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Plant responses against invasive nucleic acids: RNA silencing and its suppression by plant viral pathogens

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Abstract

RNA silencing is a common strategy shared by eukaryotic organisms to regulate gene expression, and also operates as a defense mechanism against invasive nucleic acids such as viral transcripts. The silencing pathway is quite sophisticated in higher eukaryotes but the distinct steps and nature of effector complexes vary between and even within species. To counteract this defense mechanism viruses have evolved the ability to encode proteins that suppress silencing to protect their genomes from degradation. This review focuses on our current understanding of how individual components of the plant RNA silencing mechanism are directed against viruses, and how in turn virus-encoded suppressors target one or more key events in the silencing cascade.

Keywords

RNA silencing; siRNA; Viral suppressors; DICER LIKE proteins; ARGONAUTE

1. Introduction

As is known for some years now, plants use post-transcriptional gene silencing (PTGS) to combat virus infections [1]. PTGS is an ancient RNA silencing mechanism mainly used for the directed, sequence-specific degradation of invasive RNA, via the generation of short-interfering RNAs (siRNAs) [2], and for the regulation of endogenous gene expression by generation of microRNAs (miRNAs) [3].

RNA silencing functions by a set of core reactions initiated by double-stranded RNA (dsRNA) structures that are processed into 21–24 nt RNA duplexes by a DICER-LIKE RNase III enzyme [4]. The small dsRNA is recruited into an RNA-induced silencing complex (RISC) containing an ARGONAUTE (AGO) protein and one of the siRNAs strands remains associated to bind complementary RNA which is then hydrolyzed, presumably by the AGO protein [5]. In certain instances the cycle is self-perpetuated by the action of RNA dependent RNA polymerase (RDR) and other components that amplify the silencing signal resulting in the induction of RNA silencing in the cells distant from the infection site [6].

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Plant viruses encode specific proteins that function as suppressors of RNA silencing; many of these were known as “pathogenicity factors” involved in symptom development during infection [7]. In general, viral suppressors interfere with one or more steps in the RNA silencing pathway, with a plethora of versatile mechanisms. Here we will describe the consecutive steps in the RNA silencing mechanism as it is understood today, especially in the context of an antiviral response, and will provide examples of how different components are blocked by viral suppressors.

2. Mechanism of RNA silencing against invasive nucleic acids

2.1. Detection and processing of dsRNA

RNA silencing is triggered by the presence of dsRNA which are commonly generated during plant virus replication. In the case of some single-stranded RNA (ssRNA) viruses, the viral RDR copies a plus-sense ssRNA into a minus-sense ssRNA generating a dsRNA molecule, whereas in others the two strands do not anneal but can fold into highly structured molecules that in essence have dsRNA regions [8]. Viroids also form hairpin structures, resembling precursors of miRNAs, which contain intervals of dsRNA [9]. For geminiviruses, the RNAs transcribed from their circular genomes can act as a source of dsRNA [10].

The plant defense surveillance system detects the presence of dsRNA and directs DICER-LIKE (DCL) proteins to cleave these into short 21 to 24 nucleotide (nt) duplexes. DCLs are large multi-domain proteins categorized as type III endonucleases [4]. They encode an RNase III domain which cleaves the dsRNA to yield characteristic 5' phosphate groups and two-nucleotide overhanging 3' ends [11]. There are three classes of RNase III endonucleases [12, 13]. Bacteria and yeast possess Class 1, which contain a single RNase III domain and a dsRNA binding domain. Fungi, plants, vertebrates and invertebrates possess Class 2 and Class 3 with two tandem RNase III domains. In addition most of Class 3 encode an N-terminal RNA helicase with a closely related PAZ (PIWI/ ARGONAUTE / ZWILLE) domain that is shared between DCL and ARGONAUTE (AGO) proteins [13].

Primarily based on genomics research with *Arabidopsis thaliana*, it is thought that plants contain several DCLs in contrast to other eukaryotes that encode one or two of these proteins. Presently four DCLs are identified in *Arabidopsis*, each with specific roles in the biogenesis of different classes of endogenous small RNAs [11]. For example, DCL3 cleaves 24-nt repeat associated siRNAs that target transposons, retroelements and repetitive DNA, DCL4 produces 21-nt trans-acting siRNAs (tasiRNAs) that guide silencing of endogenous mRNAs [14,15]. DCL1 is responsible for the generation of 21-nt miRNAs [3], and together with DCL2 produces antisense transcripts-derived siRNAs induced by environmental stresses [16,17].

Arabidopsis DCL4 and DCL2 show evidence of hierarchical antiviral activities, DCL4 acts as primary sensor producing 21-nt viral siRNAs; if DCL4 is functionally compromised DCL2 produces 22-nt siRNAs with antiviral activities [16,18]. Mutated *Arabidopsis* plants with the corresponding genes (*dcl4 dcl2*) inactivated exhibit hyper-susceptibility symptoms when infected with *Tobacco rattle virus* (TRV, genus *Tobravirus*), in contrast to the normal TRV-associated silencing response in non-mutated *Arabidopsis* that results in much milder symptoms [16]. This antiviral defense role for DCL4 and DCL2 was also shown for *Cauliflower mosaic virus* (CaMV; genus *Caulimovirus*, family *Caulimoviridae*) on *dcl*-mutated *Arabidopsis*, whereas DCL3 was also implicated in the silencing response [19].

However, the role of host silencing factors cannot easily be uncovered with wild-type viruses encoding viral suppressors that mask the role of host proteins. For this reason it can be advantageous to use viruses impaired for suppressor activity, as has been illustrated with suppressor-compromised mutants of *Turnip crinkle virus* (TCV, *Carmovirus*,

Tombusviridae) [16,18] and *Cucumber mosaic virus* (CMV, *Cucumovirus*, *Bromoviridae*) [20]. Evidence was obtained that certain *dcl Arabidopsis* mutant plants are more susceptible to virus infection than wild-type plants, once again demonstrating the antiviral defense roles of DCL2 and DCL4.

In general, DCLs associate with dsRNA binding proteins (DRB) that mainly assist in the biogenesis of short RNAs [21,22]. The use of T-DNA insertion mutant lines enabled reverse genetic studies of the five encoded *Arabidopsis* DRB like proteins [23]. When these lines were challenged with *Tomato spotted wilt virus* (TSWV; *Tospovirus*, *Bunyaviridae*), *Turnip mosaic virus* (TuMV, *Potyvirus*, *Potyviridae*), and CMV, no clear antiviral roles were evident for DRB2, DRB3 and DRB5. However, DRB1 functions exclusively with DCL1 in the biogenesis of miRNAs, and DRB4 operates with DCL4 in the production of tasiRNAs and 21-nt siRNAs from viral RNA [23]. Genetic studies provided support for the physical interaction *in vitro* and *in vivo* of DRB4 with DCL4 in the production of viral siRNAs [18,24].

2.2. RNA silencing signal amplification by secondary viral induced gene silencing

Once RNA silencing is triggered, siRNA may act as a signal to systemically alert other tissues [25]—presumably its small size allows for its rapid transport through plasmodesmata and phloem [26]. However, the phloem traffic of small RNAs is not passive and may require the assistance of transporter proteins [27]. Irrespective of the mechanism, for this signaling to operate effectively it seems that the signal needs to be amplified by host encoded RDRs [28–30]. By amplifying mobile silencing signals the antiviral machinery in non-infected tissues can be conditioned to respond quickly and to target incoming foreign nucleic acids [31].

Arabidopsis encodes six RDR proteins. RDR1, RDR2 and RDR6, all share the C-terminal catalytic DLDGD motif of eukaryotic RDR with direct orthologs in many plant species [29]. The other three contain an atypical DFDGD amino acid motif (RDR3a, RDR3b and RDR3c) with unknown function. The siRNA amplification by RDR occurs via two mechanisms. In the first, primary small RNAs are recruited by an RDR complex to prime the synthesis of new dsRNA. Support for this role is based on transitivity, the generation of siRNAs via antisense transcription [32]. From this, viral induced gene silencing (VIGS) is initiated by a viral small RNA of one part of the targeted mRNA leading to the accumulation of siRNA corresponding to the non-overlapping part [30]. In *Arabidopsis*, this amplification mechanism involves the activities of RDR6 and SDE3, a protein with RNA-helicase activity [30,33].

A second amplification mechanism detects the presence of an aberrant RNA that accumulates as a result of erroneous transcription of sense transgene-derived transcripts [34], transposons, or viruses [35]. The aberrant RNA is then copied *de novo* and dsRNA is produced by the combined action of AGO1, the coiled-coil protein SGS3, RDR6 and SDE3 [30,33–35]. Previous studies suggest that SGS3 binds and stabilizes RNA templates to initiate RDR6-mediated dsRNA synthesis. These two proteins, SGS3 and RDR6, co-localize and interact in specific cytoplasmic granules referred to as SGS3/RDR6-bodies; these are different from processing-bodies (P-bodies) where mRNA is degraded or stored [36].

The importance of the above-mentioned proteins in antiviral silencing was demonstrated with a series of *Arabidopsis* mutants impaired for one of these AGO1-RDR6-SGS3 proteins. The mutated plants became hypersensitive to CMV and exhibited a reduction in viral DNA induced gene silencing to *Cabbage leaf curl virus* (CaLCuV, *Begomovirus*, *Geminiviridae*) [37–39]. Moreover, hypersusceptibility of *N. benthamiana* plants to *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) is caused by a natural mutation that disrupts the function of a salicylic acid (SA) responsive RDR. Complementation studies using a *Medicago truncatula* SA responsive RDR ortholog confers *Nicotiana benthamiana* plants with resistance to TMV but not to CMV or *Potato virus X* (PVX, *Potexvirus*, *Flexiviridae*) [40].

2.3. Stabilization of siRNA by methylation or adenylation

RNA silencing-associated short RNAs are methylated at the 2'-OH group of the 3'-ribose by the methyltransferase HUA enhancer 1 (HEN1) [41,42] that protects the 3' ends from uridylation (addition of one to five U residues) and subsequent degradation [43,44]. In comparison, ten different miRNA families in the cambium zone of *Populus trichocarpa* were adenylated, and *in vitro* experiments revealed a delay in degradation of adenylated miRNAs [45], pointing to the idea that this stabilization mechanism can also be used by siRNAs.

2.4. Cytoplasmic export of nuclear siRNA

An additional step required for maturation of short RNAs processed in the nucleus such as miRNAs and some siRNAs, is their translocation to the cytoplasm in order to activate RISC. In *Arabidopsis*, HASTY [46] an ortholog of the mammalian nuclear export receptor protein (EXP5), carries out this export function. Not much is known about this particular process but it is possible that HASTY transports the miRNA duplex along with RISC from the nucleus to the cytoplasm [47]. Although *hasty* mutants show a reduced accumulation of most miRNAs, there is no obvious effect on transport of tRNA, endogenous siRNAs or on transgene silencing, suggesting the existence of multiple nuclear export pathways for these RNAs.

2.5. Recognition of siRNA by RISC and cleavage of cognate RNA

Both siRNAs and miRNAs are used to program RISC whereby one strand of the duplex is incorporated into the RISC holocomplex. This RISC-associated short RNA molecule functions as a piloting primer to direct nucleotide sequence-specific recognition of the targeted transcripts and their subsequent enzymatic degradation or translational repression [48]. The base pairing interaction that occurs between short and target RNAs is responsible for the high sequence specificity of RNA silencing [49,50]

As presently understood, the universal components of RISC are short RNAs and proteins belonging to the ARGONAUTE (AGO) family. AGOs are proteins of about 90 to 100 kDa, containing one variable N-terminal domain and three conserved C-terminal PAZ, MID and PIWI domains [48]. The MID domain, binds to the 5' phosphate of short RNAs and the 3' end is recognized by the PAZ domain [48]. The PIWI domain folds similarly to RNaseH enzymes and displays endonuclease activity through an active site carrying an Asp-Asp-His (DDH) motif [51]. Most AGO proteins cleave the target mRNAs at the center of their short complementary RNA sequence, but other AGOs, especially those associated with miRNA regulate their targets by repression of translation [52–54]. Both miRNAs and AGO proteins are associated with polysomes and localize to cytoplasmic processing bodies (P-bodies) [55] which contain deadenylase and decapping complexes, suggesting that target mRNA decay follows RISC activity.

The *Arabidopsis* genome encodes 10 AGOs, each with characteristic functional properties for interaction with specific short RNAs. AGO1 plays a role in the miRNA pathway since *ago1* mutants exhibit reduced levels of miRNA and concomitant increased amounts of target mRNAs [56]. Immunoprecipitation experiments showed that AGO1 binds to miRNAs and to several classes of siRNA, including transgene siRNA and tasiRNA [57,58], but failed to detect viral siRNAs association with AGO1 [57]. However, a mutant virus inactivated for its suppressor (TCV-ACP) accumulated to higher levels in *ago1* plants than in wild type plants; thus, implicating AGO1 in viral RNA silencing [18]. In addition to AGO1, AGO7 may contribute to viral defense [18]. AGO1 is preferentially associated with short RNAs containing a 5'-terminal uridine but how this relates to antiviral silencing is not clear [59,60].

AGO10 displays functional redundancy with AGO1 in the *Arabidopsis* Columbia ecotype [48,61]. Conversely, mutations of these genes in *Arabidopsis* Landsberg *erecta* ecotype,

produce exacerbated phenotypes when compared to Columbia ecotypes, pointing to potential ecotype-specific modifiers of AGO1 and AGO10 [48]. AGO5 localizes to the nucleus and the cytoplasm, and immunoprecipitation experiments found it preferentially associated with short RNAs containing a 5'-terminal cytosine [59]. No evident role during plant development or in antiviral responses has been assigned to this protein. However, mutations in one of the six rice AGO5 paralogs caused sterility, pointing to functional variation in AGO proteins of different species [62].

AGO2 and AGO3 are the only *Arabidopsis* AGOs without a DDH motif within the PIWI domain; instead, they have acquired a degenerate DDD motif similar to that present in bacterial RNaseH enzymes [63]. No clear function is evident for these AGO proteins, but AGO2 localizes to nucleus and cytoplasm, and associates with short RNAs containing 5'-terminal adenosine [59,60]. AGO4 is the slicer protein operating during transcriptional gene silencing (TGS) controlling the maintenance of epigenetically silent states at repeated loci, transposons and heterochromatin through the action of 24-nt siRNAs [48]. AGO4 colocalizes to nuclear Cajal bodies and to nuclear A-B bodies [64]; immunoprecipitation of AGO4 revealed its association with 24-nt small RNAs, which mainly contain 5'-terminal adenosine [65]. Recent evidence suggests a novel role of AGO4 in translational regulation in virus gene expression [54].

Although the precise role of individual AGOs to antiviral silencing remains to be elucidated, their differential distribution and preference for short RNAs with different 5'-terminal nucleotides suggests they may operate at different levels. Regardless, from a functional perspective it appears that viral RNAs are cleaved by one or more AGOs associated with RISC.

2.6. Processing of cleaved target RNA

Once the target RNA is cleaved, the endonuclease products need to be processed and presumably recycled. The 5' end of the cleaved transcripts is rapidly turned over by the exosome 3'-to-5' exonucleolytic activity [66] and the 3'-end is degraded by specific nucleases. AtXRN4, a homolog of the major yeast mRNA degrading exonuclease Xrn1, is involved in the miRNA-mediated decay pathway of selected targets [67]. The cytoplasmic XRN4 is expressed in many tissues and its mutation does not cause critical growth defects, indicating the absence of a critical role in general mRNA turnover [68]. XRN4 displays 5'-to-3' exonucleolytic activities, as demonstrated in experiments where XRN4 was mutated to yield plants that accumulated 3'-end products of the miRNA-RISC targeted RNA [67]. Moreover, *xrn4* mutations caused increased rates of sense PTGS probably as a result of higher RDR activity on the *xrn4* stable cleaved targeted RNA. This suggests XRN4 and RDR may compete for substrates [68].

The nuclear exoribonucleases XRN2 and XRN3 are also involved in processing of 3'-end cleaved target RNAs. Mutant plants affected in these genes display two different phenotypes represented by accumulation of precursors of miRNA for *xrn2*, and embryo lethality for loss of *xrn3* function. XRN3 might be a general exonuclease and its absence dramatically affects plant development [69]. Moreover, the nucleotidase/phosphatase FIERY1 (FRY1) was found to suppress endogenous PTGS presumably by a co-repression mechanism of XRN2, XRN3 and XRN4 [67,69]. Mutations in *fry1* recapitulate mutations of *xrn2*, *xrn3* and *xrn4*. These enzymes act as endogenous suppressors of RNA silencing, and the levels of these proteins can interfere with host endogenous gene silencing and viral suppression of silencing. This was illustrated with the *fry1* phenotype that included hyper-resistance to CMV, probably by means of increasing the substrate for RDR polymerases in silencing signal amplification [69].

2.7 Concluding Remarks on RNA silencing

Our knowledge of the RNA silencing pathway accumulates at an accelerating pace. Focusing on antiviral silencing, specific regulators are likely intricately regulated and recruited by the host plant to control viral dsRNA recognition and cleavage, siRNA amplification, modulation, protection, and transport (inter- and intracellularly), target viral RNA recognition and cleavage, and removal of the viral RNA. However, identifying precisely which of the specific factors that display functional redundancy (*e.g.*, DCL or AGO) are involved in antiviral silencing against different viruses requires substantially more attention.

Moreover, the RNA silencing field has virtually restricted its focus on *Arabidopsis* because of the genetic resources. It is crucial that this information be translated to deciphering the antiviral components of other plants, a necessary challenge for the future [70]. Considering the ancestral nature of innate immunity, antiviral silencing and the gene redundancy of several silencing components, it is tempting to speculate that specific plants adapted silencing effectors that are related to but different from those used by *Arabidopsis*.

3. Suppression of RNA silencing by viral proteins

A decade ago it was shown that viruses encode proteins to counteract the effect of RNA silencing [7]. Many were previously identified as pathogenicity factors that aided in systemic invasion [7,71]. Of course, viral suppressors are no longer limited to plant viruses—animal and human viruses also encode suppressors [72]. The different sections below aim to provide examples of how plant viral suppressors affect separate steps of the RNA silencing pathway. In certain circumstances, the same viral protein can act at different stages in the silencing process which reaffirms the efficient and pleiotropic nature of many virus proteins.

Many virus-encoded suppressors seem to have an RNA binding property and often display preference for a specific molecule [73,74]. Even when not explicitly stated in sections below, in many cases suppression may be assisted at varying degrees by the general ability of these proteins to bind RNA.

3.1 Suppressors affecting the processing of dsRNA

The P38 coat protein of TCV was shown to effectively suppress RNA silencing [75,76]. Interestingly, P38 failed to function as a suppressor when committed to viral capsid formation since the required region is embedded within the interior of the virion [75]. *Arabidopsis* plants expressing TCV P38 did not exhibit a distinctive morphological phenotype [77] in contrast to what was shown for other suppressors transgenically expressed in *Arabidopsis* and *Nicotiana* plants [78,79]. This suggested that the mechanism of silencing suppression used by P38 was specific to viral attack without disturbing endogenous host processes. Subsequently, it was observed that siRNAs were undetectable after P38-mediated suppression of RNA silencing induced by either sense or inverted repeat transgenes indicating that P38 suppressed DCL activity [80]. The accumulation of siRNA was monitored in single, double and triple *dcl* *Arabidopsis* mutants infected with a *p38*-deficient TCV, where P38 was replaced by GFP (TCV-GFPΔp38) [16]. In infected *dcl4* mutants 22-nt siRNAs were predominantly found, but not 21-nt siRNAs. In *dcl2-dcl4* plants the RNA levels of TCV-GFPΔp38 were elevated at levels comparable to those upon infection with wild-type TCV in the same mutants. This corroborated that P38 suppression is mainly exerted on DCL4 and DCL2 activity because if other silencing factors were targeted by P38 then the levels of TCV-GFPΔp38 viral RNA should have decreased.

The accumulation of 21, 22 and 24 -nt siRNAs was reduced in CMV infection, due to the effects of the 2b protein. This suggested that 2b also interferes with the activity of DCL4, DCL2 or DCL3 [20]. An indirect block of DCL function was caused by the CaMV P6 protein, because

its transgenic expression in *Arabidopsis* reduced levels of DCL4-dependent 21-nt siRNAs, similar to what occurred upon inactivating *Arabidopsis* DRB4. Furthermore, immunoprecipitation assays demonstrated that P6 physically interacts with DRB4 and the ability of P6 to move within cellular compartments (nucleus and cytoplasm) was important for full silencing suppression activity [81].

3.2. Suppressors of the silencing signal amplification

The V2 protein of *Tomato yellow leaf curl virus* (TYLCV; *Begomovirus*, *Geminiviridae*), binds to SGS3 which is a coiled-coil protein involved in siRNA signal amplification, and consequently interferes with RNA silencing [82]. A point mutation on V2 that impairs its binding to SGS3 also abolishes its ability to suppress RNA silencing, indicating that the V2-SGS3 interaction is important for suppression [82]. In other experiments where PTGS of transgenes was blocked by mutations on SGS2 and SGS3, the ability of the plant to restrict virus infection was compromised, demonstrating a connection between the activity of SGS proteins and antiviral silencing [34].

3.3 Suppressors of the stabilization of siRNA

3.3.1. Sequestration of siRNA—The potyvirus-encoded viral suppressor HC-Pro is a versatile protein with roles in different crucial steps of the viral infection process such as, viral replication, systemic and cell-cell movement and proteolytic cleavage of the viral polypeptide [83]. HC-Pro also displays different modes of action when acting as a viral suppressor of RNA silencing, including possible interference with DCL proteins [84], but in addition HC-Pro might be involved in sequestration of 21-nt siRNA duplexes containing 3' 2-nt overhangs [73]. A conserved FRNK box in HC-Pro was found to be involved in siRNA and miRNA duplex sequestration, and mutations of FRNK to FINK caused attenuation of symptoms upon infection and a decrease in miRNA accumulation [85]. This led to the hypothesis that interactions of the FRNK box with the different plant miRNAs may influence their accumulation levels that contribute to symptom development [85].

In vivo immunoprecipitation experiments of HA tagged P21 encoded by *Beet yellows virus* (BYV; *Closterovirus*, *Closteroviridae*) in transgenic *Arabidopsis* plants demonstrated that this suppressor interacts with miRNA duplexes and hairpin RNA derived siRNAs [78]; interactions later confirmed with RNA binding assays [73]. Also a mutant form of P21 (8A-21), failed to suppress RNA silencing *in vivo* or bind siRNA [73]. For another closterovirus, *Citrus tristeza virus*, three different suppressors have been identified but their individual roles are not yet known [86].

The role of P19 as a viral suppressor of gene silencing was discovered upon agroinfiltration of GFP-expressing cultures onto GFP-transgenic plants (Fig. 1). It was shown that infection of these plants with PVX expressing P19 interfered with the systemic onset of silencing [7]. Subsequent X-ray crystallography studies suggested an elegant mode of action for P19 whereby caliper tryptophan residues on two subunits of P19 dimers “measure” 21 nt siRNA duplexes which are bound in a non-sequence specific manner [87,88]. The biological relevance of this observation was evident when the association between P19 and siRNAs was shown to occur *in vivo* in infected plants [89], and that the suppression ability of P19 only operates effectively when this protein is produced at normal high levels [90]. P19 specifically mutated to avoid siRNA binding no longer suppressed silencing in infected *N. benthamiana* as inferred by the absence of a virus-mediated lethal necrosis [91]. Such P19 mutants are also compromised for invasion of some hosts, however not all host-dependent biological activities of P19 strictly correlate with siRNA binding [91].

It was suggested that miRNA binding by P19 may contribute to symptom induction [78], but some mutants inactivated for short RNA binding are rather symptomatic suggesting that other properties and perhaps host factors are contributing to disease outcome [92]. Nevertheless, the results in *N. benthamiana* provided support for a model whereby P19 sequesters circulating tombusvirus-derived siRNAs to prevent their use in RISC programming and thus avoid targeting and degradation of cognate viral RNA. Verification for this model was obtained during experiments where *N. benthamiana* was challenged with *p19*-deficient tombusvirus mutants causing the assembly of a high molecular weight RISC-like complex, which contains virus derived siRNAs and exhibits virus-specific ribonuclease activity [93,94].

The NS3 protein of *Rice hoja blanca virus* (RHBV, *Tenuivirus*) along with NSs of TSWV were the first identified suppressors encoded by minus-/ambi-sense RNA viruses [95]. Standard tests (Fig. 1) showed that NS3 suppresses GFP silencing presumably by affecting the accumulation of GFP siRNAs; however, suppression of GFP silencing by NSs was not due to interference with siRNA [95]. Subsequently, NS3 was found to bind ds-siRNA *in vitro* without sequence preference while exhibiting the highest affinity for 21-nt siRNAs with or without 2-nt overhangs and a lesser affinity for 26-nt siRNAs [96].

3.3.2. Interference with methyltransferase HEN1—P21 of BYV, P19 of TBSV, or P1/HC-Pro of *Turnip mosaic virus* (TuMV; *Potyvirus*, *Potyviridae*) interfere with short RNA stabilization by blocking HEN1 methylation, and even miRNA duplexes are affected by these suppressors [97,98]. This raises questions about where and when methylation processes occur, since miRNA progenitors are supposedly cleaved in the nucleus, yet they are blocked for methylation by cytoplasmic viral suppressors [98]. It might be that methylation occurs in both the nucleus and cytoplasm or that viral suppressors are able to enter the nucleus by attaching to a protein that is transported to the nucleus (as suggested from interactions between P19 and ALY proteins [99]).

Two independent studies revealed that the 126 kDa replicase protein of TMV exhibits suppressor activity [100,101]. Subsequent investigations showed that its suppression mechanism was linked to interference with HEN1 methylation of siRNA [102]. Presumably, the effects of TMV replicase protein could be on demethylation itself, since the enzyme has methyltransferase activity, or alternatively is caused by binding of the protein to siRNAs thereby excluding these from HEN1 methylation [102].

3.4 Suppressors of RISC activity

The P0 protein encoded by *Beet western yellows virus* (BWYV; *Polerovirus*, *Luteoviridae*) was identified as a strong silencing suppressor based on its ability to inhibit suppression of GFP silencing (Fig. 1) in *N. benthamiana* plants [103]. Subsequent studies on two *Arabidopsis*-infecting poleroviruses revealed that P0 contains a conserved minimal F-box motif that interacts with homologues of S-phase kinase related protein 1 (SKP1), a core subunit of the multi-component SCF family of ubiquitin E3 ligases. Mutations in the F-box motif disrupted the interaction between P0 and an SKP1 homolog in *N. benthamiana*, causing a decrease in virus pathogenicity [104]. When P0 was expressed in transgenic *Arabidopsis* plants it caused severe developmental defects, similar to defects observed in mutants affected in miRNA pathways. Down regulation of a SKP1 homolog in *N. benthamiana* resulted in plant resistance to polerovirus infection. These results suggested that P0 functions as an F-box protein that targets a key component of RNA silencing machinery [104], and subsequently it was revealed that P0 physically interacts with AGO1 to trigger AGO1 protein decay *in planta* [105].

The CMV 2b protein was one of the first identified suppressors [106]. Recent experiments performed with two cucumovirus strains, the severe FNY strain and the attenuated Q2 strain,

revealed that 2b from both interacted with AGO1 alluding to a precise biochemical function. However, the Q2-2b protein stability seemed compromised resulting in reduced protein levels and consequently lower suppressor activity [107]. The interactive region of AGO1 mapped to a PAZ module that encompasses the RNA-binding groove and part of the PIWI-box, and most importantly the interaction caused inhibition of the RNA cleavage activity in RISC reconstitution assays [107]. In addition to revealing important mechanistic data on the mode of action of 2b, this study also illustrated that homologous suppressors from strains of the same virus not necessarily exhibit the same effect on RNA silencing. As mentioned before, suppressors have pleiotropic effects and since the 2b protein also interacts with DCR, it may be that different strains have adapted specificity towards targeting DCR or AGO1.

3.5. Viral suppressors with unspecified function

The triple gene block protein 1 (TGBp1) of PVX suppresses RNA silencing [7,108], but a recent study to examine the suppressor ability of TGBp1 proteins encoded by other potexviruses revealed intriguing differences [109]. No specific functional domain was identified in connection to RNA silencing suppression [109,110], but instead it was concluded that the precise amino acid sequence is of secondary importance as long as the proper folded structure is adopted by the protein [109]. However, no clear correlation between suppression and infectivity is yet evident [109].

Cysteine-rich proteins encoded by hordeiviruses, tobnaviruses, furoviruses, pecluviruses and carlaviruses share no significant identity, but they have predicted structural resemblance and function as virus pathogenicity determinants [111,112]. Among the most studied are *Tobravirus* 16K, the *Hordeivirus* γ b, and the *Pecluvirus* P15. Inactive TRV 16K can be complemented by co-expressing the CMV 2b suppressor, indicating that both exert similar functions in RNA suppression [111]. Indeed, studies specifically designed to examine suppressor function using agroinfiltration of GFP-transgenic *N. benthamiana* plants with GFP-expressing cultures (Fig. 1), confirmed that the 16K is a suppressor [113]. Another study indicated that 16K operates downstream of dsRNA formation since its effect was compromised when dsRNA inducer was present in higher amounts suggesting oversaturation of 16K activity [114].

The *Barley stripe mosaic virus* (BSMV; *Hordeivirus*) γ b protein is involved in viral pathogenesis but dispensable for virus replication [115]. Its suppression activity was demonstrated in *Agrobacterium*-mediated transient assays (Fig. 1) [112]. The γ b protein of a related hordeivirus localized in the cytoplasm and peroxisome, but its suppression activity was not associated with its presence in the peroxisome. Similarly to γ b, the cysteine-rich pecluviral P15 suppressor forms coiled-coil structures required for protein-protein interaction and silencing activity. The coiled-coil sequence rather than the peroxisome localization motif is essential for its silencing suppression activity.

3.6. Viral RNA-mediated control

In some cases, siRNAs derived from the viral genome can play a role in down regulating expression of host genes through RNA silencing. For instance, prediction of all possible derived siRNAs on the TMV-Cg sequence and a search for the presence of cleaved products of the putative targets, identified two host targets that were silenced [28]. Another interesting finding is that suppression of RNA silencing associated with a dianthovirus is not only associated with a protein, namely the virus-encoded movement protein [116], but the replication complex and perhaps the RNA in particular may interfere with DCR activity [100].

4. Discussion and Conclusion

RNA silencing in plants (Fig. 2) is as complex and elaborated as any other biological processes. Adding to this complexity are plant virus-encoded suppressors of host silencing (Fig. 2). Commonalities in suppression of silencing do exist but there is also great variation likely driven by evolution and fitness, even to yield viral strains with different properties. For example, the CMV 2b protein targets DCR and AGO1 activities, and even sequesters siRNAs, all in a strain-dependent manner [117]. Another example is illustrated by strain-dependent suppressor activities observed for the TGBp1 of potexviruses [109]. The expression levels of host endogenous silencing suppressors also affect viral infection and viral suppression strategies. For instance, *fry1* mutant plants are hyper-resistant to CMV due to a decrease in activity of XRN4, XRN3 and XRN2 which leaves targeted cleaved RNAs available for amplification of secondary siRNAs by RDR. From this, it is likely that differences also will be found when plant viruses and their suppressors are tested in several plant species. This will provide us with a greater understanding of the parameters associated with the natural host range of a virus and possibly lead to new strategies for crop protection.

It can be anticipated that virus suppressors are actively in the process of adaptation as fortuitous or successful encounters of viruses and plants occur daily. A major technical challenge sketched at various places in this review is that viral RNA silencing and plant endogenous gene regulation have many intertwined players. As has already surfaced in a few instances, interference with virus silencing or suppression may have unwanted, deleterious effects on endogenous host processes. The key will be to find the means to attenuate such host effects while drastically repressing virus proliferation *in planta*.

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Figure 1. Common tests for viral suppressors

A. Transgenic *Nicotiana benthamiana* plants expressing GFP (1) are infiltrated (syringe) with an *Agrobacterium* suspension containing a GFP construct that induces GFP-RNA silencing. After 10 to 17 days the GFP signal is silenced and chlorophyll red fluorescence is observed under ultraviolet (UV in purple star) light at 474 nm (2). A second infiltration or inoculation is performed on the plant with an infectious clone or a transcript of a virus expressing a putative viral suppressor (3). If the viral suppressor is able to revert silencing the plant will show GFP fluorescence again under uv light, especially in the upper tissue. This routine method can be used to test for differences in viral strains, monitoring simultaneously virus movement and suppressor activity.

B. Coinfiltration of *Agrobacterium* expressing GFP with a culture containing a construct encoding the “putative viral suppressor” onto non-transgenic *N. benthamiana*. The illustration shows leaf images to be expected upon UV illumination of a mock-infiltrated leaf with an *Agrobacterium* culture without the GFP construct (1), a leaf infiltrated with the GFP expressing construct (2), and a leaf coinfiltrated with constructs expressing GFP and the candidate suppressor (3). At 10 days after infiltration the GFP signal usually decays due to RNA silencing (red fluorescence of chloroplasts only), but if the protein expressed from the coinfiltrated construct has suppressor activity, the GFP signal (green fluorescence) will remain strong as illustrated in 3.

Figure 2. Plant RNA silencing pathway and suppressor targets

A double-stranded (ds) RNA molecule is targeted by a DICER_LIKE protein (DCL) with assistance from a dsRNA binding protein (DRB), and cleaved (indicated by scissors) into 21-24-nt siRNAs with the signature 2-nt overhang (short staggered duplex arrows). Addition of methyl groups (CH₃) to the ends of the siRNA is performed by HEN1, siRNAs that are not methylated end up polyuridylated and degraded (curved white arrow) by the exosome. The methylated siRNA is recognized by the RISC-AGO holocomplex and one of the siRNA strands is incorporated into the complex (short white arrow). The programmed RISC targets the cognate mRNA (thick blue arrow) and hydrolyzes it (scissors) at a central location on the siRNA sequence. The cleaved products are either used as templates for siRNA amplification (curved blue arrow) or they are degraded by the XRN4 5'-3' exonuclease; blue lines without

arrows indicate RNA remnants. The amplification cycle requires the activity of RDR, SDE3, SGS3, DCL-DRB and AGO (in RISC). This amplified siRNA then presumably serves as a silencing signal. For each step a list of suppressors (brown characters) that interfere with silencing is provided in brackets (see text for details on origin and function); FIERY1 represents an endogenous suppressors. A group of viral (and the host rgs-CAM) suppressors for which other than RNA binding a specific function has not yet been precisely identified, is listed at the very right. Also is indicated that viral siRNAs can be used to program RISC against host RNA.