. For instance, constitutive expression of disease resistance (*R*) genes is in some cases detrimental to the host [43] and could well be normally dampened by the action of small RNAs, both at the transcriptional and posttranscriptional levels. At least three arguments support this model. First, the complex organization of *R*-gene loci [44] is prone to *de novo* generation of miRNAs through inverted gene duplication events [17*], potentially bringing entire *R*-gene families under the posttranscriptional control of a few discrete small RNA species (Figure 3a). Second, some *R*-gene loci are subject to epigenetic modifications that lead to TGS [45] and that might well be promoted by *cis*-acting siRNA populations (Figure 3b). And third, it has been shown that siRNA-directed epigenetic control of transposable elements can impact on the expression of many cellular genes that are within or close to them [46**,47**]; such genes might encode disease resistance proteins or effectors of defense pathways (Figure 3c). Taking all of these possibilities into account, we can anticipate that virus infections and the resulting interference with RNA silencing will have major impacts on small-RNA-regulated defense pathways, potentially leading to enhanced broad-spectrum resistance (Figure 3a–c). We note that some of the above hypotheses can be tested experimentally by measuring the susceptibility of *Arabidopsis* RNA-silencing mutants to various classes of pathogens.

## Host and viral small RNAs could direct the evolution of viral genomes

The recent findings highlighted in this review have profound implications for our current understanding of host–virus interactions and provide a new frame with which to investigate the impact of cellular or host polymorphisms on viral susceptibility and viral genome evolution. First, the antiviral potential of cellular small RNAs ([38**]; Figure 2) could explain, at least in part, why

specific tissues are often more permissive to viruses than others: the repertoire of expressed small RNAs is likely to vary from one cell type to another [48]. At one extreme, some cell types might be immune to a given virus if they express high levels of small RNAs or multiple small RNAs that match the pathogen's genome (Figure 2a). At the other extreme, cells that lack a specific small RNA profile could provide an optimal infection ground (Figure 2b). This principle is also applicable to entire organisms and could, in some instances, contribute to the poorly understood process of non-host viral resistance [49]. In most infections, however, cellular small RNAs might provide only a minimal layer of protection because most viruses produce silencing suppressors that directly inhibit small RNA activities. Furthermore, the high mutation rates of viruses might allow them to evade small-RNA-mediated defenses through rapid modification of their small RNA complementary regions [50,51]. This means that the cellular small RNA profile might not only form a defense layer but also guide the evolution of viral genomes and promote the emergence of novel viral quasi-species on a cell-specific or tissue-specific basis (Figure 2c).

The realization that vsRNAs contribute to infection efficacy and symptom expression through homologies to host mRNAs ([26**,35**]; Figure 2d) also has important evolutionary implications because it suggests that the nucleotide composition of viral genomes has intrinsic adaptive values, regardless of whether genome sequences are expressed as proteins. This might explain why synonymous mutations in viral open-reading frames are often beneficial to viruses, a phenomenon that is not easily reconciled with a protein-based effect [52]. This could also provide an explanation for the increased viral fitness that sometimes arises from modifications of nontranscribed viral sequences. In both cases, mutations in viral genomes could merely contribute to optimal recognition of host mRNA sequences by vsRNAs, independently of viral protein expression. It follows, therefore, that polymorphism at the host mRNA level (e.g. between related host species) could also impact on viral genomes and favor the emergence of quasi-species (Figure 2e).

Finally, it also is possible that the molecular dialog that is based on small RNAs will contribute to the evolution of host genomes. It is now clear that small RNAs that are derived from viroids or plant viruses not only promote the cleavage of host transcripts but can also direct the cytosine methylation of the corresponding host DNA [53–55], a phenomenon that occasionally results in C→T transitions. In the case of somatic infections (as seen with most plant viruses), this process could become a source of cellular mosaicism [56] by affecting genetic loci that are homologous to the vsRNAs. However, a much more profound effect can be anticipated if similar events are triggered by viruses that access meristems and gameto-

Figure 3

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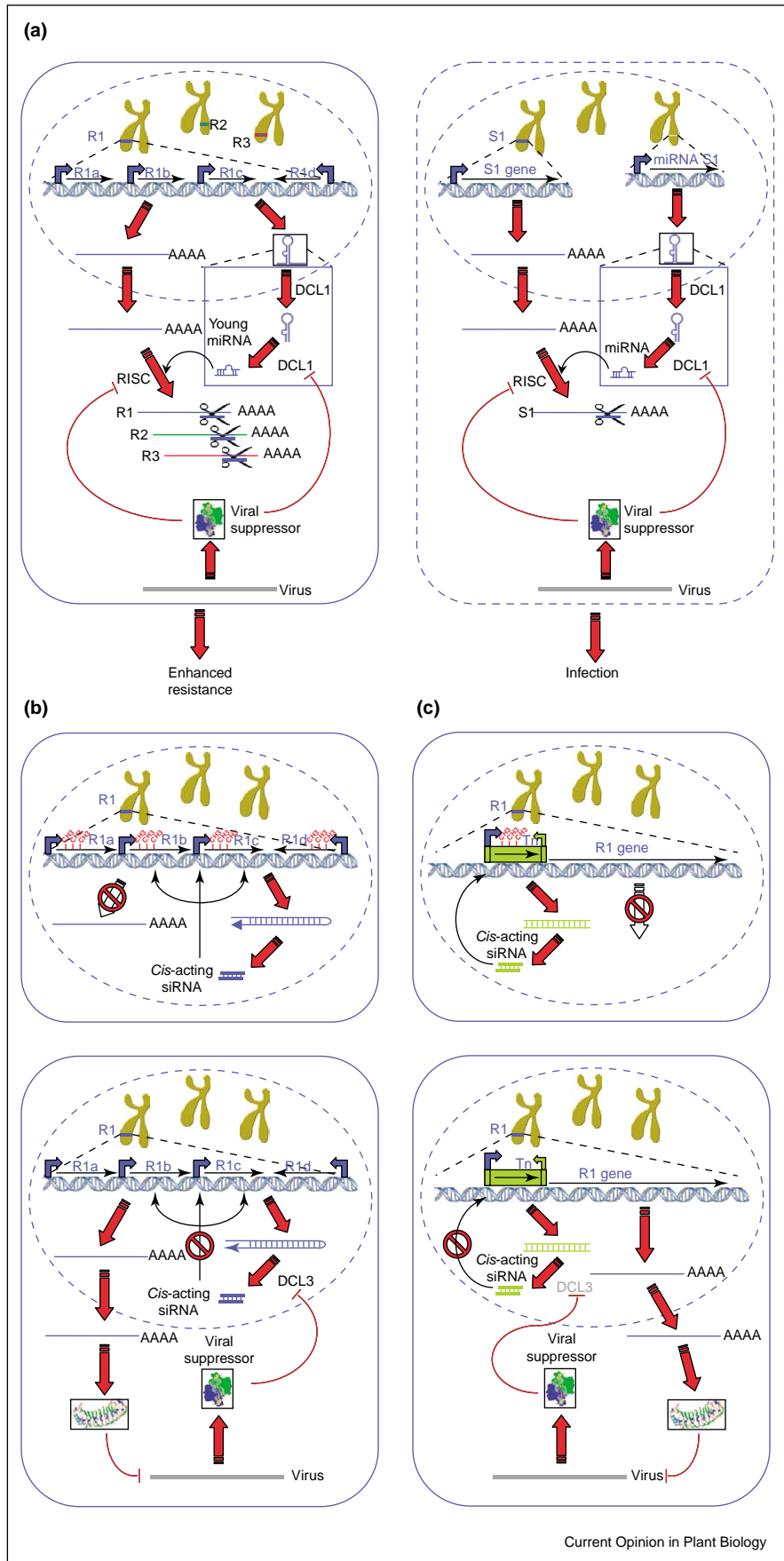
phytes, because these events might effectively generate epialleles that are uniformly transmitted to the progeny. Even more appealing is the demonstration, with the meristem-infecting tobacco rattle virus (TRV), that vsRNA-directed cytosine methylation of tobacco promoter DNA could be inherited and maintained in nearly 100% of the progeny, independently of the viral trigger [57]. Taking into account that cytosine methylation can result in stable C→T transitions, this observation raises the possibility that sequence homology between vsRNAs and host DNA could promote heritable and permanent changes in host genomes. Because many viruses infect plants without producing any obvious signs of disease, such phenomena might, in fact, be widespread and could constitute an important but not yet appreciated source of plant genetic variation.

Conclusions

Although several of the ideas evoked in this review remain to be formally tested through experimentation, they provide a glimpse of the extraordinary complexity that can be expected as a result of viral interference and usurpation of host silencing pathways. In addition, the concepts discussed here might not be restricted to viruses but could, in principle, apply to other types of pathogens that exploit foreign nucleic acids as part of their infection strategy. The challenge now will be to fully appreciate the extent to which classical defense and RNA-silencing pathways overlap in plants and, perhaps, in animals.

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(Figure 3 Legend) (a) The complex genetic organization of *R*-gene loci (here represented as an array of four paralogs R1a, b, c, d [left panel]) could generate, through inverted gene duplication events, new miRNA loci that could potentially *trans*-regulate entire *R*-gene families (including the unlinked paralogs R2 and R3). Upon viral infection and production of silencing suppressors, miRNA-mediated downregulation of *R* genes will be compromised, leading to enhanced resistance of the cell to a broad range of pathogens. In the right panel, the gene controlled by the miRNA encodes a susceptibility factor, which is required for virus infection. **(b)** Some *R*-gene loci might be transcriptionally repressed through epigenetic modifications (represented here as DNA methylation [CH_3]) that are mediated by *cis*-acting siRNAs. A silencing suppressor could interfere with this pathway, leading to transcriptional de-repression of *R*-gene loci. **(c)** The expression of endogenous genes can also be influenced by the regulation of transposable elements inserted close to them. Here, a transposon promoter (in blue) provides the transcriptional activity to an *R* gene located downstream. The transposon locus would normally be transcriptionally repressed by *cis*-acting siRNA, and this could be compromised by the expression of a silencing suppressor, leading to co-reactivation of the transposon and the *R1* gene.

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