CURRENT REVIEW

Antiviral Role of Plant-Encoded RNA-Dependent RNA Polymerases Revisited with Deep Sequencing of Small Interfering RNAs of Virus Origin

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Submitted 1 June 2010. Accepted 23 June 2010.

Several recent studies profiled virus-specific small interfering RNAs (vsRNAs) using next generation sequencing platforms and compellingly implicated plant-encoded RNA-dependent RNA polymerases (RDR) in vsRNA biogenesis and vsRNA-mediated antiviral defense. Specifically, both RDR1 and RDR6 were found to contribute to the accumulation of vsRNAs in virus-infected cells. While RDR1 was responsible for the majority of vsRNAs in plants infected with three different viruses, RDR6 acted as a surrogate when RDR1 function was disrupted. Mechanistically, vsRNAs associated with RDR1 mostly mapped to viral RNA regions close to the 5' ends, whereas those associated with RDR6 mapped to more 3' regions and appeared to be dependent on higher viral RNA concentrations. Knocking out both *RDR1* and *RDR6* led to drastically diminished vsRNA levels concomitant with enhanced viral RNA accumulation. In conclusion, these studies established that RDR1 and RDR6 function synergistically to contain RNA virus infections through the RNA silencing-based antiviral defense.

Shortly after the characterization of the first RNA-dependent RNA polymerase (RDR) from tomato (Schiebel et al. 1998), plant-encoded RDR were shown to participate in antiviral defense mechanisms that target RNA viruses. Genetic screening of *Arabidopsis* plants for mutants that restored the expression of a silenced transgene identified RDR6, one of the six RDR encoded by the *Arabidopsis* genome, as a key factor needed for maintenance of transgene silencing (Dalmay et al. 2000; Mourrain et al. 2000). These authors further showed that *rdr6* (initially called *sde1* or *sgs2*) mutant plants displayed enhanced susceptibility to *Cucumber mosaic virus* (CMV) infections but not to other viruses tested. Independently, Xie and associates (2001) observed that knocking down the expression of tobacco *RDR1* with an antisense transgene allowed for the systemic spread of *Potato virus X* (PVX), a virus known to be restricted in inoculated leaves of wild-type tobacco plants. A follow-up study also implicated *Arabidopsis* RDR1 in an antiviral defense process (Yu et al. 2003).

A few additional studies have examined the antiviral defense activities of RDR in other plant species (Qu et al. 2005; Schwach et al. 2005; Yang et al. 2004; Ying et al. 2010). Qu

and associates (2005) and Schwach and associates (2005) independently exposed a unique role of RDR6 in shielding the shoot apices of *Nicotiana benthamiana* plants from virus invasion. By contrast, Yang and associates (2004) discovered that *NbRDR1* is naturally nonfunctional due to a short insertion that disrupts the *NbRDR1* reading frame. Notably, a more recent study by Ying and associates (2010) suggested that restoring the functionality of RDR1 in *N. benthamiana* might render the plants more susceptible to virus infections. Together, these studies seemed to suggest that RDR played minor antiviral roles that were virus-specific. However, due to technical difficulties, these studies did not carefully examine whether production of small interfering RNAs (siRNAs) of virus origin, a hallmark of RNA silencing targeting RNA viruses, was influenced by the activities of RDR.

Most eukaryotic organisms encode a number of mechanistically related protein families that detect and process double-stranded (ds)RNAs in the cells and use the sequence information derived from dsRNAs to silence homologous single stranded (ss)RNAs or genomic DNA (Chapman and Carrington 2007). This surveillance system, dubbed RNA silencing, employs several key proteins that function at different steps of the RNA-silencing cascade. An RNase III-like, dsRNA-specific nuclease, named Dicer in animals and Dicer-like (DCL) in plants, initiates this cascade by processing dsRNAs and yielding small dsRNAs of discrete sizes (21 to 25 nucleotides [nt]) called microRNAs (miRNAs) or siRNAs. These small RNAs are then incorporated into one or more members of the Argonaute (AGO) protein family and direct sequence-specific degradation or translational repression of complementary ssRNAs or transcriptional repression of homologous genomic DNA. Depending on the source of dsRNA, the size of the miRNAs or siRNAs, and the mode of the RNA-silencing action, at least four different endogenous RNA-silencing pathways exist in the model plant *Arabidopsis* (Vazquez et al. 2010). Two RDR are needed to generate or amplify the dsRNA template of DCL in three of these pathways: RDR2 is essential for the biogenesis of *cis*-acting siRNAs, whereas RDR6 participates in the production of transacting siRNAs and natural antisense siRNAs.

RNA silencing plays a critical role in defending plants against virus invasion, as evidenced by the very fact that most of the prevalent plant viruses encode potent suppressors of RNA silencing. Indeed, under most experimental conditions RNA silencing-based antiviral defense is so effectively counteracted by virus-encoded suppressors of RNA silencing (VSR), only mutant viruses with inactivated VSR could be detectably

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subdued by antiviral silencing (Deleris et al. 2006; Diaz-Pendon et al. 2007; Garcia-Ruiz et al. 2010; Qu et al. 2008; Wang et al. 2010). However, since most VSR do not directly interfere with the first step of RNA silencing, which is viral siRNA (vsRNA) biogenesis mediated by DCL, vsRNAs are readily detectable in virus-infected plants. Hence, the antiviral role of plant-encoded RDR could be inferred by assessing whether the accumulation levels of vsRNAs, their distribution on the viral genome, or both are influenced by the functionality of one or more RDR.

A number of next-generation sequencing platforms have become available during the last few years, permitting in-depth profiling of vsRNA populations generated by a variety of viruses, in plants with distinct genetic backgrounds. The purpose of this minireview is to highlight the findings of several of the most-recent publications that used these high-throughput sequencing technologies as the primary tools to address the antiviral role of plant RDR. These vsRNA profiling attempts have implicated at least two RDR (RDR1 and RDR6) in vsRNA biogenesis and plant antiviral defense.

Does the production of *Cymbidium ringspot virus* (CymRSV)-derived vsRNAs bypass RDR?

The first attempt to assess the sequence characteristics of vsRNAs was reported by Molnar and associates (2005), who sequenced 228 vsRNAs cloned from *N. benthamiana* plants infected with CymRSV. Although the vsRNA count was by far unsaturated due to the low-throughput nature of their procedure, the authors were able to make several interesting observations: i) vsRNAs derived from the genomic or positive (+) strand far outnumbered those derived from their negative (–) strand counterpart; ii) most vsRNAs were concentrated in a limited number of hotspots in the CymRSV genome; and iii) double-stranded vsRNAs bound to the p19 suppressor proteins appeared to contain mismatches, as they were more sensitive to RNase A digestion than fully double-stranded controls. Together, these observations led the authors to suggest that vsRNAs were derived from the processing of highly structured regions of genomic RNA rather than from the perfectly paired dsRNAs resulting from RDR activities or viral replication. This conclusion was further reinforced by a recently published study by the same group, using next-generation deep-sequencing technologies, prompting the argument that, at least for some viruses, plant RDR or double-stranded replication intermediates do not contribute significantly to the biogenesis of vsRNAs (Szittyá et al. 2010).

CymRSV is a small RNA virus of family *Tombusviridae*, with a (+) sense RNA genome of about 4,800 nt in length, encoding five different proteins. Its 3'-proximal open reading frame (ORF) encodes p19, a potent VSR that suppresses RNA silencing by binding to vsRNA duplexes and preventing their loading into AGO-based effector complexes (i.e., RNA-induced silencing complexes [RISC]). In the newer study by Szittyá and associates (2010), the authors further extended their vsRNA profiling efforts to the mutant virus that does not encode p19 (Cym19stop). An interesting new observation from this study is that the ratio between vsRNAs of (+) and (–) strand origins decreased dramatically in Cym19stop-infected plants. In fact, it dropped from 16:1 in CymRSV infections to 3:1 in Cym19stop infections. The authors interpreted this as reflecting the lower +/- ratio of viral genomic and subgenomic RNAs in Cym19stop infections and considered this as further evidence supporting their view that vsRNAs were primarily derived from highly structured regions of single-stranded viral RNAs. However, it is important to note that these new data are also consistent with a scenario in which perfectly paired dsRNAs resulting from RDR activities or viral RNA repli-

cation become a more important source of vsRNAs in the absence of VSR.

An important lesson from the study by Szittyá and associates (2010) is that data generated with the newer generation high-throughput sequencing equipment should be interpreted with caution. Specifically, they have encountered drastic, yet repeatable differences between the vsRNA datasets obtained with the 454 and the Solexa platforms. Significantly, a very prominent (+) vsRNA hotspot between nt 600 and 800 of CymRSV RNA, which accounted for about 20% of total vsRNA reads identified with the Solexa platform, was completely absent in the 454 dataset (Szittyá et al. 2010). Although the authors were able to partially correct this bias by comparing the sequencing datasets with results of dot blot hybridizations, the cause of such a discrepancy remains to be resolved.

In light of these inconsistencies, it may not be surprising that yet another CymRSV-specific vsRNA dataset obtained by a different team of investigators appears to deviate from both datasets mentioned above (Donaire et al. 2009). For example, while the overall ratios of vsRNAs of (+/–) strand origins were similar, the data by Donaire and associates (2009) seemed to contain a larger number of hotspots with similar numbers of vsRNA reads. These variations could have been caused by differences in plant growth conditions, which are known to influence the activity of RNA silencing (Szittyá et al. 2003; Qu et al. 2005).

Notably, Donaire and associates (2009) were able to show that several hotspots were associated with the high density of vsRNAs of both polarities, suggesting that these vsRNAs might have been derived from perfectly paired dsRNAs. They then tested the idea that the majority of + sense vsRNAs derived from secondary structures within the (+) strand viral RNAs by sorting vsRNAs within the nine hotspots into predicted secondary structures of CymRSV (+) strand RNA. They found that, while some of the most abundant vsRNAs lay within stem-loop structures predicted to permit the release of vsRNAs in a manner similar to the biogenesis of miRNAs, most others either occupied regions of stem loops unfavorable for DCL processing or were present in RNA segments that are not predicted to be highly structured. These findings led to the conclusion that processing of highly structured regions within (+) strand viral RNA did not account for the occurrence of all vsRNA hotspots (Donaire et al. 2009). Therefore, perfectly paired dsRNAs synthesized by either plant RDR or viral replication could be a source of some vsRNAs in CymRSV infections.

Deep-sequencing datasets of vsRNAs from other viruses predict a more significant role for plant RDR.

Donaire and associates (2009) also profiled the vsRNAs produced by eight other viruses, including CMV, PVX, *Melon necrotic spot carmovirus* (MNSV), *Tobacco rattle tobravirus* (TRV), *Pepper mild mottle tobamovirus* (PMMoV), *Watermelon mosaic potyvirus*, *Turnip mosaic potyvirus* (TuMV), and *Tomato yellow leaf curl begomovirus*, a DNA virus. They found that similar to CymRSV, TRV and PMMoV were associated with vsRNAs that were biased towards (+) strand origin, although the difference was less pronounced than for CymRSV. The +/- ratios of vsRNAs derived from the other six viruses were closer to 1, implying that perfectly paired dsRNAs likely contributed a larger portion of the vsRNA pools. This interpretation is based on the premise that for all of these viruses, (+) strand viral RNA levels are expected to be much higher than (–) strand viral RNA levels. Notably, MNSV, a small virus similar to CymRSV in structure and replication strategy, yielded a vsRNA population with a very different strand bias. Therefore, it appears that the unique vsRNA profile of CymRSV

does not necessarily rule out the involvement of RDR in their biogenesis.

Furthermore, vsRNA hotspots were identified in genomes of all viruses analyzed, regardless of the +/- ratio of vsRNAs (Donaire et al. 2009). This observation, combined with the revelation that a majority of hotspot vsRNAs derived from viral RNA regions without extensive structures, led to the speculation that vsRNA hotspots may represent unknown sequence features other than secondary structures of viral RNAs. These features could enhance the processibility, stability, RISC accessibility of vsRNAs, or even, preferential ligation of certain subpopulations of vsRNAs to the adaptors used to accommodate different sequencing platforms, as illustrated by the different sequence profiles obtained with 454 and Solexa platforms (Szittyá et al. 2010). Indeed, a recent report showed that siRNAs of a green fluorescent protein (GFP) transgene and an inverted-repeat transgene containing partial GFP sequence, both expected to produce siRNAs that are evenly distributed throughout the source RNA with roughly equal reads from both strands, were also concentrated in a few hotspots on the sense strand of the GFP mRNA (Molnar et al. 2010). Together, these data illustrate that vsRNA hotspots concentrated on the (+) strand of viral RNA do not necessarily disqualify perfectly paired dsRNAs as the source of vsRNAs. Nevertheless, more compelling evidence was needed to directly implicate RDR in vsRNA biogenesis.

Direct involvement of RDR in vsRNA biogenesis.

Donaire and associates (2009) documented that PMMoV-specific vsRNAs showed a strong strand bias with (+) strand vsRNAs accounting for about 85% of total reads as well as 80% of total unique reads, and the reads clustered into a limited number of hotspots. Similar observations were made in an independent study by Qi and associates (2009), who profiled vsRNAs produced in *Arabidopsis* plants shortly after being infected by a crucifer strain of *Tobacco mosaic virus* (TMV-cg), a virus closely related to PMMoV. Importantly however, Qi and associates (2009) simultaneously analyzed the TMV-cg vsRNA profiles in *rdrl*- and *rdr6*-null mutant plants (the *rdrl-1* and *rdr6-15* alleles, respectively). Surprisingly, they found that, compared with wild-type *Arabidopsis* plants, the *rdrl-1* plants contained dramatically fewer vsRNA reads (13.3% versus 2%, respectively, of total small RNA reads). *rdr6-15* plants also contained fewer vsRNA reads, although the drop was less dramatic (9.2% of total reads). When the normalized vsRNA reads were compared, it was clear that RDR1 activity accounted for over 80% of the vsRNAs in the wild-type plants. This was the first report that clearly implicated both RDR1 and RDR6 in the biogenesis of vsRNAs.

Even more striking was the finding by Qi and colleagues (2009) that (+) strand-specific siRNAs were relatively more sensitive to the loss of RDR1 and RDR6 functions. They examined the +/- ratio of vsRNAs in the three kinds of plants and found that, while both (+) and (-) strand vsRNA reads were lower in the mutant plants, the drop of + strand vsRNA reads were more pronounced. While the ratio between (+) and (-) vsRNAs was 80:20 in wild-type *Arabidopsis* plants, this ratio changed to 65:35 in the *rdrl-1* plants and 66:34 in *rdr6-15* plants. These results indicate that RDR functions are partly responsible for the strand bias of vsRNAs, which further suggests that at least a subpopulation of vsRNAs of (+) strand polarity are inherently more stable. Hence, it is cautioned that a higher number of reads is not always correlated with more robust biogenesis.

The conclusions of Qi and colleagues (2009) are further supported by a more recent report using a different virus. Through an exhaustive hybridization effort, Garcia-Ruiz and

associates (2010) showed that TuMV-specific vsRNAs were severely reduced in both inoculated and systemically infected leaves of *rdrl Arabidopsis* plants. Importantly, they further demonstrated that a TuMV mutant incapable of silencing suppression regained the ability to move to uninoculated upper parts of the plants when both *RDR1* and *RDR6* were knocked out (*rdrl-1 rdr6-15* double mutants). Together, these recent studies point to a critical role for RDR in vsRNA biogenesis and in antiviral defense.

Complementary roles of RDR1 and RDR6.

With Qi and associates (2009) and Garcia-Ruiz and associates (2010) independently establishing the involvement of both RDR1 and RDR6 in vsRNA biogenesis, the next question was how these two RDR function in antiviral silencing. The more recent paper by Wang and associates (2010) appears to shed some light on this puzzle. Prior to this study, the same group reported that a mutant of the Q strain of CMV (CMVq-Δ2b), in which the VSR of CMV (2b) was truncated and thus non-functional and produced much less vsRNA than the wild-type CMVq in infected *rdrl* plants, thereby establishing a critical role for RDR1 in CMV vsRNA accumulation (Diaz-Pendon et al. 2007). However, they were unable to confirm a direct antiviral role for RDR1, because CMVq viral RNA levels seemed unchanged (Diaz-Pendon et al. 2007). In the recent update by Wang and associates (2010), the authors introduced a similar mutation into the 2b gene of the Fny strain of CMV to create CMVf-Δ2b. The CMVf-Δ2b virus did accumulate significantly higher levels of viral RNA in *rdrl* plants, concomitant with drastically reduced levels of vsRNAs. Surprisingly, no increased accumulation of viral RNAs was observed in *rdr6* plants infected with CMVf-Δ2b, even though an earlier study had implicated *RDR6* in antiviral defense against another strain of wild-type CMV (Mourrain et al. 2000).

Wang and colleagues (2010) then examined the double and triple *rdrl* mutants and they discovered a synergistic interaction between *RDR1* and *RDR6*. While the *rdr6* mutant allele did not significantly influence the viral RNA or vsRNA accumulation in CMVf-Δ2b infections, *rdrl rdr6* double mutant accumulated drastically elevated levels of CMVf-Δ2b RNAs when compared with the *rdrl* or *rdr6* single mutants (Wang et al. 2010). Notably, when *rdrl rdr6* plants were compared with *rdrl* plants, vsRNAs derived from RNA1 and RNA2 of CMV were relatively unchanged, despite the higher levels of viral RNAs that could provide more template for vsRNA production. However, vsRNAs from CMV RNA3 were severely reduced in *rdrl rdr6* plants versus in *rdrl* plants. This result strongly suggests that functional RDR6 disproportionately uses RNA3 and RNA4 (a subgenomic RNA coterminal with RNA3) as templates to produce dsRNA templates for DCL. However, it is important to point out that this function of RDR6 can be complemented by RDR1 as similar reductions in RNA3-specific siRNA levels were not detected in the *rdr6* single mutant. By contrast, RDR6 was unable to complement the loss of RDR1 function. In conclusion, RDR1 and RDR6 both contribute to the production of vsRNAs and the antiviral defense conferred through these vsRNAs, yet they seem to differ in template usage and, hence, are only partially redundant.

The analysis of accumulation profiles of CMVf-Δ2b siRNA sequences from different mutant plants provided a number of novel insights (Wang et al. 2010). First, in all three kinds of plants (wild-type *Arabidopsis*, *rdrl*, and *rdrl rdr2 rdr6*), RNA3-borne vsRNAs were at least four times more abundant than RNA1- or RNA2-borne vsRNAs, despite the smaller size of RNA3. This finding is consistent with the generalization that vsRNA levels are proportional to the accumulation levels of viral RNAs, as RNA3 and RNA4 are the most abundant viral

RNAs in infected cells. When these vsRNA reads were plotted onto the respective genomic RNAs, it became obvious that, despite the presence of multiple hotspots on both (+) and (–) strands, vsRNA reads decreased gradually from 5′ to 3′ of the genomic RNAs. This is true even for RNA3-borne vsRNA, despite a spike of vsRNA reads in its central portion, possibly reflecting the 5′ end of RNA4. This important trend has not been associated with other viruses, probably because a VSR-less mutant virus was used in this study.

More importantly, the bias of vsRNA reads to the 5′ ends of viral RNAs was dependent on the function of RDR1, as this bias disappeared in *rdr1* mutant plants. However, on CMV RNA3, the loss of 5′ end bias is accompanied by a sizable increase of vsRNA reads within the 3′ half of the RNA, corresponding to the capsid protein ORF (Wang et al. 2010). This RNA3-specific increase of vsRNA reads was apparently dependent on RDR6, as evidenced by the lack of this RNA3-specific peak in the vsRNAs from *rdr1 rdr2 rdr6* plants and Northern blots showing that RNA3-borne vsRNAs diminished in *rdr1 rdr6* but not *rdr1* plants.

To summarize, the findings of Wang and associates (2010) not only definitively demonstrated the roles of RDR1 and RDR6 in vsRNA biogenesis, they also established a causal relationship between lower levels of RDR1/RDR6-dependent vsRNAs and higher levels of viral RNAs, thus compellingly implicating both RDR in antiviral defense. They further demonstrated that while RNA1- and RNA2-specific vsRNA were mostly dependent on RDR1 activity, RNA3-specific vsRNAs were more dependent on RDR6 activity. However, this contribution of RDR6 appears to be relatively minor when RDR1 is present, since both vsRNAs and viral RNAs remained at similar levels in wild-type and *rdr6* plants. Together these results suggest that the biogenesis of RDR6-dependent vsRNAs is in response to higher viral RNA levels as a consequence of RDR1 inactivation. These secondary vsRNAs, in turn, target viral RNAs to prevent their further proliferation. Thus, based on these results, one could argue that RDR1 is the primary responder to the presence of viral RNA, whereas RDR6 senses viral RNAs when their levels reach above a certain threshold. Indeed, their deep-sequencing data support such a model by showing that RDR1-dependent vsRNAs tend to concentrate at the 5′ proximal of viral genomic RNAs. This suggests a prompt engagement of viral RNAs by RDR1 as soon as they emerge from protective entities such as invading viral capsids or membrane-shielded viral replication complexes (Schwartz et al. 2002).

Conversely, RDR6-dependent vsRNAs were concentrated in more 3′ areas of genomic RNAs and were most enriched for RNA4, the highest accumulating viral RNA. This observation is in agreement with earlier results showing that RDR6 may contribute to the defense against wild-type viruses (CMV and PVX) (Mourrain et al. 2000; Qu et al. 2005; Schwach et al. 2005). These wild-type viruses would successfully suppress RDR1-mediated antiviral silencing leading to high levels of viral RNAs in the cell that alert RDR6. This model is also consistent with the vsRNA profiles of TMV and TuMV, which identified RDR1 as a primary contributor of vsRNA biogenesis and unraveled a synergistic interaction between RDR1 and RDR6 (Garcia-Ruiz et al. 2010; Qi et al. 2009).

The exceptions.

A critical evaluation of recent studies, especially those that employed deep-sequencing techniques for vsRNA profiling, leads to the conclusion that RDR1 and RDR6 are both involved in antiviral silencing against most of the viruses examined. Based on these newly published data, I argue that certain characteristics of vsRNAs, such as vsRNA hotspots on viral RNA templates and bias of vsRNA reads towards (+) strand viral

RNA, do not preclude the involvement of RDR in their biogenesis. That being said, there is also compelling evidence that some subviral parasites, such as satellite (sat)RNAs and defective interfering (DI) RNAs, likely serve as templates for siRNA production without significant participation of RDR.

SatRNAs are small virus-associated RNA parasites (usually less than 1,000 nt in length) that depend on plant RNA viruses (helper viruses) for their replication and encapsidation but are distinct from their helper viruses at the RNA sequence level. DI RNAs, on the other hand, are parasitic RNAs of RNA viruses that are derived from the helper viral RNAs. Usually DI RNAs are composed of several discontinuous segments of helper virus RNAs that allow them to be replicated and packaged by the helper viruses. Both satRNAs and DI RNAs are known to assume compact structures that are presumably resistant to host defenses (Simon et al. 2004).

CMV is known to be associated with satRNAs. Wang and associates (2010) found that, while in different single, double, or triple *rdr* mutants, a satRNA of CMV replicated to levels that correlated with the RNA levels of CMV, the levels of siRNAs of both (+) and (–) strand origin mimicked those of their cognate satRNA templates, regardless of the genetic background of the plants. This result suggests that, unlike its helper virus (CMV), the biogenesis of satRNA-specific siRNAs does not require the function of RDR. Although sequence data are not available, it was speculated that DI RNAs are likely also resistant to RNA silencing-mediated defense, so that they serve as a source but not a target of vsRNAs (Szittyta et al. 2002).

It is worth noting that siRNAs derived from viroids, which are small circular RNAs that infect plants independent of viruses, appear to be partially influenced by RDR6 functionality (Di Serio et al. 2010). Viroid RNAs are known to form highly compact secondary structures thought to be resistant to siRNA-mediated degradation (Itaya et al. 2007). Nonetheless, viroid-borne siRNAs are abundant in infected plants, and they cluster in a limited number of hotspots in viroid genomes (Navarro et al. 2009). However, for the same reasons outlined above, these characteristics do not exclude the involvement of RDR in generating the dsRNA precursors for DCL. Instead, recent data showed that, at least for some viroids, siRNAs of both (+) and (–) strands are at similar levels (Martinez et al. 2010), which suggests that fully paired dsRNAs are the source of siRNAs. Finally, the study by Di Serio and associates (2010) illustrated clearly that RDR6 of *N. benthamiana* is critically important in excluding viroids from the apical meristems of viroid-infected plants.

Future directions.

In this review, I have summarized key findings from the recent literature that support an active role for RDR1 and RDR6 in antiviral defense against a number of viruses (TMV, CMV, TuMV). Although the vsRNA sequencing data suggest that RDR1 may be able to target viral RNAs as soon as the 5′ end of viral RNA is sensed, exactly how this is accomplished awaits further investigation. It would be interesting to determine if RDR1 is associated with or is in the close vicinity of the cellular location of viral replication. Another important observation made years ago is that RDR1 expression is induced by virus and viroid infections or salicylic acid treatment (Ji and Ding 2001; Xie et al. 2001). Consequently, it is now critical to determine if induced upregulation of RDR1 is needed for its function. This could be addressed by swapping the promoter elements of RDR1 and RDR6. Additionally, it will be imperative to elucidate the structural basis for the template preference of RDR1 and RDR6.

Secondly, while vsRNA biogenesis appears to involve RDR in most plant virus infections, the possibility that highly struc-

tured stem loops present in (+) strand RNAs of most viruses serve as source for some vsRNAs deserves further detailed evaluation. These structures may be highly accessible at the later stage of infection, when (+) strand viral RNAs are often at a very high concentration. In addition, it remains to be evaluated whether the dominance of (+) stranded vsRNAs is functionally relevant. The evidence so far seems to suggest that removal of VSR leads to preferential decline of (+) strand viral RNA levels, which would suggest that (–) strand vsRNAs are more frequently used to target (+) stranded viral RNAs.

Finally, very little is known about the function of RDR3a, RDR3b, and RDR3c, initially named RDR3, RDR4, and RDR5. The genes of these three RDR are clustered on *Arabidopsis* chromosome II (At2g19910, At2g19920, and At2g19930), with an overall sequence similarity of over 80% (Yu et al. 2003). Preliminary annotation appears to suggest that they all have uninterrupted coding capability. It would be informative to learn whether they contribute to the production of a basal level of vsRNAs, as both CMV and TuMV are associated with a low level of vsRNAs even in the *rdrl rdr2 rdr6* triple mutant (Garcia-Ruiz et al. 2010; Wang et al. 2010). Careful examination of these questions is expected to advance our understanding of the antiviral function of RDR and provide new means for managing viral diseases of crop plants.

ACKNOWLEDGMENTS

Space limitations prevented a comprehensive discussion of all relevant results; thus, apologies are extended to authors whose studies were not included in this review. I thank S.-W. Ding for fruitful discussions and K. A. White for critically reading the manuscript. Work in my lab is supported by seed grants from Ohio Agricultural Research and Development Center, Ohio Plant Biotechnology Consortium, and Ohio Soybean Council.

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