Review

Virus-derived small interfering RNAs at the core of plant–virus interactions

César Llave

Department of Environmental Biology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

Once a virus enters a cell, viral double-stranded RNA (dsRNA) is targeted by the RNA silencing machinery to initiate a cascade of regulatory events directed by viral small interfering RNAs (vsiRNAs). Recent genetic and functional studies along with the high-throughput sequencing of vsiRNAs have shed light on the genetic and structural requirements for virus targeting, the origins and compositions of vsiRNAs and their potential for controlling gene expression. The precise nature of the triggering molecules of virus-induced RNA silencing or the targeting constraints for viral genome recognition and processing represent outstanding questions that will be discussed in this review. The contribution of vsiRNAs to antiviral defense and host genome modifications has profound implications for our understanding of viral pathogenicity and host specificity in plants.

RNA silencing: plant viruses on the target

In eukaryotes, RNA silencing controls gene expression to regulate development, genome stability, stress-induced responses and defense against molecular parasites [1–3]. RNA and DNA plant viruses activate RNA silencing through the formation of viral RNA with double-stranded features and vsiRNAs [4]. Virus-induced RNA silencing occurs in three steps: initiation, amplification and spreading [5]. Silencing is initiated when the trigger dsRNA is recognized by the same consortium of Dicer-like (DCL) ribonucleases responsible for the biogenesis of endogenous siRNAs and processed into 21 to 24 nt primary vsiRNAs [6]. Amplification involves the activity of one or more cellular RNA-dependent RNA polymerases (RDRs) that use single-stranded RNA (ssRNA) to synthesize long, perfect dsRNA [7,8], which serves as a substrate for the DCL-dependent formation of secondary vsiRNAs [9]. This secondary pool of vsiRNAs supports the systemic silencing that spreads throughout the plant [10,11]. vsiRNAs associate with distinct ARGONAUTE (AGO)-containing effector complexes where they provide specificity for RNA or DNA targeting through a sequence homology-dependent mechanism [12,13]. In principle, the functional interaction of vsiRNA-containing AGO complexes with complementary target RNAs leads to the endonucleolytic cleavage and/or translational inhibition of the cognate RNAs [14,15], whereas the interaction with target DNA molecules causes transcriptional repression through the modification of DNA and/or histones [16,17] (Figure 1).

Here, I will dissect the most relevant events that occur during the initiation and amplification of virus-induced RNA silencing. The molecular bases of vsiRNA targeting and the potential of vsiRNAs to modulate viral and host gene expression during viral infections in plants are also discussed.

DCL enzymes as sensor factors for virus-induced RNA silencing activation

The *Arabidopsis* genome encodes four *DCL* genes [18,19]. Genetic studies have revealed the hierarchical access of DCL4, DCL2 and DCL3 to viral dsRNA in the biogenesis of distinct vsiRNA size classes [20–29]. DCL4 is the primary sensor of viral dsRNAs and produces 21 nt vsiRNAs, the most abundant size class in infected tissues. DCL2 acts as a DCL4 surrogate to generate 22 nt vsiRNAs and DCL3 targets viral dsRNA efficiently in the absence of DCL4 and DCL2 to produce 24 nt vsiRNAs.

DCL1 is a minor contributor to vsiRNA formation in plants infected with RNA viruses [22,23,25]. Nevertheless, low amounts of vsiRNAs have been detected in *dcl2 dcl3 dcl4 Arabidopsis* loss-of-function triple mutants infected with *Turnip mosaic virus* (TuMV), suggesting that DCL1 might gain access to viral dsRNA substrates when other DCL activities are compromised [27]. By contrast, DCL1 is thought to excise hairpin-like structures from primary transcripts in dsDNA *Cauliflower mosaic virus*-infected plants, thereby facilitating access by the other DCLs [28].

Deciphering the triggering dsRNA molecule of virus-induced RNA silencing

Two nonmutually exclusive models can rationalize the initial silencing triggers upon virus infection (Figure 1). First, genomic segments with either extensive or local intramolecular dsRNA-forming capacities can be computationally predicted along viral ssRNA for most, if not all, plant viruses tested [30]. Initial processing events might then involve the DCL-mediated cleavage of folded viral ssRNA to generate a discrete subset of primary vsiRNAs as described for microRNA (miRNA)-like precursors in the herpesvirus family [31,32]. This hypothesis is plausible but controversial, because stable miRNA precursor-like structures using criteria established for canonical miRNA precursors are rare in plant viruses [33]. Furthermore, vsiRNA-producing DCL4, DCL2 and DCL3 are unlikely to provide the required processing activity given that they normally dice perfectly complementary dsRNA [34–36]. Alternatively, a residual DCL1 or other uncharacterized DCL activities containing different types of RNA-binding

Corresponding author: Llave, C. (cesarllave@cib.csic.es).

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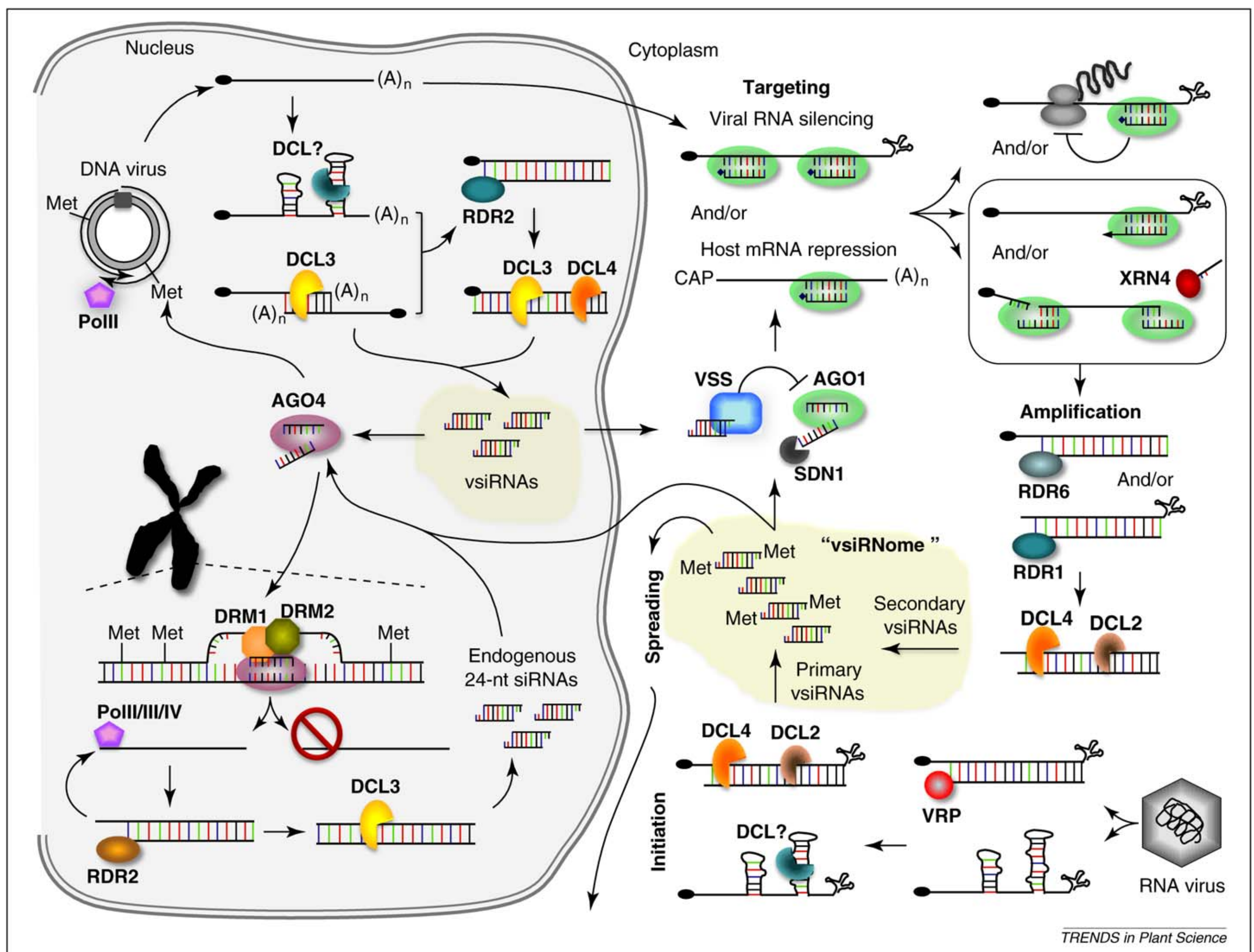

Figure 1. Schematic overview of virus-induced RNA silencing in *Arabidopsis*. dsRNAs derived from the structural features of the viral ssRNA or the intermolecular base pairing of viral RNA strands synthesized by the viral RNA polymerase (VRP) (RNA viruses) or the host RNA polymerase II (PolII) (DNA viruses) are hierarchically processed by DCL4, DCL3, DCL2 and, perhaps, an unknown DCL (DCL?) into vsiRNAs of 21 to 24 nt. vsiRNAs are recruited into AGO-containing complexes where the guide strand provides specificity for target recognition (RNA or DNA) and the passenger strand is degraded by small RNA degrading nucleases (SDN1). Virus-encoded silencing suppressors (VSS) can sequester vsiRNAs to prevent assembly into AGO complexes. AGO1 mediates the slicing and, possibly, the translational repression of the target host and/or viral RNA. Aberrant RNA cleavage products are either degraded by exoribonucleases (XRN4) or recruited by RDRs (RDR1, RDR2, RDR6) to synthesize dsRNA. Furthermore, vsiRNAs associated with AGO1 might serve as primers for RDR. RDR-dependent dsRNA is targeted by various DCLs to generate a pool of secondary vsiRNAs. AGO4 promotes DNA/histone methylation in the nucleus by attracting cytosine methyltransferases (DRM1, DRM2). vsiRNAs corresponding to promoter sequences cause the transcriptional repression of the target gene that can be maintained when a host RNA polymerase (PolII/III/IV) transcribes ssRNA from silenced, methylated genomic DNA and serves as a substrate for RDR2 amplification. vsiRNAs act as mobile silencing signals between plant cells.

modules in place of the canonical PAZ domain might exist in plants providing targeting specificities toward undefined secondary structures [37].

Second, dsRNA can be generated by viral RNA polymerases from RNA or DNA plant viruses either as an intermediate in genome replication and transcription (RNA viruses) or via converging bidirectional transcription (DNA viruses) [38]. The subsequent processing of dsRNA intermediates might result in the production of primary vsiRNAs as described for RNA viruses in vertebrate and invertebrate systems [39–41]. The derivation of long dsRNA is relatively easy to envisage during virus replication in the infected cell if the nascent negative viral RNA strand remains annealed to the positive strand from the infecting virus, or the progeny positive RNA remains annealed to the negative RNA templates [42]. Another possibility is the transient generation of shorter hybrids of cRNA strands during the transcription of subgenomic mRNAs of some positive RNA and DNA viruses. Nevertheless, all these possible scenarios need to be experimentally corroborated in plant-infecting viruses. Direct *in situ* evidence for the production of significant amounts of intracellular dsRNA has not yet been reported for plant viruses [43]. Furthermore, given that RNA replication occurs in intracellular membrane structures, double-stranded replication intermediates are likely to be transient in time and might be inaccessible to host RNA silencing components [42].

RDR-mediated amplification of virus-induced RNA silencing

The amplification of virus-induced silencing evokes a third mechanism of dsRNA formation, which entails the activity of several host RDRs (Figure 1). Indeed, RDR-dependent

dsRNA is the preferential substrate for DCL4, DCL3 and DCL2 cleavages [36]. *Arabidopsis* contains six identified RDRs [44] with specialized, albeit presumably interconnected, functions in the biogenesis of distinct siRNA classes [6,36,45].

RDR1, RDR6 and, perhaps, RDR2 also seem to exhibit different specificities in targeting virus genomes for vsiRNA production. Such specificity is poorly understood, although it might reflect different sensitivities to the action of virus-encoded silencing suppressors [27,46,47]. For instance, RDR1 alone seems to be dispensable for the production of vsiRNAs from several RNA viruses including *Cucumber mosaic virus* (CMV) [23,48]. However, infection with a 2b silencing suppressor-defective CMV reveals that the accumulation of vsiRNAs is RDR1-dependent [24,46]. RDR1 is also a major contributor to vsiRNAs in plants infected with TuMV because TuMV-derived vsiRNAs are significantly reduced in mutant combinations harboring the *rdr1* gene [27]. Likewise, vsiRNAs are reduced in *Arabidopsis rdr1* or *rdr6* single mutants infected with *Tobacco mosaic virus* (TMV) [49], whereas RDR1, RDR2 and RDR6 are simultaneously required for the biogenesis of nearly 90% of vsiRNAs in *Tobacco rattle virus*-infected *Arabidopsis* [25].

Nevertheless, a basal but consistent level of vsiRNAs can be detected in *rdr1 rdr2 rdr6* triple mutants infected with several RNA viruses [25,27,46], bringing to light a dsRNA-generating mechanism that is independent of RDR1, RDR2 and RDR6. This subset of vsiRNAs in the *rdr1 rdr2 rdr6* background might derive from the activity of RDR3, RDR4 or RDR5, although none of them has been shown to be functional. More likely, they indicate RDR-independent primary vsiRNAs formed by the initial cleavage of the dsRNA activator. The finding that RDR-independent vsiRNAs also spread throughout the entire viral genome points toward long dsRNA replicative forms as the primary source of DCL substrates as opposed to isolated hairpin-like regions of viral ssRNA [25,46]. The stringent DCL-dependency in vsiRNA biogenesis precludes a significant contribution of alternative mechanisms involving direct RDR-mediated single in-phase biosynthesis events from long RNA templates, such as those described in *Caenorhabditis elegans* for secondary siRNAs [50,51].

RDR activities in the amplification cycles of silencing are presumably linked to the perception of viral ssRNA that lacks features of host cellular transcripts [5′ cap or poly-(A) tail] and to which RDRs could be directly or indirectly attracted [7,52,53]. High levels of viral replication in infected cells or initial processing events directed by primary vsiRNAs on virus genomic RNAs might conceivably supply viral RNA species with aberrant signatures. Therefore, the predominant route of RDR-directed RNA silencing amplification in plants involves both unprimed (5′ to 3′) and primed (3′ to 5′) RNA synthesis starting at the 3′ end of the targeted transcripts [9].

vsiRNA populations: an issue of great complexity

High-throughput sequencing has contributed to markedly expanding our previously knowledge of vsiRNA populations based on RNA hybridization to depict a more accurate scenario about vsiRNA abundance, complexity and diversity in infected tissues [54]. It is now known that vsiRNAs from plants infected with RNA or DNA viruses are extraordinarily abundant and diverse and occur widespread in near saturation at any region of either the positive and negative genomic RNAs [27,30,46,49,55–57]. This particular overlapping configuration with vsiRNA sequences setting at 1 nt intervals is remarkable and predicts DCL targeting events at any nucleotide position along the virus genome.

However, it is arguable whether the sequenced set of vsiRNAs offers an absolute picture of the entire vsiRNA population that results from the cleavage of the various dsRNA precursors. Sequenced datasets usually under-represent certain vsiRNA species because: (i) vsiRNAs are not equally stabilized into AGO-containing complexes because of thermodynamical constraints [58] as well as 5′ end sequence identities [59,60]; (ii) a subset of vsiRNAs is subjected to chemical and structural modifications [61,62] that might affect stability and prevent ligation to adaptors used for sequencing; or (iii) vsiRNAs are predisposed to small RNA degrading nucleases [63] and sequestration by viral silencing suppressors [64] that limit their accumulation in the infected tissue. These are possible scenarios that might account for the bias in vsiRNA strand polarity that has been observed for some plant–virus interactions and whose basis is poorly understood. The infection of some viruses is associated with a near equivalent abundance of vsiRNA positive and negative strands consistent with vsiRNA deriving from the cleavage of long stretches of dsRNA, which has an equimolar ratio of both strands. By contrast, several other viruses show asymmetrical distribution with a dominance of sense vsiRNAs compared with antisense species, which is difficult to rationalize if they were generated through the DCL processing of a long dsRNA [30,49,57]. To explain this apparent inconsistency it has been suggested that chemical modifications affecting the nascent RDR-dependent viral RNA strands might prevent the modified vsiRNA strand from selective incorporation into an AGO complex [49]. This idea is sensible because the strand favoritism of TMV-derived vsiRNA is reduced in loss-of-function *rdr1* or *rdr6* mutants in agreement with a major contribution of replicative dsRNA intermediates to vsiRNA biogenesis in these mutants [49]. But, why should these (and other) factors alter the vsiRNA strand ratio for certain virus species, whereas others remain unaffected? Interestingly, some infectious animal viruses yield a vsiRNA profile with a bias toward positive strand vsiRNAs where the same viruses in other host environments produce equal amounts of both strands [41]. It would be interesting to investigate using a broad range of plant infectious systems whether the strandedness of vsiRNAs is also sensitive to both viral and host characteristics, including a possible differential tissue-specific contribution of the distinct mechanisms of viral dsRNA formation.

Structural features associated with vsiRNA biogenesis

vsiRNA sequences commonly have a non-uniform spatial distribution that reflects differential vsiRNA density and diversity along the virus genome [25,27,28,30,46,49,56,57,65,66]. For instance, vsiRNAs tend to accumulate with

increased frequencies in the 3′ region in infections with viruses that generate subgenomic mRNAs at this end. More noticeable is the existence of multiple highly reproducible hotspots where various vsiRNAs are clustered in specific regions of the genome. Interestingly, hotspots of each vsiRNA size class typically colocalize within the same regions of each particular virus genome indicating similar, although hierarchical, targeting affinities among the three DCL enzymes. This punctuated pattern is not unique to vsiRNAs because endogenous extended inverted-repeat loci in plants also generate abundant siRNAs from isolated regions embedded in the midst of long dsRNA precursors [67]. However, it is not clear what structural features ultimately influence the accessibility, affinity or processing of each DCL, which in turn can be affected by its partner dsRNA-binding proteins and by the preferred starting sites of RDR-mediated amplification of ssRNA templates [29]. A reasonable possibility is that DCL activities could be favored by a higher GC content within hotspots, rendering dsRNA structures more stable [30,56,68]. Another possibility is that hotspots originate from the preferential DCL processing of secondary structures within viral ssRNA [57,66]. This idea is unlikely because a correlation between putative foldback structures and vsiRNA-generating hotspots has never been demonstrated for any viral genome [25,30,49,56].

Evidence for RNA silencing-based antiviral immunity in plants

Plant RNA silencing operates as a potent defense mechanism where DCL and RDR enzymes are sensors and amplifiers of the antiviral responses and vsiRNAs are thought to promote the autosilencing of viral RNA [3]. Accordingly, viruses produce a variety of silencing suppressor proteins that target many steps of the RNA silencing pathway to avoid or suppress its antiviral effect [47]. DCL4 is the major innate responder against positive ssRNA viruses, whereas the antiviral effect of DCL2 is often, but not always, subordinated to DCL4 [21–26,28,29]. *Arabidopsis* plants with dysfunctional DCL4 are hypersusceptible to infection with multiple RNA and DNA viruses and exhibit exacerbated viral accumulation levels and symptom severity. A recent paper reported that DCL2 is, in the absence of DCL4, sufficient to restrict the systemic infection of a suppressor-defective TuMV in *Arabidopsis* inflorescences but not in either inoculated leaves or cauline leaves [27]. These differences between DCL requirements for antiviral defense at the local (DCL4-dependent) and systemic (DCL4- or DCL2-dependent) levels support a model by which cooperative interaction between DCL4 and DCL2 is necessary during systemic antiviral silencing [27]. For dsDNA caulimoviruses and ssDNA geminiviruses, DCL4 and DCL3 are the most active sensors of RNA silencing-based antiviral defense [21,28]. Finally, DCL1 represses antiviral RNA silencing by acting as a negative regulator of the expression of DCL4 [29].

Several studies have also proposed a function for RDR in antiviral silencing because plants with compromised RDR activities show enhanced susceptibility to infection by some RNA and DNA viruses [25,29,48,69–73]. It has only recently been shown that RNA silencing-based systemic immunity is strongly dependent on the cellular RDR1-, RDR2- or RDR6-mediated amplification of vsiRNAs [25,27,46]. Interestingly, the inactivation of a single RDR only causes changes in vsiRNA accumulation and susceptibility to infection with a few viruses, whereas other viruses tested are unaffected by the presence of a single *rdr* mutant gene. This is in part because of functional redundancy or specificity among RDR enzymes; thus, more than one RDR has to be inactivated to alter the outcome of the RDR-mediated antiviral response. Nevertheless, several examples have been reported whereby a correlation between the RDR-dependent accumulation of secondary vsiRNAs and antiviral silencing is not obvious [24,74]. For instance, RDR1, RDR2 and RDR6 are complementary, or act in coordination, to confine TuMV infection in inoculated leaves; RDR1 and RDR6 are both needed to prevent the viral systemic infection of cauline leaves, and RDR1 and RDR6 either alone or in combination restrict infection in inflorescence tissues [27]. However, only RDR1, but not RDR2 or RDR6, seems to be crucial for TuMV-derived vsiRNA biogenesis. This observation is difficult to explain and might be related to the different sensitivities of the RDR-dependent pathways to silencing suppressors [27,46]. It might also be influenced by possible tissue-specific variations in the basal level of RDR-independent antiviral silencing provided by primary vsiRNAs. All viruses tested accumulated to much lower levels in *rdr1 rdr2 rdr6* triple mutants than in *dcl2 dcl3 dcl4* triple mutants (which produce barely detectable levels of vsiRNA), indicating that primary vsiRNAs in the *rdr1 rdr2 rdr6* triple mutant guide antiviral silencing responses [25,27,46].

Molecular basis of vsiRNA-based antiviral defense

It is commonly held that antiviral immunity is substantiated by vsiRNAs that direct AGO complexes against their complementary target viral RNAs (Box 1) (Figure 1). Based on the known requirement of AGO1, and perhaps AGO7, for virus resistance and its binding affinity for 5’ terminal uridine-enriched vsiRNAs, a reasonable expectation is that vsiRNAs guide the AGO1-driven slicing of target viral RNA [29,75–77]. Paradoxically, molecular evidence is only partial, and the remarkable abundance of vsiRNA in the infected tissues cannot be an accurate reflection of antiviral silencing activities. The dicing of viral replication intermediates or virus-derived dsRNA is not sufficient to halt

Box 1. AGO proteins and vsiRNA function in *Arabidopsis*

Ten AGO proteins have been identified in *Arabidopsis*, but only four have been characterized to date. AGO1 and AGO7 possess slicer activity by promoting the siRNA-guided endonucleolytic cleavage of the target RNA at the center of the complementary region [60,76]. AGO4, and probably AGO6, operate during transcriptional gene silencing in plants and are required for the maintenance and establishment of DNA methylation [16,90]. The association of siRNAs with a particular AGO protein in plants is primarily dictated by the identity of the 5′ terminal nucleotide of the siRNA [59,60,77]. vsiRNAs exhibit a preference to begin with uridine or adenine, and to a lesser extent, cytosine [27,30,46,49,56] and, therefore, could be selectively loaded into multiple AGO complexes to provide sequence-specificity for RNA or DNA target recognition. AGO1, AGO2 and AGO5 immunoprecipitates isolated from several virus-infected plants have been shown to selectively bind vsiRNAs [75,77].

virus accumulation and virus-induced gene silencing in plants as demonstrated by the inability of the DCL3-dependent dicing reaction alone to limit RNA virus infection [23,25,27]. Recently, an antiviral vsiRNA-programmed AGO-containing complex capable of directing sequence-specific cleavage, rather than translational inhibition, of a transiently expressed sensor construct containing viral segments has been reported [57,78]. Interestingly, cleavage occurs at privileged sites along the viral target sequence at positions that do not necessarily coincide with hotspots of vsiRNA formation. Furthermore, the positive-strand genomic RNA seems to be cleaved in infected plants at roughly the same cleavage positions as those found for the non-replicating sensor constructs [78]. Surprisingly, the cleavage of negative-strand viral RNA has not been observed in these infected plants even though vsiRNAs of sense polarity are more abundant than antisense vsiRNAs [78]. Although these findings are compelling, further studies are needed to confirm that these viral products are *bona fide* cleavage products and assess the impact of vsiRNA-guided viral RNA degradation in resistance responses.

RNA silencing against DNA viruses has a strong transcriptional component. Members of the Geminiviridae family replicate their circular ssDNA genomes to generate minichromosomes of dsRNA [79]. DCL3-dependent, 24 nt vsiRNAs associated with AGO4 are likely to restrict viral transcription by inducing chromatin condensation of nuclear viral episomes and minichromosomes. *Arabidopsis* plants harboring inactivating mutations in methylation pathway components, including *ago4,* are hypersusceptible to geminivirus infection [80].

Do vsiRNAs specifically regulate host gene expression?

Recent reports have suggested that vsiRNAs could be inhibitors of host gene expression [28,30,49,81] (Figure 1). This hypothesis is credible because sorting vsiRNAs into distinct AGO complexes can facilitate functional interaction with host RNA and chromatin, and vsiRNAs from recombinant viruses inhibit the expression of homologous cellular transcripts *in trans* [59,77,82]. By using computational procedures that take into account the thermodynamics and 5′ seed pairing requirements for optimal siRNA and miRNA activity in plants [83,84], it has been possible to identify hundreds of potential mRNA targets for a given plant virus. However, functional interactions between host mRNAs and vsiRNAs resulting in the vsiRNA-guided cleavage of host mRNAs have only been experimentally validated for a couple of genes among the bulk of predicted targets and for which a role in the virus infectious cycle has not been determined [28,49]. Most of the candidate targets tested failed experimental validation using either sensor constructs containing the predicted target sequence or modified RNA ligase-mediated rapid amplification of cDNA ends [15]. Nevertheless, high-throughput technologies based on target enrichment followed by microarray hybridization or deep sequencing should facilitate the identification of authentic RNA targets of vsiRNAs on a genome-wide scale [85–87].

vsiRNAs might also interact with host chromosomes to interfere with gene expression at a transcriptional level. For instance, vsiRNAs with a 5′ terminal adenine might associate to AGO4 and direct DNA methylation and transcriptional gene silencing at specific genomic loci that share sequence complementarity with the vsiRNA. In support of this hypothesis, virus vectors engineered to carry promoter rather than transcribed sequences trigger transcriptional gene silencing through the sequence-specific epigenetic modifications of DNA and chromatin [88,89].

Outstanding questions

The potential of plant viruses to generate complex populations of vsiRNAs has profound implications in the crosstalk interactions between plants and viruses. Outstanding advances have recently been made in our comprehension of vsiRNA metabolism and function. The current picture of vsiRNA-directed silencing pathways is, however, far from complete and many important questions remain. Further studies should clarify whether dsRNAs directly arising from structural features or the intermolecular base pairing of viral RNA strands synthesized by viral RNA polymerases exist in the infected tissue and to what extent they contribute to the formation of primary vsiRNAs. A satisfactory explanation for the uneven spatial and polarity distribution of vsiRNAs in the infected tissue will require fine-tuned analyses of the structural features that determined preferential DCL and RDR activities taking into account the influence of both host and viral factors. Another fundamental question is related to the function and mode of action (endonucleolytic cleavage versus translational arrest) of vsiRNAs in RNA silencing-based immunity because little is known about the molecular bases of viral RNA targeting by vsiRNAs. Genome-scale approaches for vsiRNA host target identification followed by functional analyses are needed to assess the biological significance of vsiRNA-mediated control in gene regulation, viral pathogenesis or host responses.

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