Small RNAs in viral infection and host defense

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Small RNAs are the key mediators of RNA silencing and related pathways in plants and other eukaryotic organisms. Silencing pathways couple the destruction of double-stranded RNA with the use of the resulting small RNAs to target other nucleic acid molecules that contain the complementary sequence. This discovery has revolutionized our ideas about host defense and genetic regulatory mechanisms in eukaryotes. Small RNAs can direct the degradation of mRNAs and single-stranded viral RNAs, the modification of DNA and histones, and the inhibition of translation. Viruses might even use small RNAs to do some targeting of their own to manipulate host gene expression. This review highlights the current understanding and new insights concerning the roles of small RNAs in virus infection and host defense in plants.

Small RNA pathways in antiviral defense

RNA silencing is an inducible defense pathway that uses small interfering RNAs (siRNAs) to specifically target and inactivate invading nucleic acids. The role of siRNAs in defense against plant viruses became clear nearly a decade ago in the wake of two discoveries. First, virus infection induced destruction of RNAs related to the invading viral genome, conferring resistance against the virus and suggesting the existence of an RNA-directed antiviral defense mechanism [1]. Second, plant viruses were found to encode potent suppressors of silencing, an effective counter-defensive strategy [2–4]. Today, we know that plants and other eukaryotic organisms have a variety of endogenous small RNAs that share many common features with the siRNAs produced during virus infection. These small RNAs include microRNAs (miRNAs), trans-acting siRNAs, repeat- or heterochromatin-associated siRNAs, and natural antisense transcript siRNAs [5,6]. These endogenous small RNAs have important roles in many aspects of gene regulation in plants, controlling developmental programming in addition to stress responses. Here, we focus on the role of small RNA pathways in defensive and counter-defensive mechanisms during viral infection in plants.

The workhorses of the small RNA pathways are two families of proteins that both have ribonuclease activity: ‘dicers’ and ‘slicers’ (Figure 1). Members of the Dicer-like (DCL) family have RNaseIII-type activity and process double-stranded (ds) RNAs to generate siRNAs (Figure 1a). In Arabidopsis thaliana, siRNAs are 21-, 22- or 24-nucleotides (nt) long, depending on whether they were produced by DCL4, DCL2 or DCL3, respectively. DCL1 also produces 21-nt small RNAs and serves primarily in the production of miRNAs. Members of the Argo-naut (AGO) family, dubbed ‘slicers’, have RNaseH-type activity and cleave single-stranded (ss) RNA. A single AGO protein bound to a single strand of siRNA forms the core of the sequence-specific effector complex (i.e. the RNA-induced silencing complex [RISC]) that targets and cleaves ssRNA (i.e. post-transcriptional silencing) or methylates DNA and histones (i.e. transcriptional silencing) (Figure 1b). There are ten AGO genes in plants, and recent reports indicate that at least some of these have evolved to selectively bind to small RNAs and to participate in specific small RNA pathways [7,8]. Many excellent reviews cover dicers, slicers and silencing complexes in great detail [5,6,9].

The importance of systemic silencing in antiviral defense

There are two major ways in which RNA silencing thwarts viral multiplication in plants. The first is to limit accumulation of viral RNA in initially infected cells. This is achieved by processing viral dsRNAs into viral siRNAs, which are then used to target destruction of additional viral RNA molecules. The second is to prime distant tissues for a rapid antiviral response by producing a systemic silencing signal that moves along the same routes as the virus, spreading silencing to all parts of the plant. Two phenomena that were described early on by classical plant virologists suggested that the major antiviral impact of silencing is in the systemic response. The first phenomenon, called recovery, refers to the complete loss of viral disease symptoms and virus accumulation in the upper, systemically infected parts of an infected plant. The second phenomenon, called plant viral synergism, refers to the greatly increased disease symptoms and virus accumulation that sometimes occurs when two viruses infect the same host, particularly in systemically infected leaves (Figure 2) [10,11]. We now know that recovery is the result of highly effective antiviral silencing, which enables the host to effectively block systemic infection (Figure 2a) [12]. By contrast, synergism is the result of very effective suppression of silencing by one virus, resulting in a dramatic increase in the accumulation of the co-infecting virus, again primarily in the systemic leaves (Figure 2b) [2]. In many plant–virus synergisms, a member of the potyvirus...
group provides potent suppression of silencing, resulting in increased accumulation of a broad range of heterologous viruses, such as potato virus X (PVX). Interestingly, PVX infection of a transgenic plant expressing high levels of P1/HC-Pro, the potyviral suppressor of silencing, produced the same pattern of synergistic symptoms as the dual infection, displaying severe symptoms only in the systemic leaves [13,14]. Thus, the major impact of suppression of silencing on infection was in systemic tissues, even in plants in which silencing had been suppressed at the onset of infection. In the case of RNA viruses, which constitute the vast majority of plant viruses [15], the importance of the systemic response probably reflects the importance of viral replication in the induction of silencing [16]. Thus, in initially infected tissues, the onset of silencing for RNA viruses necessarily lags behind the onset of viral replication. By contrast, the systemic signal gives distant tissues the ability to target an incoming virus before it replicates, thereby improving the plant’s chances of blocking infection in those tissues.

**Origin of viral siRNAs**

Viral siRNAs can be grouped into three classes based on the source of the dsRNA from which they arise. dsRNA can be produced through different mechanisms during virus infection, and siRNAs from the different sources might well have different roles. All four *Arabidopsis* DCL enzymes appear to be involved – directly or indirectly – in the production of siRNAs from plant DNA viruses, whereas the activities of DCL4 and DCL2 dominate the production of siRNAs from plant ssRNA viruses [17–19].

**Primary siRNAs**

This class of siRNAs derives from processing the dsRNA produced by one of two routes: replication of viral RNA genomes in the cytoplasm; or bi-directional transcription of DNA viruses in the nucleus (Figure 3a). These long dsRNAs are considered to be the major natural substrate for the formation of viral siRNAs, because approximately equal amounts of sense and antisense viral siRNAs accumulate in the case of several virus species [20–22]. High-throughput sequencing of small RNAs from virus-infected maize revealed approximately equal amounts of sense and antisense viral siRNAs in thousands of viral siRNAs in the case of three different ssRNA viruses (V. Vance, unpublished), providing further evidence that viral siRNAs derive primarily from long dsRNA replication intermediates.

**Figure 1.** Dicer versus Slicer function. (a) In plants, Dicer function is performed by the DCL family of enzymes, which cleave dsRNA to produce siRNAs. (b) A single siRNA strand bound by a member of the AGO family of enzymes is incorporated into an RNA-induced silencing complex (RISC). Depending on the identity of the AGO protein, and possibly other proteins, the complex can target either ssRNA or DNA complementary to the bound strand of siRNA.

**Figure 2.** Recovery and synergism. (a) Recovery: tobacco plants infected with tomato black ring nepovirus (TBRV) show pronounced symptoms on the initially infected lower leaves, whereas upper leaves show no symptoms, owing to induction of highly effective silencing. (b) Synergism: tobacco plants infected singly with either potato virus X (PVX) or potato virus Y (PVY) show mild symptoms, whereas doubly infected plants develop a severe synergistic disease in systemically infected leaves. The synergistic disease results from suppression of silencing, mediated by the P1/HC-Pro suppressor of silencing encoded by PVY. This leads to a dramatic increase in PVX accumulation in the systemically infected leaves.
Secondary siRNAs

These siRNAs arise by a process known as transitive silencing, in which dsRNA is produced from ssRNA templates by cellular RNA-dependent RNA polymerase (RDR) activity (Figure 3b). Plant virologists noted three decades ago that host RDR activity was stimulated in virus infection [23,24], but the likely function of this activity was not understood until the discovery of RNA silencing. In Arabidopsis, the RDR gene family has six members, two of which have been implicated in antiviral defense. RDR1 is induced by the defense regulatory molecule salicylic acid and is likely to have an important role in virus-induced silencing [25]. RDR6 is required for transitive silencing of transgenes, and it also enables the plant to respond to the systemic silencing signal, suggesting that transitivity has a key role in antiviral defense [26–29]. Consistent with a role for host RDRs in antiviral defense, loss of cellular RDR activity increased susceptibility to virus infection, and systemic infection is especially affected in some cases [25,28–31]. In addition, several different viral suppressors of silencing specifically block accumulation of the secondary siRNAs that arise through transitive silencing during transgene-induced RNA silencing, again pointing to the importance of transitive silencing in antiviral defense [32,33]. Although viruses have long been known to induce production of secondary siRNAs from homologous transgene messages [34], the accumulation of virus-derived secondary siRNAs has only recently been demonstrated [35]. Viral primary siRNAs were expected to have an initiating role in the production of the secondary siRNAs, either as a primer for RDR or through siRNA–RISC-mediated cleavage of ssRNA [36,37]. Biochemical characterization of RDR6 activity, however, showed that the enzyme does not use a primer and does not preferentially recognize the expected products of RISC cleavage [38]. Thus, the details of secondary siRNA production are not yet fully understood.

Structure-associated siRNA

Several studies have reported that imperfectly base-paired intramolecular hairpins in viral genomes or transcripts contribute to the viral siRNA pool in infected plants (Figure 3c) [19,22,39,40]. In contrast to primary and secondary siRNAs that derive from dsRNA, (+) strand siRNAs are disproportionately represented in this class of siRNAs [40,41], reflecting the large differential accumulation of the (+) strand viral genomic RNA versus the (–) strand, which serves as a replicative intermediate. Cauliflower mosaic virus (CaMV) siRNAs that were predicted to target the Arabidopsis genome derive from a highly structured region of the CaMV 35S promoter [19].

Roles for viral siRNAs in host defense

The beauty of RNA silencing as a defense mechanism is that it couples the destruction of the dsRNA triggers of silencing with the use of the resulting siRNAs as specificity...
factors to target other nucleic acid molecules that have complementary sequence. siRNAs can be used in several different ways as specificity factors in antiviral defense.

Cleavage of viral transcripts through siRNA–RISC
The most obvious role of viral siRNAs in antiviral defense is to direct the RISC complex to viral genomic and sub-genomic ssRNAs, thereby targeting those molecules for destruction (Figure 4a). Recent studies have provided evidence suggesting that viral siRNA-containing RISC complexes are present in virus-infected tissues [42,43]. Further evidence suggests that RISC-directed cleavage has an important role in conferring antiviral immunity [44]. The hyper-susceptibility of Arabidopsis ago1 mutants to virus infection supports the idea that RISC-directed cleavage is important in antiviral defense and suggests that AGO1 is the antiviral slicer in plants [45]. Consistent with this hypothesis, AGO1 recruits viral siRNAs in vivo [46]. However, recent work indicates that other AGO proteins (including both AGO2 and AGO5) also bind to viral siRNAs, suggesting a more complicated picture [8]. To date, there is no evidence indicating that viral siRNA-directed translational inhibition occurs in plants.

Transcriptional silencing
In Arabidopsis, transcriptional silencing of transposons, retro-elements and repetitive DNA is associated with DNA methylation and histone modification directed by 24-nt cellular siRNAs produced by DCL3 [47–49]. Given that the transcriptionally active forms of many DNA plant viruses are histone-associated minichromosomes located in the nucleus, viral siRNA-directed transcriptional silencing could potentially provide an additional defense against these viruses (Figure 4b). To date, there is only indirect evidence that this mechanism is used as an anti-viral defense in plants: (i) both RNA and DNA plant viruses induce promoter methylation and transcriptional silencing of transgenes expressed from promoters homologous to those carried on the viruses [50,51]; (ii) plants infected with a member of the geminivirus group of DNA viruses recover from infection after treatment with dsRNA targeting a viral promoter [52]; and (iii) the geminivirus silencing suppressor, AL2, inactivates adenosine kinase, a host enzyme important in maintaining methylation capability [53].

A possible role for viral siRNAs in systemic silencing
The mobile silencing signal moves along the same route as the virus infection: initially it moves from cell to cell through plasmodesmata; then it enters the phloem for long-distance movement; and finally it exits the phloem and again moves from cell to cell (Figure 4c) [54–57]. To be an effective deterrent, the signal must move out of initially infected cells in advance of the movement of the infectious viral RNA, thereby establishing antiviral silencing ahead of the viral infection front. Although many studies have focused on the appealing idea that siRNAs are the signal, the identity of the systemic silencing signal has remained elusive.

Given that transgene silencing is thought to mirror the viral defense pathway [58], studies of non-cell autonomous silencing induced by transgenes offer insights into the genetics of systemic antiviral silencing. Early work based on transient silencing assays suggested that 24-nt siRNAs were correlated with vascular spread of silencing, whereas the cell-to-cell spread of silencing was correlated with 21-nt siRNAs [59]. However, grafting experiments in tobacco (Nicotiana tabacum) argued against this idea, showing...
that rootstocks that contained no detectable siRNAs mediated robust systemic silencing [60]. The role of siRNAs in systemic silencing has remained a controversial issue, especially with regard to systemic silencing. Experiments in Arabidopsis provide further support for the idea that 21-nt siRNAs have a role in cell-to-cell movement of silencing [61]. In this study, silencing of an endogenous gene by phloem-specific expression of a hairpin transgene was reported to exit the vascular cells and move from cell to cell for distances of up to ~15 cells. DCL4 was required for this cell-to-cell movement of silencing; however, given that DCL4 was also required for cell autonomous silencing in this system, a specific role for siRNAs in movement was not actually demonstrated. Two studies have shown that RDR6 is required for response to the mobile silencing signal, but not for production of the signal, arguing that secondary siRNAs do not have a role in signaling [26,29]. Indeed, grafting experiments in Arabidopsis failed to identify any genes required for the generation and/or sending of the mobile silencing signal [26]. Even del2–del3–del4 triple mutant rootstocks, which are defective for all three DCL genes involved in siRNA production, sent a silencing signal – again arguing that siRNAs are not required in signaling. Thus, the identity of the systemic signal remains unclear.

Endogenous small RNAs in antiviral host defense

There is emerging evidence indicating that endogenous small RNAs have regulatory roles in plant defense responses. Several studies implicate miRNAs in the regulation of plant defenses: miRNA repression of auxin signaling increased bacterial resistance [62], and several newly identified Arabidopsis miRNAs have been shown to target genes that have predicted roles in disease resistance [63,64]. Evidence for endogenous siRNA regulation of plant defenses comes from the discovery of a bacterially induced endogenous siRNA that represses a negative regulator of a host resistance pathway in Arabidopsis [65]. This raises the possibility that a similar process exists in antiviral defense.

In contrast to mammals, in which a direct antiviral effect of a cellular miRNA was reported [66], no direct antiviral activity of plant-encoded small RNAs in natural plant virus infections has been observed to date. Several studies, however, have demonstrated that miRNAs can be used to confer virus resistance in plants. These studies have either inserted miRNA target sequences into a virus or used artificial miRNAs designed to target natural virus sequences [67–69]. The engineered viruses rapidly accumulated mutations within the miRNA target sequence [69], suggesting that the high evolution rates of plant RNA viruses prevent the use of miRNA targeting as a natural antiviral strategy in plants. However, it remains to be seen whether wild type viruses targeted by artificial miRNA constructs undergo similar mutations to evade miRNA targeting.

Small RNA pathways in counter-defensive mechanisms

Lessons from viral suppressors of silencing

Since the discovery of the first viral suppressors of silencing in 1998, the literature has been replete with reports of plant viral proteins that block silencing and with clues as to their mechanism of action [70–72]. Two major mechanistic themes for suppressors have emerged: some interfere with biogenesis of siRNAs, and some interfere with siRNA function. Here, we focus on some recent insights from studies of viral suppressors of silencing.

Suppressors and siRNA biogenesis

Much of the early work on plant viral suppressors examined their role in transgene-induced silencing. Those studies did not discriminate between primary and secondary siRNAs, and this led to confusion in the literature about whether a given suppressor did or did not block siRNA production. The seemingly contradictory results have now been resolved, with the finding that some viral suppressors (i.e. P1/HC-Pro, P38, P19 and P122) specifically block accumulation of secondary siRNAs and leave primary siRNA accumulation unimpaired, whereas other viral suppressors (i.e. P15 and P25) block accumulation of primary siRNAs [32,33,73]. The specific blockage in secondary siRNA accumulation might be produced simply by inhibiting primary siRNA function. However, P1/HC-Pro and P38 eliminate accumulation of secondary siRNAs in sense transgene-induced silencing, a system in which there are no known primary siRNAs [32,60]. This suggests that the blockage is independent of primary siRNAs, at least in some cases. Given that transitive silencing is required for the response to the systemic silencing signal [26,29], the fact that viral suppressors eliminate the accumulation of secondary siRNAs (which arise through transitive silencing) provides additional evidence for the importance of the systemic antiviral response.

Suppressors and small RNA function

The finding that primary siRNAs accumulate to high levels in the presence of certain suppressors, but that degradation of target RNAs is blocked, indicates that the primary siRNAs are not functional. In this regard, there are many reports of viral suppressors of silencing binding to small RNA duplexes, thereby sequestering them and preventing their incorporation into the RISC effector complex [72,74–76]. Indeed, sequestration of siRNAs has been proposed as a general mode of action for viral suppression of silencing [74]. Viral suppressors can also alter the biochemical structure of siRNAs, and this might well have a part in blocking their function. Previous studies have shown that plant endogenous small RNAs and transgene siRNAs are methylated at their 3’ termini, a HUA ENHANCER 1 (HEN1)-dependent step in their biogenesis [77,78]. Methylation of viral siRNAs has also been demonstrated in plants infected with either DNA or RNA viruses, and several viruses and viral suppressors have been shown to interfere with both siRNA and miRNA methylation [73,79–82]. Furthermore, virus alteration of host miRNA accumulation and function is thought to underlie at least some symptoms of plant virus infection [83]. Although most such studies have focused on the role of viral suppressors, a recent study has shown that expression of other viral proteins can also affect miRNA accumulation and function [84].
Multifunctional nature of suppressors

Whereas many suppressor studies focus on understanding a particular mechanism of action, it should be noted that, as with all viral proteins, the viral suppressors have multiple roles. The cucumber mosaic virus (CMV) suppressor 2b is a prime example of a viral suppressor of silencing that interferes with multiple aspects of silencing. Early studies demonstrated that CMV 2b blocked delivery of the systemic silencing signal [70,85]. Subsequently, CMV 2b was shown to interact directly with AGO1, inhibiting slicer activity and thereby blocking RISC function [46]. Finally -- for now anyway -- CMV 2b has been shown to block silencing indirectly by interfering with the salicylic acid-mediated defense pathway: CMV 2b interferes with the activity of RDR1, a salicylic acid-inducible RNA-dependent RNA polymerase that has a role in producing viral siRNAs [35].

Do viral siRNAs target host genes?

The idea that viruses encode siRNAs that target host RNAs is an intriguing one. Some animal viruses encode miRNAs that have been shown to target host genes [86–88], but evidence for a similar strategy in plant virus infection is only beginning to emerge. The first indication came from studies on viroids, subviral entities with highly structured ssRNA genomes that do not encode proteins. Viroid infections induce host-specific disease symptoms associated with accumulation of siRNAs. The viroid siRNAs in these infections have minimal effect on the pathogen genome, raising the possibility that they serve instead to target host genes [89]. The same authors showed that transgenic expression of a non-infectious viroid hairpin RNA induced siRNA accumulation and symptoms similar to viroid infection, again suggesting that viroid siRNAs modulate host gene function. To date, however, no direct evidence that a viroid siRNA interferes with host gene expression has been reported. More recently, an in silico analysis indicated that the highly structured leader region of CaMV 35S viral transcripts gives rise to two siRNAs with the potential to target more than a hundred host genes [19]. During CaMV infection, three of the predicted target transcripts, in addition to a sensor transcript carrying one of the 35S leader siRNA sequences, were downregulated, suggesting that viral siRNAs were targeting host genes (Figure 4d) [19]. However, a null mutation in one of the putative gene targets had no effect on CaMV infection. Thus, it remains to be determined whether viral siRNA-mediated downregulation of host gene expression is a bona fide viral counter-defensive mechanism.

Future challenges

Progress in understanding small RNA pathways has proceeded at a rapid pace in the past ten years or so, thanks in part to the discovery that silencing is an antiviral defense mechanism and to insights obtained from using viral systems. The major impact of silencing in antiviral defense appears to be at the level of systemic spread of the virus. However, the identity of the systemic silencing signal and the biogenesis of this molecule(s) are key points that have eluded capture to date. These questions, in addition to the many others that remain to be answered, promise to keep the hunt interesting for some time to come.

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