CHAPTER 2

RNA Silencing: An Antiviral Mechanism

T. Csorba*, **V. Pantaleo**† and **J. Burgyán***,†

Contents

Abstract

RNA silencing is an evolutionarily conserved sequence-specific gene-inactivation system that also functions as an antiviral mechanism in higher plants and insects. To overcome antiviral RNA silencing, viruses express silencing-suppressor proteins which can counteract the host silencing-based antiviral process. After the discovery of virus-encoded silencing suppressors, it was shown that these viral proteins can target one or more key points in the silencing machinery. Here we review recent progress in our understanding of the mechanism and function of antiviral RNA silencing in plants, and on the virus's counterattack by expression of silencing-suppressor proteins. We also discuss emerging

* Agricultural Biotechnology Center, Plant Biology Institute, P.O. Box 411, H-2101 Gödöllõ, Hungary
† Istituto di Virologia Vegetale del CNR, Torino, Italy

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evidence that RNA silencing and expression of viral silencing-suppressor proteins are tools forged as a consequence of virus–host coevolution for fine-tuning host–pathogen coexistence.

I. INTRODUCTION

Viruses are obligate intracellular pathogens that infect almost all living organisms. They are mostly composed of two, sometimes three components: the viral genome of either DNA or RNA, a protein coat, which protects the genome, and a not obligatory third part, the envelope, which surrounds the virus particles originating mostly from the host (e.g. Rhabdoviridae, Tospoviridae) (Matthews, 1991). Subviral RNAs such as satellite RNAs (satRNAs), defective RNAs (D-RNAs) or defective interfering RNAs (DI-RNAs) are frequently found associated with plant viruses; these RNAs can be distinguished from the viral genome by their dispensability for normal virus propagation. Subviral RNA replication is completely dependent on enzymes encoded by their helper virus and thus amplification is limited to coinfected cells (Simon *et al.*, 2004). Plants are also occasionally infected with viroids, the smallest self-replicating plant pathogens known to date. Their genomes consist of short, naked, circular, single-stranded RNA with a high degree of secondary structure, and without any protein coding capacity (Flores *et al.*, 2005).

The genomes of plant viruses show huge diversity having genomes of DNA, RNA, linear, circular or segmented, single- or double-stranded, positive (+), negative (−) or ambisense (+/−). These differences between viral genomes imply differences in the respective viral replication strategies. RNA viruses encode their own RNA-dependent RNA polymerase (RdRp). The DNA genome of pararetroviruses is replicated by reverse transcription involving RNA and DNA intermediates, through the action of reverse transcriptase encoded by the viral genome; viroids use host DNA-dependent RNA polymerases and replicate via RNA intermediates (Hull, 2002).

The presence and replication of viruses in the host induce diverse mechanisms for combating viral infection at the level of single cells and the whole organism. These mechanisms range from RNA interference a mechanism mainly found in plants and lower eukaryotes, to the sophisticated interferon-regulated gene response of higher animals. Since all types of viruses at a given point of their replication reach the stage of ssRNA or dsRNA, they actively provoke RNA-induced silencing-based host defense responses (Ding and Voinnet, 2007).

RNA silencing relies on small RNA (sRNA) molecules, approximately 21–24 nucleotides long, so-called short interfering RNAs (siRNAs) and micro RNAs (miRNAs) (Hamilton and Baulcombe, 1999; Hamilton *et al.*,

2002; Kim, 2005; Plasterk, 2002). Biochemical and genetic analyses have shown that the core mechanisms of RNA silencing are shared among different eukaryotes (Baulcombe, 2004; Hannon and Conklin, 2004; Meister and Tuschl, 2004; Plasterk, 2002; Voinnet, 2002; Zamore, 2002). RNA silencing is triggered by double-stranded (ds) or self-complementary foldback RNAs that are processed into 21–24 nt short siRNA or miRNA duplexes by the RNase III-type DICER enzymes (Bartel, 2004; Baulcombe, 2004; Bernstein *et al.*, 2001). These miRNAs and siRNAs activate a multiprotein effector complex, the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000; Tomari and Zamore, 2005), of which Argonaute protein (AGO) is the slicer component showing similarity to RNase H (Liu *et al.*, 2004a; Song *et al.*, 2004; Tomari and Zamore, 2005). RISC is the executioner of RNA silencing, inhibiting target RNA expression. The specific recognition of target sequences is guided by the sRNAs through a base-pairing mechanism, whereas the slicing of target RNA is carried out by the AGO proteins at the post-transcriptional or transcriptional levels (Almeida and Allshire, 2005; Bartel, 2004; Brodersen *et al.*, 2008; Eamens, *et al.*, 2008).

Short RNAs can also guide another effector complex, namely the RNA-induced transcriptional gene silencing (RITS) complex to direct the chromatin modification of homologous DNA sequences (Verdel *et al.*, 2004).

RNA silencing regulates several biological processes via down-regulation of gene expression by miRNAs and siRNAs such as developmental timing and patterning, transposon control, DNA methylation and chromatin modification as well as antiviral defense.

One of the best-established functions of RNA silencing is antiviral defense, which was first discovered in plants (Dougherty *et al.*, 1994; Lindbo *et al.*, 1993; Ratcliff *et al.*, 1997). The antiviral functions of RNA silencing are supported by the following observations: first, virus-derived siRNAs (viRNAs) accumulate at high level during viral infections and can effectively target the viral RNA. Second, most if not all plant viruses have evolved virulence factors called viral suppressors of RNA silencing (VSRs) to overcome the RNA silencing-based host defense.

II. RNA-BASED ANTIVIRAL IMMUNITY

The first indications that RNA-mediated responses play an important antiviral role came from observations that transgenic expression of viral sequences protected plants from homologous viruses by conferring a sequence-specific degradation of challenging viral RNAs (Dougherty *et al.*, 1994; Lindbo and Dougherty, 1992). Later it was shown that viruses are potentially both initiators and targets of gene silencing (Ratcliff *et al.*, 1997). Subsequently, it has been shown that several viruses encode

proteins, which suppress RNA silencing-mediated defense (Voinnet *et al.*, 1999) indicating that these pathogens have evolved counter-defensive strategies against RNA silencing.

A. Mounting the antiviral defense

The main steps of mounting antiviral silencing are: (i) activation of RNA silencing in the cell by the incoming viral RNA, where structured or double-stranded RNA molecules are recognized by plant Dicer-like (DCL) enzymes, producing vsiRNAs; (ii) the protection of vsiRNAs by 2′ O-methylation. These vsiRNAs are then recruited by AGO-containing complexes to target cognate viral RNAs. Alternatively these vsiRNAs can enter the plant RNA-dependent RNA polymerase (RDR)-mediated amplification cycle to enhance the antiviral silencing response (Fig. 1).

1. Activation of RNA silencing and production of vsiRNAs

The majority of known plant viruses have RNA genomes and replicate via double-stranded replication intermediates, at first suggesting that these molecules are the main trigger of RNA silencing. However it turned out that induction of the silencing response is much more complex. The probability that viral RNAs are present in a naked form in the plant cell is very small. The majority of viral RNAs are in encapsidated form or in complexes for replication or movement. Moreover viral replication usually takes place inside a specialized replication compartment and the viral dsRNA replication intermediates can immediately be unwound by viral or host RNA helicases (Ahlquist, 2002). It is more likely that the highly structured single-stranded viral RNAs with stem-loop structures are recognized by the silencing machinery, and the double-stranded regions directly chopped by plant DCLs into virus-derived siRNAs (vsiRNAs) (Fig. 1). The sequencing and experimental data of vsiRNAs strongly support this model since the resulting vsiRNA molecules are imperfect duplexes (Molnar *et al.*, 2005) that have a non-random distribution along the viral genome, and they map asymmetrically to the positive strand of the viral RNA (Donaire *et al.*, 2009; Ho *et al.*, 2006; Molnar *et al.*, 2005; Qi *et al.*, 2009; Szittya *et al.*, unpublished results). Similarly, in the case of *Cauliflower mosaic virus* (CaMV) the 35S polycistronic transcript of this dsDNA virus contains an extensive secondary structure, which is the major vsiRNA source (Moissiard and Voinnet, 2006). In viroid-infected plants the strandness of viroid-specific siRNAs is also asymmetrical and they are preferentially derived from the highly structured plus sense viroid RNA sequence (Itaya *et al.*, 2007), although recent deep sequencing data have shown more symmetrical origin of viroid siRNAs (Navarro *et al.*, unpublished results). Furthermore, in plants infected by the *Potyvirus Turnip mosaic virus* (TuMV),

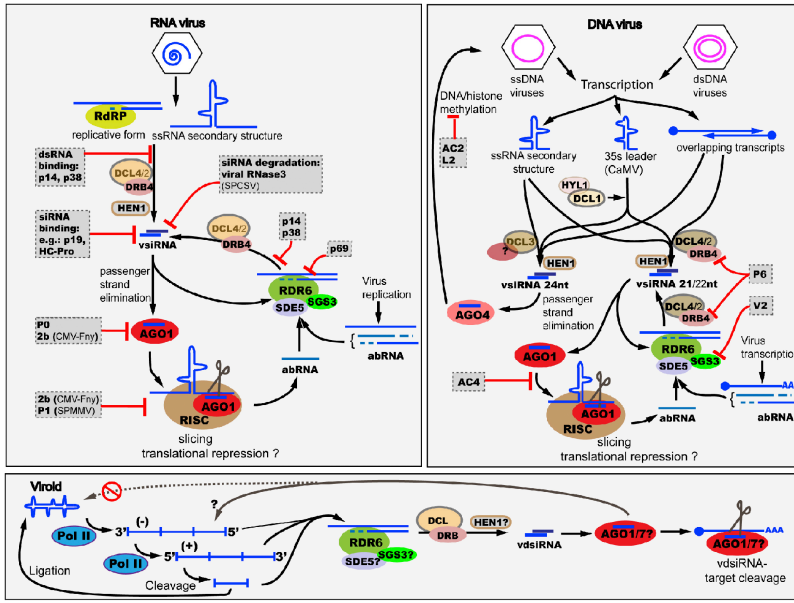

FIGURE 1 Current model of antiviral RNA silencing and its suppression in plants. RNA silencing is initiated by the perception of viral dsRNA or partially double-stranded hairpin RNA, which are processed to 21 nt viral siRNAs (vsiRNAs) by dsRNA-specific RNases called Dicer like 4 (DCL4) in association with dsRNA-binding protein 4 (DRB4). If DCL4 is suppressed or inactive DCL2 can replace it, generating 22 nt vsiRNAs. The vsiRNA are stabilized by 2′-O-methylation by HUA ENHANCER1 (HEN1) and afterward incorporated into Argonaute1 (AGO1) protein, the major antiviral slicer. Other members of the AGO family like AGO7 may be also involved. Plant RISC may also inhibit viral gene expression by translational arrest, although this has not yet been proved to be an active antiviral mechanism. In addition to incorporation into RISC, may vsiRNAs also take part in amplification of the silencing response through the action of RNA-dependent RNA polymerase 6 (RDR6) and its cofactors. The viral RNA molecules cleaved by RISC and viral RNAs lacking 5′- or/ and 3′- end are likely recognized being aberrant RNAs (abRNA) and converted to dsRNA by RDR6 action. This dsRNA processed again vsiRNAs, that leads to generation of more vsiRNA and amplification of the silencing response. In DNA virus infections all four DCLs are involved in the production of 21–23 nt long vsiRNAs. In the case of circular ssDNA geminiviruses highly structured regions of viral ssRNA transcripts or dsRNA molecules formed by overlapping complementary viral RNA transcripts recognized by DCL3/4/2 and processed vsiRNAs. DCL1 has only a very limited role in ssDNA virus-derived vsiRNA production (Blevins *et al.*, 2006). In dsDNA virus infection such as CaMV RNA silencing triggered mainly by the highly structured 35S leader sequence of viral mRNA transcript. DCL3 and DCL4, are the most important dicers implicated in the production of vsiRNAs derived from CaMV transcripts. DCL2 activity is evident especially when DCL4 is inactive. DCL1 has a facilitating role, possibly making the 35S leader sequence more accessible for the other dicers (Moissiard and Voinnet, 2006). The DCL3-dependent 24 nt vsiRNAs are incorporated into AGO4, and may direct DNA/histone methylation of the DNA virus genome in the nucleus. DCL1- and DCL4-dependent 21 nt vsiRNAs are recruited by AGO1 to direct slicing or are implicated in RDR6 pathway-mediated amplification (Ding and Voinnet, 2007). (see Page 3 in Color Section at the back of the book.)

which has a positive ssRNA genome that expresses a polyprotein, the sequenced vsiRNAs showed similar amounts of (+) and (−) strand vsiRNAs (Ho *et al.*, 2006). This result may suggest that the TuMV derived vsiRNAs are processed from dsRNA.

In the case of circular ssDNA geminiviruses a part of vsiRNAs are likely derived from dsRNAs formed by overlapping sense–antisense transcripts (Akbergenov *et al.*, 2006; Blevins *et al.*, 2006; Ding and Voinnet, 2007). These findings demonstrate that the perfect dsRNAs can also be a substrate for vsiRNAs indicating that plant DCLs are adapted to different viral replication and expression strategies and are able to recognize the different RNA structures, which are formed during virus life cycles.

The *Arabidopsis thaliana* genome encodes four DCLs for sRNA processing: DCL1 to DCL4. Specific DCLs have major functions in specific silencing pathways but functional redundancy exists between members: DCL1 contributes to miRNA production and has no or little role in the antiviral response. DCL2, DCL3 and DCL4 are able to recognize viral structures and, respectively, generate vsiRNAs of 22, 24 and 21 nt in length (Blevins *et al.*, 2006; Deleris *et al.*, 2006).

Biogenesis of vsiRNAs needs the coordinated and hierarchical action of DCL enzymes (Moissiard and Voinnet, 2006). RNA virus infection is mainly affected by DCL4 and to a lesser extent by DCL2. Inactivation of DCL4 reveals the subordinate antiviral role of DCL2. Deactivation by mutation of both DCL2 and DCL4 was necessary and sufficient to restore systemic infection of a suppressor-deficient virus, indicating the crucial role of DCL4 and DCL2 in the antiviral response (Bouche *et al.*, 2006; Deleris *et al.*, 2006).

Upon DNA virus infection, the production of 24 nt vsiRNA by DCL3 is also sufficient for virus-induced gene silencing (Blevins *et al.*, 2006). DCL3-dependent 24-nt long vsiRNAs have also been detected in *Tobacco rattle virus* (TRV) and *Cucumber mosaic virus* (CMV) infected wild-type (wt) plants or *Turnip crinkle virus* (TCV) infected *dcl4/dcl2* double mutant *Arabidopsis* plants (Deleris *et al.*, 2006; Qu *et al.*, 2008).

The participation of DCL1 in the antiviral silencing induced by RNA viruses is slight since DCL1-dependent vsiRNAs are hardly detected in the *dcl2/dcl3/dcl4* triple mutant plants (Blevins *et al.*, 2006; Bouche *et al.*, 2006; Deleris *et al.*, 2006). However, DCL1 promotes DCL3- and DCL4-derived vsiRNA accumulation upon dsDNA (CaMV) or ssDNA (geminiviruses) infection. Very likely DCL1 excises the stem-loop structures of 35S leader transcripts, which are very similar to pre- or pri-miRNAs and renders them more accessible to other DCLs (Moissiard and Voinnet, 2006). An opposite effect of DCL1 was found in plants infected with TCV: the disruption of DCL1 function led to higher expression of DCL4 and DCL3, and enhanced antiviral response, suggesting that these proteins are under DCL1-negative control (Qu *et al.*, 2008).

Plant dsRNA-binding proteins (DRBs) have been found associated with DCLs, facilitating their production of sRNAs (Vaucheret, 2006) (Fig. 1). In *Arabidopsis* plants, five DRBs have been identified. While DCLs act redundantly and hierarchically, there is little or no redundancy or hierarchy among the DRBs in their DCL interactions. HYPONASTIC LEAVES1 (HYL1) is a DRB protein that cooperates with DCL1 and is required in processing of miRNA precursors in the plant cell nucleus (Hiraguri *et al*., 2005). DCL4 operates exclusively with DRB4 to produce trans-acting (ta) siRNAs (Adenot *et al*., 2006; Nakazawa *et al*., 2007) and 21 nt siRNAs from viral RNAs (Hiraguri *et al*., 2005). DRB proteins associated with DCL2 and DCL3 are likely involved in vsiRNAs and natural siRNAs generation.

Whether DRBs are also associated with heterochromatic siRNA production has not yet been reported. Co-localization of DCL1 and HYL1 or DCL4 and DRB4 partners suggests that they could form heterodimer complexes (Hiraguri *et al*., 2005). In *drb4* mutant plants, a high level of silencing-suppressor mutant TCV-ΔCP RNA was detected compared to wt plants, but less than in *dcl4* plants, and the vsiRNA accumulation of TCV-ΔCP in *drb4* mutant plants was slightly decreased compared to wt plants. These findings suggest that DRB4 may not be involved directly in vsiRNA production but rather in vsiRNA stabilization or delivery to effector complexes (Qu *et al*., 2008).

2. Protection of sRNAs by 3′ end methylation

The biogenesis of sRNA in plants requires an additional step apart from DCL-mediated processing (Fig. 1). This is a methylation reaction catalyzed by HUA ENHANCER 1 (HEN1) methyltransferase, which links a methyl group to the ribose of the 3′ last nucleotide of the sRNA duplex in a sequence-independent manner (Yu *et al*., 2005). The 2′-O-methylation of 3′end appears to protect sRNA molecules against uridylation (Li *et al*., 2005) and against the exoribonuclease activity of small RNA degrading nucleases (SDN1-3) (Ramachandran and Chen, 2008). All types of endogenous sRNAs are methylated in plants whereas in insects and vertebrates only the germline-specific piRNAs are methylated (Ohara *et al*., 2007). Resistance to β-elimination has proved that plant virus-derived vsiRNAs are also methylated (Akbergenov *et al*., 2006; Blevins *et al*., 2006; Csorba *et al*., 2007; Lozsa *et al*., 2008).

Hen1 mutant plants accumulate less vsiRNAs from both RNA and DNA viruses and exhibit reduced levels of silencing (Blevins *et al*., 2006). HEN1 is the only methyltransferase involved in methylation of vsiRNAs and miRNAs (Csorba *et al*., 2007). Experiments using siRNA-binding suppressors suggest that vsiRNAs are methylated in the cytoplasm while miRNAs are methylated in the nucleus and in the cytoplasm (Lozsa *et al*., 2008). The finding that the p122 suppressor expressed by cr-TMV could

interfere only partially with miRNA methylation supports this scenario. This also suggests that miRNAs are exported from the nucleus in both methylated and non-methylated forms. Strikingly the methylation of miRNAs could not be inhibited in cr-TMV infected HASTY mutant plants (*hst-15*), where the export of miRNA from nucleus to cytoplasm is compromised (Csorba *et al.*, 2007). In line with this observation HEN1 is reported to be present in both the nucleus and the cytoplasm (Fang and Spector, 2007).

B. Effector steps of antiviral silencing

1. Antiviral RNA-induced silencing complexes

The *Arabidopsis* genome contains ten AGO proteins, AGO1 to AGO10, and they are the catalytic components of RNA silencing effector complexes. They interact with small RNAs to effect gene silencing in all RNAi-related pathways known so far (Fig. 1). AGO proteins are characterized by two principal domains: the sRNA-binding PAZ domain at the N-terminus (Ma *et al.*, 2004) and the PIWI domain with its metal-coordinating DDE catalytic triad at the C-terminus, responsible for RNaseH-like "slicer" activity on target ssRNAs complementary to the sRNA loaded within the AGO (Tolia and Joshua-Tor, 2007). The functional equivalent in HsAGO2 contains the DDH motif (Rivas *et al.*, 2005). The presence of the catalytic triad does not necessarily imply slicer activity, indeed miRNA-loaded AGOs can silence gene expression through translational arrest without slicing (Bartel, 2004).

The DCL-mediated processing of viral dsRNA regions into vsiRNA in theory could be enough for viral RNA degradation. However, *dcl2*/*dcl3*, *dcl2*/*dcl4* and *dcl3*/*dcl4* mutant plants infected with TRV had approximately equivalent levels of vsiRNAs but only *dcl2*/*dcl4* plants showed strong viral symptoms and high virus titer (Deleris *et al.*, 2006) suggesting that dicing *per se* is not sufficient for defense against virus infection, and additional effector complex action is required.

AGO1 was suggested to be involved in antiviral silencing, as hypomorphic *ago1* mutants are hypersensitive to CMV infection (Morel *et al.*, 2002). Pull-down experiments revealed that AGO1 recruits miRNAs, tasiRNAs, transgene-derived siRNAs and that AGO1-sRNA complex had slicer activity *in vitro* (Baumberger and Baulcombe, 2005; Qi *et al.*, 2005). Subsequently Zhang *et al.* (2006) have shown that AGO1 also recruits vsiRNAs and the AGO1–vsiRNA complex is a major player in antiviral defense. In addition, very recent studies demonstrated that both AGO2 and AGO5 can bind CMV-derived vsiRNAs, selecting for short RNAs having 5′- A and C nucleotides, respectively (Mi *et al.*, 2008; Takeda *et al.*, 2008).

More direct evidence of the existence of antiviral RISC comes from studies with the positive-strand RNA *Cymbidium ringspot virus*. Two vsiRNA-containing silencing complexes, which co-fractionated with miRNA-containing complexes were detected in infected plants: the smaller one at approximately the AGO1-siRNA size (150 kDa), the so-called minimal-RISC, and a high molecular weight (670 kDa) multiprotein complex (Pantaleo *et al.*, 2007) probably homologous to animal RISC (Pham *et al.*, 2004). A similar complex was isolated in separate experiments involving another tombusvirus. This complex contained vsiRNAs and exhibited *in vitro* nuclease activity, which preferentially targeted homologous viral sequences (Omarov *et al.*, 2007). Strikingly, viral RNA was targeted in a non-random fashion in hotspots by the antiviral RISC in Cym19stop suppressor mutant virus-infected plants (Pantaleo *et al.*, 2007).

Those regions of viral RNA that show hotspots for vsiRNA generation probably form strong secondary structures, which are selectively recognized by DCLs. However, these hotspots are poor targets for RISC-mediated cleavage, since RNA sequences possessing strong secondary structures are not accessible for RISC (Ameres *et al.*, 2007; Pantaleo *et al.*, 2007; Szittya *et al.*, 2002). The accessibility of the viral RNA is probably also influenced by encapsidation, formation of replication complexes containing host and viral proteins and compartmentalization of virus replication. It has been shown recently that there is asymmetry in the strandness of virus-derived siRNAs, showing that the majority of viral siRNAs have plus-stranded viral sequences (Donaire *et al.*, 2009; Ho *et al.*, 2006; Molnar *et al.*, 2005; Qi *et al.*, 2009; Szittya *et al.*, unpublished results). This finding suggests that viral siRNA-guided RISC should target more frequently the viral strand having negative polarity than the plus-stranded viral RNA. Indeed, in previous experiments strand-specific sensors were used for sensing antiviral RISC-mediated cleavages and the sensor RNAs carrying (−) strand sequences were better target than the (+) strand sensors (Pantaleo *et al.*, 2007). It is worth noting that the amount of negative-strand viral RNA is a rate-limiting factor for viral replication; thus preferential targeting of the negative viral strand makes the antiviral silencing response very efficient and very attractive for plant defense. The analysis of 5′-RNA cleavage products of sensor RNAs and viral RNAs reveals the presence of non-templated U residues at the cleavage site (Pantaleo *et al.*, 2007), this is the signature of RISC action (Shen and Goodman, 2004), confirming the presence of RISC-mediated slicing.

According to the current model of virus-induced RNA silencing (Fig. 1) a large amount of vsiRNA originates from partially base-paired regions of plus-stranded viral RNAs (Ding and Voinnet, 2007; Molnar *et al.*, 2005; Szittya *et al.*, unpublished results). Thus plus-stranded vsiRNAs could also potentially target plus-stranded viral RNA through translational arresting. Indeed, recent findings suggest that translational arresting could also be a

widespread way to inhibit gene expression by plant miRNAs and siRNAs (Brodersen *et al.*, 2008; Lanet *et al.*, 2009). Moreover, a novel role of AGO4 has been suggested for specific translational control of viral RNA (Bhattacharjee *et al.*, 2009). AGO7 was shown to function as a surrogate slicer in the absence of AGO1 in the clearance of viral RNA of TCV, and favors less structured RNA targets (Qu *et al.*, 2008).

Another possibility for antiviral defense occurs at the transcriptional level, and is encountered with DNA viruses. *De novo* asymmetric cytosine methylation occurs on *Tomato leaf curl virus* DNA and restricts its replication (Alberter *et al.*, 2005; Bian *et al.*, 2006).

2. Amplification of silencing response

The third family of proteins involved in silencing in plants is the RDR family. In plants there are six RDR paralogs: RDR1, RDR2, RDR3a (RDR3), RDR3b (RDR4), RDR3c (RDR5) and RDR6 (SDE1/SGS-2). The putative catalytic domain is the DLDGD motif, which is highly conserved among all RDRs identified (Wassenegger and Krczal, 2006). In the silencing pathways RDRs synthesize cRNA from the 3′- terminal nucleotides of the template RNA. Then the template and the cRNA remain bound forming a perfectly base-paired dsRNA molecule, which is later processed by DCLs into siRNAs.

Plant RDRs have important homeostatic and defensive functions. The major cellular function of RDR is its involvement in the trans-acting siRNA biogenesis. The process is initiated by miRNA-directed cleavage of non-coding trans-acting siRNA primary transcripts (TAS) (Allen *et al.*, 2005; Howell *et al.*, 2007) and the cleaved TAS RNA is converted to dsRNA by RDR6. The resulting dsRNA is processed by DCL4 to in-phase 21 nt tasiRNAs, which regulate endogenous targets that may control organ development and juvenile-to-adult transition (Hunter *et al.*, 2006).

The other important role of RDRs is defense against selfish and foreign nucleic acids like transposons, transgenes or viruses through the amplification and spreading of RNA silencing. In plants, amplification of the silencing response occurs in at least two different ways (Fig. 1). In the priming-dependent mechanism, viral or transgene-derived primary siRNAs recruit RDRs to the cognate ssRNA, which is converted to dsRNA through synthesis of complementary RNA. This dsRNA is then processed to secondary siRNA by DCLs (Voinnet, 2005). Plant RDRs can also amplify the silencing response in a primer-independent manner, in which RDRs detect the somehow aberrant (different from normal cellular and viral RNAs) RNA molecules deriving from viruses, transgenes or transposons, convert it into dsRNA which becomes the substrate for DCLs, and produce secondary siRNAs. Recent studies have demonstrated experimentally the generation and accumulation of secondary vsiRNAs in plants infected with CMV (Diaz-Pendon *et al.*, 2007). These

siRNAs, upon incorporation into RISC complexes, execute effector steps of silencing and also direct further amplification rounds by releasing the cleaved target RNAs, additional templates for RDR enzymes (Vaucheret, 2006; Voinnet, 2005). These vsiRNA were able to act both in cell-autonomous and non-cell-autonomous fashion (Dunoyer *et al.*, 2005).

De novo dsRNA synthesis mediated by the host RDR pathway may play an important role in antiviral silencing against some viruses such as CMV, since *Arabidopsis* mutants lacking components of the AGO1-RDR6-SGS3-SDE5 pathway show enhanced disease susceptibility (Vaucheret, 2006; Voinnet, 2005). Tobacco plants in which RDR6 activity was silenced are also hypersusceptible to several unrelated (+) ssRNA viruses (Qu *et al.*, 2005; Schwach *et al.*, 2005). However, this is not a general phenomenon for all plant viruses since other studies showed that loss-of-function mutations in RDR6 have no detectable impact on the production of vsiRNAs and virus accumulation in *Arabidopsis* plants infected with TRV, TCV and cr-TMV (Blevins *et al.*, 2006; Dalmay *et al.*, 2000, 2001; Deleris *et al.*, 2006).

The existence of six RDRs suggests redundancy and specialization between RDRs in the different pathways. The nuclear-localized RDR2 is involved in DCL3-AGO4 dependent heterochromatic silencing (Matzke and Birchler, 2005), and RDR1 has a role in defense against tobamoviruses, tobraviruses and potexviruses (Yang *et al.*, 2004; Yu *et al.*, 2003). RDR1 is strongly induced by salicylic acid (Xie *et al.*, 2001), a defense-signaling hormone, whereas RDR6 expression is controlled by the stress hormone abscisic acid (Yang *et al.*, 2008). Recently RDR2 was also implicated in the antiviral defense against TRV, where the major contributors are RDR1 and RDR6 (Donaire *et al.*, 2008). vsiRNA production is strongly reduced in triple *rdr1/rdr2/rdr6* mutants, pointing to the importance of RDR action in generating substrates for DCLs.

It has also been suggested that the RDR-dependent secondary vsiRNAs can drive a more effective antiviral response, against some but not all virus infections (Vaistij and Jones, 2009). These findings indicate that although there are very conserved steps in the silencing-based antiviral response, plants are able to respond specifically to different viruses, demonstrating the versatility of this antiviral surveillance mechanism. Plants attacked by viruses can thus activate alternative pathways to counteract the invasion with an appropriate strategy; this system has likely evolved to face the many different viruses possessing their ample portfolio of replication, infection, transmission and silencing suppression strategies.

III. SILENCING SUPPRESSION STRATEGIES

A decade ago the discovery of VSRs provided the most convincing evidence for the antiviral nature of RNA silencing and revealed the

pathogen counter-defensive strategy of active suppression of host surveillance (Voinnet *et al.*, 1999). More than 50 individual VSRs have been identified from almost all plant virus genera (Table 1), underlining the need of their expression for successful virus infection (Diaz-Pendon and Ding, 2008; Ding and Voinnet, 2007). Available data suggest that virtually all plant viruses encode at least one suppressor, but in many cases viruses encode more than one (e.g. carmo-, clostero-, crini- and begomoviruses; see Table 1).

Viral suppressors are considered to be of recent evolutionary origin, often encoded by out-of-frame ORFs within more ancient genes. They are surprisingly diverse within and across kingdoms with no obvious sequence homology (Ding and Voinnet, 2007). VSRs are variously positioned on the viral genome and expressed using different strategies such as subgenomic RNAs, transcriptional read-through, ribosomal leaky-scanning or proteolytic maturation of polyproteins. Due to their evolution many of the suppressors identified to date are multifunctional: beside being RNA-silencing suppressors they also perform essential roles by functioning as coat protein, replicase, movement protein, helper-component for virus transmission, protease or transcriptional regulators. Virtually all steps of the silencing pathway have been found to be targeted by VSRs; either acting on silencing-related RNA molecules or through protein–protein interaction (Fig. 1; Table 1).

A. Suppressors targeting silencing-related RNAs

The most widely used suppression strategy, adopted by many viral genera (tospo-, cucumo-, poty-, ipomo-, tombus-, clostero-, viti-, tobamo- and hordeiviruses) is ds siRNA sequestration (Lakatos *et al.*, 2006; Merai *et al.*, 2006), which prevents assembly of the RISC effector complex (see Table 1 and the references within). Importantly, these siRNA-binding VSRs are completely unrelated proteins although they share analogous biochemical properties, suggesting their independent evolution in different viruses.

siRNA binding is exemplified by the tombusvirus p19 protein, probably the most studied viral silencing suppressor so far. Crystallographic studies have shown that the head-to-tail p19 homodimer acts like a molecular caliper, which measures the length of siRNAs and binds them with high affinity in a sequence-independent way selecting for the 19 bp long dsRNA duplex region of the typical siRNA (Vargason *et al.*, 2003; Ye *et al.*, 2003). P19 demonstrates extraordinary adaptation of a viral protein to inactivate vsiRNAs, which are the most conserved key element of the antiviral silencing response. Other VSRs such as the *Cucumovirus Tomato aspermy virus* (TAV) 2b protein or B2 of the insect-infecting *Flock House virus* also show siRNA-binding activity, however structural studies have shown that the structures of silencing-suppressor proteins and their mode of binding

TABLE 1 Identified silencing suppressor proteins encoded by plant viruses

Family	Genus	Type species	Supressor	Suppression mechanisms	Other functions	References
dsRNA						
Reoviridae	*Phytoreovirus*	*Rice dwarf virus*	**Pns10**	Upstream to dsRNA	Unknown	Cao *et al.* (2005)
ss (−) RNA						
Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus*	**NSs**	Inhibition of sense-PTGS	Pathogenicity determinant	Bucher *et al.* (2003), Takeda *et al.* (2002)
No family	*Tenuivirus*	*Rice hoja blanca virus*	**NS3**	siRNA binding	Unknown	Hemmes *et al.* (2007)
		Rice stripe virus	**NS3**	ss-,ds-siRNA and ssRNA-binding	Unknown	Xiong *et al.* (2009)
ss (+) RNA						
Bromoviridae	*Cucumovirus*	*Cucumber mosaic virus* (Fny)	**2b**	AGO1 interaction	Host specific movement	Zhang *et al.* (2006)
		Cucumber mosaic virus (CM95R)	**2b**	siRNA binding	Host specific movement	Goto *et al.* (2007)
		Tomato aspermy virus	**2b**	siRNA binding	Host specific movement	Chen *et al.* (2008)
Comoviridae	*Comovirus*	*Cowpea mosaic virus*	**S protein**	Unknown	Small coat protein	Canizares *et al.* (2004), Liu *et al.* (2004b)
Potyviridae	*Potyvirus*	*Potato virus Y*	**HC-Pro**	siRNA binding	Movement, polyprotein processing	Kasschau and Carrington (1998)

Table 1 *(Continued)*

Family	Genus	Type species	Supressor	Suppression mechanisms	Other functions	References
		Tobacco etch virus	**HC-Pro**	siRNA binding	Aphid transmission, pathogenicity determinant	Lakatos *et al.* (2006)
	Ipomovirus	*Cassava brown streak virus*	**P1**	Unknown	Serine proteinase	Mbanzibwa *et al.* (2009)
		Sweet potato mild mottle virus	**P1**	AGO1 interaction	Serine proteinase	Giner *et al.* (unpublished results)
		Cucumber vein yellowing virus	**P1b**	siRNA binding	Serine proteinase	Valli *et al.* (2008)
Tombusviridae	*Tombusvirus*	*Carnation Italian ringspot virus*	**p19**	siRNA binding	Movement, pathogenicity determinant	Silhavy *et al.* (2002), Vargason *et al.* (2003)
		Tobacco bushy stunt virus	**p19**	siRNA binding	Movement, pathogenicity determinant	Voinnet *et al.* (1999)
	Aureusvirus	*Pothos latent virus*	**p14**	dsRNA binding	Pathogenicity determinant	Merai *et al.* (2005)
	Carmovirus	*Turnip crinkle virus*	**p38**	dsRNA binding	Coat protein	Merai *et al.* (2006), Thomas *et al.* (2003)
		Melon necrotic spot virus	**p7B**	Unknown	Movement	Genoves *et al.* (2006)
			p42	Unknown	Coat protein, pathogenicity determinant	Genoves *et al.* (2006)

		Hibiscus chlorotic ringspot virus	**CP**	Downstream to RDR6	Coat protein	Meng *et al.* (2008)
	Dianthovirus	*Red clover necrotic mosaic virus*	**replication**	Host factor sequestration, e.g. DCL1	Replication	Takeda *et al.* (2005)
			MP	Unknown	Movement	Powers *et al.* (2008)
		**Satellite panicum mosaic virus*			**CP** suppressor of VSR	Qiu and Scholthof (2004)
Closteroviridae	*Closterovirus*	*Beet yellows virus*	**P21**	siRNA binding	Replication enhancer	Lakatos *et al.* (2006), Reed *et al.* (2003)
		Citrus tristeza virus	**p20**	Unknown	Replication enhancer	Lu *et al.* (2004)
			p23	Unknown	Nucleic acid binding	Lu *et al.* (2004)
			CP	Unknown	Coat protein	Lu *et al.* (2004)
	Crinivirus	*Sweet potato chlorotic stunt virus*	**RNase3**	siRNA cleavage	Pathogenicity determinant, synergism	Cuellar *et al.* (2009), Kreuze *et al.* (2005)
			p22	Unknown	Pathogenicity determinant	Cuellar *et al.* (2008)
		Cucurbit yellow stunting disorder virus	**p25**	Unknown	Unknown	Kataya *et al.* (2009)
		Tomato chlorosis virus	**p22**	Suppress local RNA silencing	Unknown	Canizares *et al.* (2008)
			CP	Unknown	Coat protein	Canizares *et al.* (2008)

Table 1 *(Continued)*

Family	Genus	Type species	Supressor	Suppression mechanisms	Other functions	References
			CPm	Unknown	Coat protein minor	Canizares *et al.* (2008)
Luteoviridae	*Polerovirus*	*Beet western yellows virus*	**P0**	AGO destabilization	Pathogenicity determinant	Baumberger *et al.* (2007),Bortolamiol *et al.* (2007)
Tymoviridae	*Tymovirus*	*Turnip yellow mosaic virus*	**p69**	Upstream to dsRNA formation	Movement, pathogenicity determinant	Chen *et al.* (2004)
Flexiviridae	*Potexvirus*	*Potato virus X*	**P25**	Inhibits systemic silencing	Movement	Voinnet *et al.* (2000)
	Trichovirus	*Apple chlorotic leafspot virus*	**p50**	Inhibits long distant movement of silencing	Movement	Yaegashi *et al.* (2007,2008)
	Vitivirus	*Grapevine virus A*	**p10**	ss-,ds-siRNA binding	RNA-binding, movement, pathogenicity determinant	Chiba *et al.* (2006), Zhou *et al.* (2006)
No family	*Tobamovirus*	*Tobacco mosaic virus*	**p126**	siRNA binding	Replicase subunit	Harries *et al.* (2008)
		Cr-Tobacco mosaic virus	**p122**	siRNA binding	Replicase subunit	Csorba *et al.* (2007)
		Tomato mosaic virus	**p130**	siRNA binding	Replicase subunit	Kubota *et al.* (2003)
	Tobravirus	*Tobacco rattle virus*	**16K**	Downstream to dsRNA	Unknown	Martinez-Priego *et al.* (2008)

	Furovirus	*Soil-borne wheat mosaic virus*	**19K**	Systemic silencing inhibition	Pathogenicity determinant	Te *et al.* (2005)
	Pecluvirus	*Peanut clump virus*	**P15**	siRNA binding	Intercellular virus movement	Dunoyer *et al.* (2002)
	Benyvirus	*Beet necrotic yellow vein virus*	**p31 (roots)**	Inhibits silencing in roots	Vector transmission, pathogenicity determinant	Rahim *et al.* (2007)
			p14	Unknown	Regulation of RNA2 and CP expression	Dunoyer *et al.* (2002), Rahim *et al.* (2007)
	Hordeivirus	*Barley stripe mosaic virus*	**γB**	siRNA binding	Pathogenicity determinant	Merai *et al.* (2006), Yelina *et al.* (2002)
	Sobemovirus	*Rice yellow mottle virus*	**P1**	Unknown	Movement, pathogenicity determinant, virus accumulation	Sire *et al.* (2008), Voinnet *et al.* (1999)
ssDNA						
Geminiviridae	*Curtovirus*	*Beet curly top virus*	**L2**	Inhibits ADK and transmethylation	Pathogenicity determinant	Wang *et al.* (2003, 2005)
	Begomovirus	*African cassava mosaic virus*	**AC4**	ssRNA binding	Movement, pathogenicity determinant	Bisaro (2006), Chellappan *et al.* (2005)
			AC2		Transcriptional transactivator	Voinnet *et al.* (1999)
		Tomato golden mosaic virus	**AL2**	Inhibits ADK and transmethylation	Synergistic genes: AC2–AC4	Wang *et al.* (2005)

Table 1 *(Continued)*

Family	Genus	Type species	Supressor	Suppression mechanisms	Other functions	References
		Mungbean yellow mosaic virus	**AC2**	Activates endogenous silencing suppressor		Trinks *et al.* (2005)
		Tomato yellow leaf curl virus	**V2**	Inhibits SGS3 activity	Unknown	Fukunaga and Doudna (2009), Glick *et al.* (2008)
		**Satellite DNAβ*	**βC1**	Unknown	Replication, movement	Saunders *et al.* (2004))
dsDNA (RT)						
Caulimoviridae	*Caulimovirus*	*Cauliflower mosaic virus*	**P6**	RDB4 interaction	Translational transactivator	Haas *et al.* (2008), Love *et al.* (2007)

siRNAs do not share any similarity (Chao *et al.*, 2005; Chen *et al.*, 2008). Recently, it was also reported that siRNA-binding suppressors (p19, HC-Pro, p122) may prevent the essential siRNA and miRNA 2′-O-methylation steps in the biogenesis of siRNA and miRNA (Csorba *et al.*, 2007; Ebhardt *et al.*, 2005; Lozsa *et al.*, 2008; Vogler *et al.*, 2007). However, it seems that this inhibitory effect requires temporal and spatial co-expression of the suppressor, endogenous or viral siRNAs and miRNAs (Lozsa *et al.*, 2008). In the presence of siRNA-binding VSRs, plants fail to confine the infection and virus spread occurs, since vsiRNAs are sequestered by the VSRs before they can be incorporated in silencing effector complexes.

A very similar outcome is achieved by adopting a completely different strategy in the case of the *Crinivirus Sweet potato chlorotic stunt virus* (SPCSV). SPCSV-encoded RNase3 endonuclease cleaves the 21-, 22- and 24-vsiRNAs into 14 bp products, which are inactive in the RNA-silencing pathways (Cuellar *et al.*, 2009). The p14 of *Pothos latent aureusvirus* and p38 of *Turnip crinkle virus* (TCV) are potent VSRs and bind long and short dsRNAs (including ds siRNAs) in a size-independent way (Merai *et al.*, 2005, 2006). p14 and p38 may interact with the ds viral RNA, inhibiting the RNA-silencing machinery on two levels: (i) by siRNA sequestration (Merai *et al.*, 2006), and (ii) by interfering with DCL4-mediated vsiRNA processing. The inhibition of DCL4 by p38 has also been confirmed experimentally (Deleris *et al.*, 2006). In contrast to dsRNA-binding VSRs, the AC4 suppressor of *African cassava mosaic virus* binds single-stranded small RNAs bound by AGOs and prevents holo RISC assembly. AC4 also inhibits miRNA-mediated negative regulation of endogenous genes (Chellappan *et al.*, 2005). *Rice stripe virus* NS3 and *Grapevine virus A* p10 proteins are also able to sequester ss-siRNA molecules (Xiong *et al.*, 2009; Zhou *et al.*, 2006) implying, in part at least, a similar strategy to AC4.

Previous studies have shown that the V2 protein from *Tomato yellow leaf curl virus* is an efficient suppressor of RNA silencing (Glick *et al.*, 2008; Zrachya *et al.*, 2007) and V2 was proposed to interact with the tomato protein SGS3 (SlSGS3) in infected plant cells (Glick *et al.*, 2008). However, recent *in vitro* studies on V2 show that it outcompetes SGS3 protein for binding a dsRNA with 5′ ssRNA overhangs, whereas a V2 mutant lacking the suppressor function *in vivo* cannot efficiently overcome SGS3 binding (Fukunaga and Doudna, 2009). These findings not only predict a new type of RNA-binding silencing suppressor but also may reveal a new RNA intermediate, which is essential for SDS3/RDR6-dependent siRNA formation in the plant (Kumakura *et al.*, 2009).

B. Suppressors interacting with silencing-related host proteins

The 2b protein of CMV was one of the first VSRs described (Brigneti *et al.*, 1998). In plants efficient virus infection requires the inhibition of either

the short or long-range silencing signal of antiviral RNA silencing. 2b prevents the spread of the long-range silencing signal, and so facilitate the systemic virus infection (Guo and Ding, 2002). Indeed, the 2b-deficient mutant virus (CMV-Δ2b) replicates in tobacco protoplasts at wt level but its accumulation is 20-fold lower in inoculated tobacco leaves and it is not detectable in the upper leaves (Soards *et al.*, 2002). In inoculated leaves CMV-Δ2b infects plant cells in small isolated spots, whereas wt CMV infects over large areas. CMV-Δ2b can be rescued by the *dcl2/dcl4* host double mutant, which is impaired in vsiRNA production, indicating that 2b is dispensable for infection and spread in a host defective in small RNA-directed immunity (Diaz-Pendon *et al.*, 2007). RDR-dependent CMV vsiRNA production is strongly reduced in the presence of 2b (Diaz-Pendon *et al.*, 2007). This suggests that 2b facilitates short- and long-distance virus spread but in the absence of 2b plant tissues can set up their antiviral machinery, which restricts further spreading of the virus.

Consistently, 2b of Fny-CMV has been found to physically interact on PAZ and part of the PIWI domain with siRNA-loaded AGO1, and inhibits its slicing activity (Zhang *et al.*, 2006). Fny-CMV 2b was found to colocalize with AGO1 protein preferentially in the cell's nucleus but also in cytoplasmic foci (Mayers *et al.*, 2000). Fny-CMV 2b protein expression phenocopies the *ago1-27* mutant phenotype and leads to the accumulation the inactive miRNA duplexes (formed by mature miRNA and the normally labile passenger strand, called miRNA*), and miRNA-target accumulation (Zhang *et al.*, 2006). The phenotype of Fny-CMV 2b expressing transgenic plants is similar to plants expressing other siRNA-binding suppressors (Dunoyer *et al.*, 2004; Lewsey *et al.*, 2007; Zhang *et al.*, 2006).

Chen *et al.* (2008) reported that 2b of TAV a cucumovirus related to CMV binds siRNA duplexes. Analysis of the crystal structure of TAV-2b-siRNA has shown that 2b adopts an alpha-helix structure to form a homodimer, and binds to siRNA by measuring its length, similarly to tombusvirus p19, although, the structures of the two VSRs (p19 and 2b) do not share any similarity. 2b of the severe CMV strain CM95R is also known to bind siRNAs (Goto *et al.*, 2007). Thus, cucumovirus 2b could have a dual mode of action, either sequestering siRNAs or interacting with AGO1.

As recently described, the 29 kDa P0 protein of the phloem-limited poleroviruses targets Argonautes, the core component of RISC for degradation (Baumberger *et al.*, 2007; Bortolamiol *et al.*, 2007; Pazhouhandeh *et al.*, 2006). This protein is indispensable for viral infection. Null mutations of P0 in *Beet western yellows virus* (BWYV) and *Potato leafroll virus* strongly diminish or completely abolish virus accumulation (Mayo and Ziegler-Graff, 1996). In contrast to the RNA-binding VSRs, P0 has no

RNA-binding activity (Zhang *et al*., 2006) (Csorba *et al*., unpublished results); instead it interacts with the SCF family of E3-ligase SKP1 (S-phase kinase-related protein 1) components orthologous to *Arabidopsis* ASK1 and ASK2, by means of its minimal F-box motif and promotes Argonaute degradation. Disruption of the F-box motif by mutation annuls P0 silencing-suppressor activity. Downregulation of SKP homologues in *Nicotiana benthamiana* plants by virus-induced gene silencing leads to resistance against BWYV infection (Pazhouhandeh *et al*., 2006).

The P0 is suggested to interact with PAZ and adjacent upstream domains of multiple Argonautes (AGO1, AGO2, AGO4-6, AGO9), however this interaction is probably transient or indirect *in vivo*. AGO degradation seems to be 26S proteasome-independent (Baumberger *et al*., 2007) probably involving other cellular proteases. Transgenic expression of P0 in *Arabidopsis* leads to severe developmental abnormalities similar to those induced by mutants affecting miRNA pathways, which is accompanied by AGO1 protein decay *in planta* and enhanced levels of several miRNA-target transcripts (Bortolamiol *et al*., 2007).

Earlier results suggested that the impact of P0 on plant endogenous silencing pathways is so devastating that it is unfavorable even for the virus itself (Pfeffer *et al*., 2002). In natural virus infection P0 expression is limited by a suboptimal translation initiation codon. Attempts to optimize the translation initiation region have failed: backward mutations restored the poor translation initiation codon characteristic to the wt or ended up in additional mutation creating a termination codon downstream (Pfeffer *et al*., 2002), showing that P0 overexpression is unfavorable for the virus.

Interestingly, large amounts of polyubiquitinated proteins accumulate upon BWYV P0 ectopic expression (Csorba *et al*., unpublished results), suggesting that BWYV P0 may have multiple targets in the cell or induces protein-based immunity; this points to a link between RNA silencing and protein-based defense strategies. This idea is supported by the fact that transient expression of P0 induces a dose-dependent cell death phenotype similar to that caused by the P0 of *Sugarcane yellow leaf virus*, another polerovirus (Mangwende *et al*., 2009).

A new type of AGO-interacting VSR was recently characterized in our laboratory. The P1 suppressor of the *Ipomovirus Sweet potato mild mottle virus*, seems to act by inhibition of siRNA and miRNA programmed RISC through targeting AGO1. Suppression activity was mapped to the N-terminal part of P1, a region containing WG/GW motifs essential both for AGO binding and for suppression (Giner *et al*., unpublished results). The conserved GW182 family of proteins has recently been identified and the family members have been shown to be associated with miRISC and to be required for miRNA-mediated gene silencing. Proteins containing WG/GW motifs have been found in

animals and plants where they are thought to bind AGOs and be required for proper RISC function. In animals, the GW182 proteins, such as P-body components, have been found essential for miRNA-induced silencing and mRNA degradation (Behm-Ansmant *et al.*, 2006; Eulalio *et al.*, 2008; Liu *et al.*, 2005). The plant RNA polymerase IVb also contains several WG/GW motifs, which are required for AGO4 binding and RNA-directed DNA methylation (RdDM) (El-Shami *et al.*, 2007). Recently, KTF1 protein containing WG/GW motifs and SPT5-like domains has been identified as AGO4-binding proteins playing an important role in RdDM (Bies-Etheve *et al.*, 2009; He *et al.*, 2009). Thus, the action of P1 represents a novel mode of RNA-silencing suppression, which might act by out-competing cellular components with similar motifs, and that this is radically different from other VSR mechanisms described.

C. Other silencing suppressor strategies

The p69 protein of the positive-strand RNA *Turnip yellow mosaic virus* (TYMV) suppresses RNA silencing induced by sense–transgenes (S-PTGS) but not silencing induced by inverted-repeat transgenes (IR-PTGS) (Chen *et al.*, 2004); the negative-strand RNA virus *Tomato spotted wilt virus* (TSWV) encodes a silencing suppressor, the NSs protein, which appears to adopt a mechanistically similar strategy. In a transient co-expression assay, NSs suppresses local and systemic S-PTGS, but not IR-PTGS (Takeda *et al.*, 2002). This suggests that these suppressors could interfere with dsRNA generation by inhibition of plant RDRs or other components of this pathway. Consistent with these observations is the fact that p69 expression leads to a phenotype characteristic for *rdr6* mutant (Chen *et al.*, 2004; Dalmay *et al.*, 2000).

Suppressors from the *Geminiviridae* family nicely exemplify that silencing suppressors may modulate endogenous biochemical pathways for virus benefit. The *Tomato golden mosaic virus* (TGMV)-encoded AL2 protein and the closely related *Beet curly top virus* (BCTV) L2 interact with and inactivate adenosine kinase (ADK), a cellular enzyme important for adenosine salvage and the methyl cycle. Plants infected with the *l2* mutant BCTV and other unrelated viruses display increased ADK activity, suggesting that ADK could be part of a plant response to virus infection (Wang *et al.*, 2003). ADK has a role in sustaining the methylation cycle. By inhibiting ADK, the AL2 and L2 proteins indirectly block this cycle, and thus could interfere with epigenic modification of the viral genome (Bisaro, 2006; Wang *et al.*, 2005). *In vitro* methylated TGMV cannot replicate in protoplasts (Bisaro, 2006), suggesting that the methylation of the viral genome could be a valid mode to combat geminivirus infection.

Evidence concerning the transcription-dependent activity of *Mungbean yellow mosaic virus* (MYMV) and *African cassava mosaic virus* (ACMV) protein AC2 has also been obtained. AC2 induces expression of more than 30 host genes, including *Werner exonuclease-like 1* (*WEL1*) an endogenous negative regulator of silencing (Trinks *et al.*, 2005). The picture is more complex since these genes also include positive regulators of silencing. AC4 of ACMV but not that of *East African cassava mosaic virus* was suggested to bind ss-siRNAs and miRNAs. Thus AC4 uses a novel mechanism different from that of AC2, to block silencing by interfering with RISC loading downstream to ds sRNA unwinding.

AC4 expression in transgenic plants leads to severe developmental defects since miRNA pathway is also disrupted (Chellappan *et al.*, 2005). In the presence of AC4 the level of miRNA targets is upregulated, but miRNA level is downregulated. This implies that AC4-mediated sequestration of ss-sRNA has a different outcome to that of the siRNA- and miRNA-binding suppressors, where the RNA duplexes are stabilized by the suppressors. The different geminiviral AC2 and AC4 proteins are not equally efficient in suppressing silencing, and the presence of two different mechanisms may explain in part the synergy observed in mixed geminivirus infections (Vanitharani *et al.*, 2004).

Host factors involved in both RNA-silencing suppression and viral replication have been proposed to play roles in RNA-silencing suppression during infection by *Red clover necrotic mosaic virus* (RCNMV). Upon RCMV infection there is a close relationship between negative-strand RNA synthesis and RNA-silencing suppression. It has been suggested that sequestration of host factors required for antiviral silencing could reduce the silencing response. The putative host factor involved in both processes could be DCL1 protein, since miRNA biogenesis is inhibited by virus replication and *dcl1* mutant plants show reduced susceptibility to RCNMV infection (Takeda *et al.*, 2005). In the suggested scenario, DCL1 and its homologues are recruited by the viral replication complex and therefore depleted from the silencing pathways.

The above examples show that plant viruses have evolved various strategies to counteract antiviral RNA-silencing mechanisms. The majority of silencing suppression strategies target conserved key elements of RNA-silencing pathways such as siRNAs or their precursors and crucial enzymes like AGO proteins; sometimes a single VSR can target more than one element in the silencing pathways.

The large variety of well-described VSRs also offers better understanding of plant silencing pathways through targeting specific steps of silencing machinery.

IV. SILENCING SUPPRESSORS AND VIRAL SYMPTOMS

Viral infection leads to various symptoms such as development of lesions, dark green islands and growth defects (Hull, 2002). Although many VSRs (Table 1) have been identified as pathogenic determinants largely responsible for virus-induced symptoms, the molecular basis for virus-induced diseases in plants has been a long-standing mystery. It is well established that the antiviral and endogenous silencing pathways share common elements, and VSRs have been shown to interfere with those pathways. siRNA-binding VSRs (e.g. HC-Pro and p19) could interact with siRNA and miRNA biogenesis at different stages. This interference may alter endogenous gene expression regulated through miRNAs or siRNAs. Similarly, long dsRNA-binding VSRs (e.g. p38 and p14) could compromise DCLs or AGO1-targeting VSRs (e.g. P0 and P1) inhibit RISC activities, which in turn may alter the expression of an unpredictable number of genes involved in plant development. Indeed expression of VSRs in transgenic plants leads to phenotypes that mimic virus symptoms (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003).

However, transgenic expression of VSRs does not necessary reflect the effects of viral infection on endogenous silencing pathways, since in natural viral infection, expression of VSRs is restricted to virus-infected tissues and compartments, and is also limited in time. In fact recent results show that inhibition of 3′ modification of vsiRNAs and miRNAs in virus-infected plants requires spatial and temporal co-expression of small RNAs and VSRs (Lozsa *et al.*, 2008).

V. CONCLUDING REMARKS

During the last few years dramatic progress has been made in understanding the roles and pathways involved in antiviral RNA silencing. A large number of new silencing-suppressor proteins have been described from almost all plant virus genera. The discovery of the molecular bases of silencing suppression for many proteins has inspired new concepts on the existence of cellular negative regulators of RNA silencing, such as silencing suppressors. In virus-infected plants the key function of RNA silencing is to protect plants against viral invasion. Surprisingly it seems that viruses may exploit this defense to keep the virus titer at a tolerable level in plant tissues through controlling the expression level of VSRs. For example in natural virus infection a suboptimal codon controls the expression of the polerovirus P0 VSR, thus the moderate inhibition of RNA silencing ensures that both the viruses and the plants survive (Pfeffer *et al.*, 2002).

It is likely that antiviral RNA silencing accelerates the continuous modification/evolution of viral genome since even a single base change

in the target site of antiviral si/miRISC could protect the viral genome against degradation. However, this protection is very temporary since the vsiRNAs produced from the modified new sequence can target the viral genome again. Therefore, this is a continuous selection pressure for the RNA genome to alter the si/miRISC target site sequence. To escape from this endless circle, viruses evolved VSRs to protect their genome. Alternatively, viruses evolved their genome to be highly structured, which is not accessible for RISC. For example the fast evolution of CymRSV DI-RNAs ended up with a short highly structured DI-RNA, which are resistant against RNA silencing (Szittya *et al.*, 2002). The highly structured rod-like form of matured viroid genome is another example for the structure-mediated resistance of a RNA molecule to RNA silencing (Gomez *et al.*, 2009). On the other hand highly structured RNA molecules are good substrates for plant DCLs. Indeed, silencing-resistant DI-RNAs of CymRSV efficiently trigger RNA silencing against their helper genomes, while the generated vsiRNAs are not able to target the highly structured DI-RNAs (Szittya *et al.*, 2002).

The fast evolution of viral genome under RNA silencing pressure was also exemplified by introducing natural or artificial miRNA target site in the viral genome (Lin *et al.*, 2009; Simon-Mateo and Garcia, 2006). The most common outcome was the deletion or modification of the target site in the viral genome. The fast mutation of the viral genome may explain why host plant derived miRNAs or siRNAs are not found to target viral genome in natural virus resistance.

An extraordinary adaptation of viruses to the antiviral silencing has been found in the CymRSV–satellite RNA system. It has been shown that the helper virus harnesses RNA-silencing mechanism to control the accumulation of the virus parasitic satellite RNA (Pantaleo and Burgyan, 2008). Thus, RNA silencing appears to be involved in many ways in this fine-tuning of plant–virus interplay for joint survival, but our knowledge is still limited about the regulation of this intimate plant–virus interaction, which remains for future exploration.

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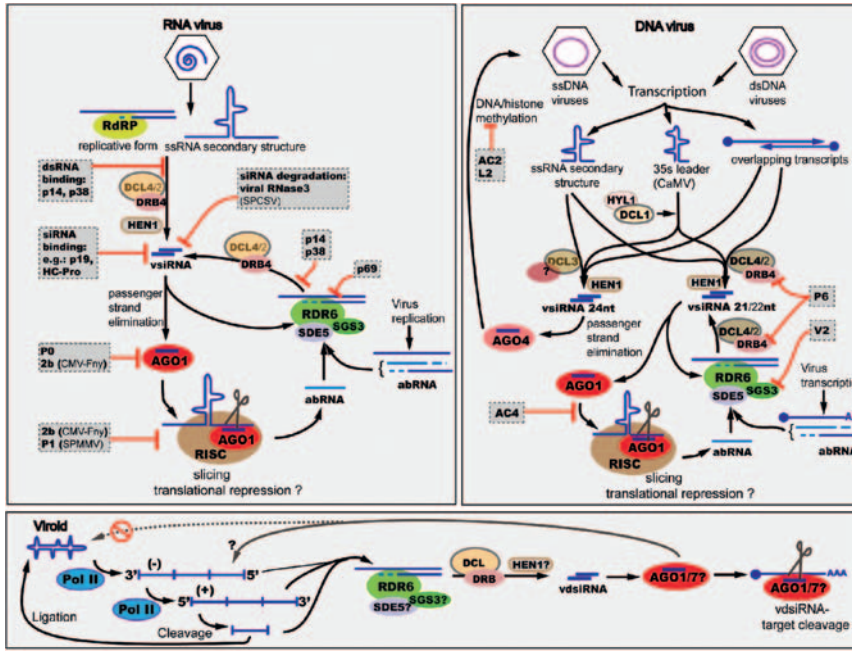

Figure 1, Csorba *et al.* (See Page 39 of this Volume)