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Viral induction and suppression of RNA silencing in plants ☆

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ABSTRACT

RNA silencing in plants and insects can function as a defence mechanism against invading viruses. RNA silencing-based antiviral defence entails the production of virus-derived small interfering RNAs which guide specific antiviral effector complexes to inactivate viral genomes. As a response to this defence system, viruses have evolved viral suppressors of RNA silencing (VSRs) to overcome the host defence. VSRs can act on various steps of the different silencing pathways. Viral infection can have a profound impact on the host endogenous RNA silencing regulatory pathways; alterations of endogenous short RNA expression profile and gene expression are often associated with viral infections and their symptoms. Here we discuss our current understanding of the main steps of RNA-silencing responses to viral invasion in plants and the effects of VSRs on endogenous pathways. This article is part of a Special Issue entitled: MicroRNAs in viral gene regulation.

1. Introduction

Eucaryotic organisms depend on networks of gene regulatory pathways. Small RNAs (sRNAs), are key components of these networks. sRNAs are short (21–24 nt in length), endogenously expressed, and are processed from double stranded (ds)RNAs or dsRNA-like precursors. In both plants and animals, sRNAs exert their functions upon incorporation into ribonucleoprotein silencing complexes and through their base-paring capacity. They are implicated in a variety of processes, including post-transcriptional regulation of mRNA, mRNA stability and availability for translation, establishment of heterochromatin and silencing of transposons [1]. Different classes of sRNAs differ in the proteins required in their biogenesis, the constitution of ribonucleoprotein complexes that mediate their regulatory functions, their type of gene regulation, and the biological functions in which they are implicated. Plants display a remarkable diversity of sRNA types and sRNA pathways, likely needed for managing multiple environmental stimuli, including biotic and abiotic stresses. Several lines of evidence suggest that plant sRNAs play critical roles in plant–pathogen interactions. Indeed, upon infection, most plant pathogens can interfere with the expression of endogenous sRNAs, thus altering the expression of specific host factors implicated in the suppression or in the activation of plant defences. Evidence for these phenomena has been reported for bacterial and fungal pathogens (reviewed by [2]). Viruses are obligate infectious agents, whose life cycle (expression of viral proteins, viral genome replication and virion assembly) is integrated with host cell functions. Plant viruses can both modify the profiles of endogenous sRNAs (in common with bacteria and fungi) and induce the production of additional sRNAs derived from their own genomes (viral sRNAs; vsiRNAs). The latter gives a clear indication of the activation of RNA silencing-based responses of the plant. In some cases, this results in reduction of the titre of the invading virus and, in recovery of upper, non-inoculated leaves [3,4]. To counteract RNA silencing, many plant viruses have evolved proteins (viral suppressors of RNA silencing: VSR) that target various components of the plant silencing machinery. Viruses can induce specific symptoms resembling developmental anomalies and affecting organs and tissues such as leaves, flowers and fruits. These anomalies are often reconcilable with virus-induced alterations of RNA silencing-based endogenous pathways, due to: i) the direct activity of VSRs on endogenous sRNAs or on silencing related effectors; ii) the abundance of vsiRNAs in competition with endogenous sRNAs; iii) the action of specific vsiRNAs entering into RNA silencing complexes and targeting specific host genes.

Here we provide an overview of the major cellular RNA silencing pathways in plants with particular reference to those involved in antiviral functions. Finally we highlight examples of the complex interactions between viral molecular processes and host RNA processes.

2. Fundamental RNA-silencing pathways in plants

Besides small non-coding RNAs that are not involved in RNA silencing (e.g. transfer (t)RNAs, small nuclear (sn)RNAs and small nucleolar (sno) RNAs), several classes of endogenous sRNAs with regulatory functions have been described in plants. Most of our knowledge on sRNA pathways in plants comes from studies carried out in *Arabidopsis thaliana*. In this section we recapitulate the major cellular pathways that are involved in

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biogenesis and functions of microRNAs (miRNAs) and other endogenous small interfering RNAs (siRNAs).

2.1. MicroRNAs

miRNAs derive from primary transcripts called pri-miRNAs, which are transcribed by RNA polymerase II (RNApol II) from specific intronic or intergenic MIRNA genes. Pri-miRNAs form fold-back structures, which are processed into stem loop precursors known as pre-miRNAs which are subsequently diced to release small RNA duplexes composed of the mature miRNA and the miRNA* (reviewed in [5]). The generation of miRNAs from their pri-miRNAs requires the sequential action of the DICER-like protein 1 (DCL1; an RNase III type endonuclease) assisted by its dsRNA-binding protein (DRB) partner HYPONASTIC LEAVES1 (HYL1 or DRB1). The mechanism by which DCL1 recognizes where to cut and the exact dynamic of miRNA liberation are still relatively poorly understood in plants, although experimental studies suggest that spacing and symmetry of bulges in imperfect fold-back structures contribute to defining DCL1 specificity [6–8]. Intriguingly, it has been shown that some miRNA precursors (pre-miRNAs) are processed from the base towards the loop of the stem, while a loop towards base mechanism can release multiple miRNA/miRNA*-like duplexes from some precursors [9]. DAWDLE protein (DDL) has been proposed to be involved in recruiting DCL1 to pri-miRNA [10,11], whereas the zinc-finger protein SERRATE is proposed to act together with DCL1 and HYL in pri-miRNA to pre-miRNA processing [12]. Unlike animal, plant miRNA/miRNA* duplexes undergo a process of maturation consisting of HUA-ENHANCER1 (HEN1)-mediated 2'-O-methylation of their 3′-ends [13–15]. Methylation protects miRNA duplexes from 3′ uridylation, thought to be a signal for degradation [16]. Mature miRNA duplexes then exit the nucleus and enter the cytoplasm with the help of the importin nucleocytoplasmic transporter protein HASTY (HST) [17]. The miRNA strand is finally loaded into ARGONAUTE (AGO)-containing complexes such as AGO1-containing, AGO7-containing and AGO10-containing RNA-induced silencing complexes (RISCs) to direct endo-nucleolytic cleavage of target transcripts [18–20] or translational inhibition of mRNAs [21]. The removal of miRNA* from the incorporated duplex is a prerequisite for target recognition. With an elegant plant *in vitro* system, Iki and collaborators have shown that removal of siRNA passenger strands requires AGO1 RNase activity, likely through siRNA guide strand-mediated cleavage (similarly to the Drosophila siRISC system [22]), whereas removal of miRNAs* is not [23].

Besides canonical DCL1-dependent 21 nt-long miRNA species, DCL3-dependend 24 nt-long miRNA species have been identified in plants. While the former usually regulate gene expression at post-transcriptional level, the latter are associated with AGO4 and can direct sequence specific DNA methylation of loci from which they are produced [24,25].

2.2. Endogenous small-interfering RNAs

Heterochromatic (hc) siRNAs, the most abundant siRNAs, are usually 24 nt long and are produced at transposon loci and DNA repeats [26–28]. Their generation requires the cooperation of RNA-directed RNA polymerase (RDR) 2, RNA polymerase IV (RNApol IV) and Dicer-like protein 3 (DCL3) (reviewed in [29]). These hc-siRNAs are incorporated into AGO4 or AGO6 and guide cytosine methylation and/or histone modifications at their target sites (RNA-dependent DNA methylation; RdDM). RdDM may cause transcriptional gene silencing (TGS) [29].

Trans acting (ta) siRNAs are generated from primary RNA transcripts from non-coding TAS genes. TAS transcripts are initially targeted and cleaved by specific miRNAs incorporated in specific AGOs (i.e. mi173/AGO1 for TAS1 and TAS2; AGO1-miR828 for TAS4; miR390/AGO7 for TAS3) [20,30–33]. The resulting 3′- or 5′ RNA fragments act as templates for RDR6-mediated synthesis of a complementary RNA strand in conjunction with suppressor of gene silencing 3 (SGS3). The resulting dsRNA is then diced by Dicer-like protein 4 (DCL4) in cooperation with DRB4 to produce phased ta-siRNAs in a register with the miRNA cleavage site. ta-siRNAs direct post-transcriptional gene silencing of mRNAs involved in plant developmental phase changes and organ polarity (reviewed in [34]).

Natural *cis*-acting (nat) siRNAs derive from cis-antisense RNA transcripts and have been shown to play important roles in biotic and abiotic stresses [35,36].

Long (l) siRNAs represent a recently discovered additional class of endogenous siRNAs. Conversely to the other siRNAs, lsiRNAs size ranges from 30 to 40 nt, are induced by bacterial pathogens and destabilize the mRNA target by its decapping [37].

3. Antiviral RNA silencing

3.1. sRNAs derived from viruses

In plants, sRNA generation (either of endogenous or exogenous sRNAs) requires at least two common biochemical steps: i) induction by dsRNAs and ii) processing of dsRNAs into sRNAs.

In the case of viruses, there are several types of RNAs that may account for dsRNA production. Positive (+) strand RNA viruses, the majority of plant viruses, accumulate several copies of (+) genomic RNA through negative-stranded RNA intermediates via the viral RNA-dependent RNA polymerase (RdRp). These replication intermediates may form perfect long dsRNAs molecules, which may constitute an obvious substrate for DCLs (Fig. 1A). However, it is well known that one of the common principles of (+) strand viruses is that viral replication associates with extensively rearranged intracellular membranes (i.e. vesicles), and that replication intermediates are assembled with many viral RdRp proteins (reviewed in [38]). These circumstances reduce the probability for dsRNA replication intermediates to be processed into vsiRNAs. As a consequence, it seems unlikely that, *in vivo*, dsRNA replication intermediates alone account for the massive amount of vsiRNAs found in virus-infected tissues. Moreover, long perfect dsRNAs should generate mainly perfect vsiRNA duplexes (Fig. 1A), whereas cloning and high-throughput sequencing in several virus-plant systems showed that the resulting vsiRNAs are not perfect duplexes [39,40] and that they map mainly to the (+) strand RNA rather than to the negative (−) strand [40–45]. The latter observation is consistent with the relative abundance of (+) strand RNA (genomic) than (−) strand (antigenomic) in such viral infections. Nevertheless, an unprecedented finding has shown that vsiRNAs from *Grapevine fleck virus* (belonging to the family *Tymoviridae*) in its own natural host, are mainly from viral (−) strand. These observations suggest that dsRNA-like secondary structures within the single-stranded viral RNA are more likely than dsRNA replication intermediates to constitute the main source of vsiRNAs in infected tissues (Fig. 1B). Similarly, plant viruses having a DNA genome (which do not possess a dsRNA replication step but, instead dsDNA intermediates) produce massive amount of vsiRNAs from RNA transcription units. Indeed, extensive secondary structures of the polycistronic 35S RNA transcript of the dsDNA virus *Cauliflower mosaic virus* (genus *Caulimovirus*, CaMV) are the major source of vsiRNA [46]. Likewise, most of the vsiRNAs associated with geminivirus infections (plant viruses with single-stranded DNA genome) likely derive from fold-back structures within RNA transcription units [47,48]. Geminiviruses offer an additional example of dsRNA molecules from which vsiRNAs originate. In these circular single stranded (ss)DNA viruses, transcription is bidirectional from the dsDNA intermediate (Fig. 1D). Indeed, sense-antisense transcript pairs of opposite polarity overlap at their 3′-ends, forming a perfectly complementary dsRNAs — at least partially explaining the generation of vsiRNAs (Fig. 1D) [47].

Plant RDRs are believed to recognize aberrant RNA products of incomplete genome replication or transcription and synthesize complementary RNAs, forming dsRNAs (Fig. 1C and see also ta-siRNA generation in the previous paragraph) (reviewed in [49]). *A. thaliana*

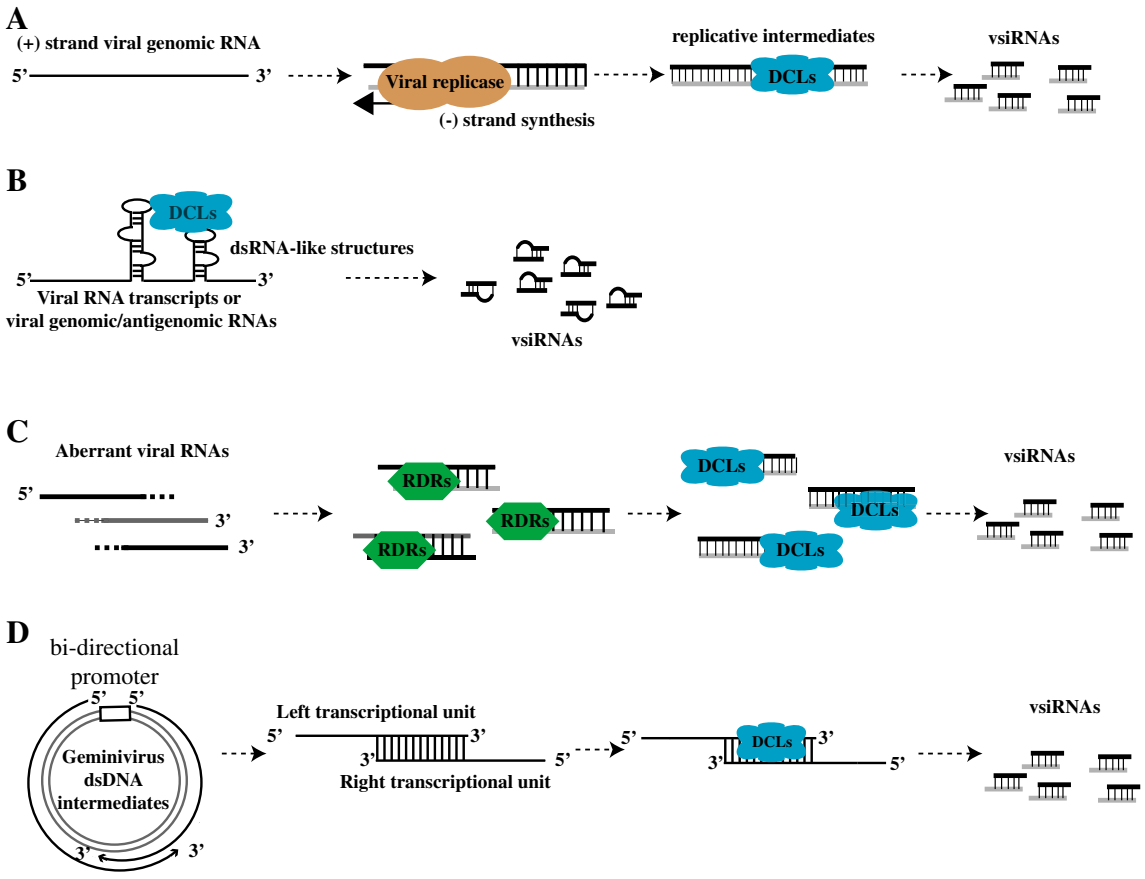

Fig. 1. Sources of double-stranded (ds) RNA for viral siRNA generation. A) The viral replicase copies the genomic RNA (+) into a complementary negative (−) strand RNA, which is subsequently used for the production of large amounts of new (+) RNAs. The complementarity of the two viral strands may promote the formation of perfectly base-paired viral dsRNA which are substrates of Dicers (DCLs). B) Secondary structures on both (+) and (−) stranded viral RNAs may form imperfect dsRNA-like structures, which can stimulate the action of DCLs. C) Plant RNA-directed RNA polymerased (RDRs) templates include aberrant RNA of viral origin, which may be converted into long perfect dsRNAs. D) Geminiviruses are circular single stranded (ss) DNA viruses having a dsDNA intermediate step. A bidirectional promoter drives the generation of long viral RNA transcripts, which overlap at their 3′ ends, thus forming a perfectly base-paired dsRNA structures.

possesses six putative RDR proteins (RDR1-6), although only RDR1, RDR2 and RDR6 are characterized in terms of function [50]. RDR1, RDR2 and RDR6 play complementary roles in generating *Tobacco rattle virus* (genus *Tobravirus*, TRV) siRNA and in limiting infection in *Turnip mosaic virus* (genus *Potyvirus*, TuMV)-inoculated leaves [42,51]. Either RDR1 or RDR6 is necessary for amplification of vsiRNAs from *Cucumber mosaic virus* (genus *Cucumovirus*; CMV) and TMV-Cg (a strain infecting members of the family *Cruciferae*, to which *A. thaliana* belongs) [44,45]. RDR6 functions require cofactors such as the putative RNA helicase SILENCING DEFECTIVE 3 (SDE3) and SGS3. Indeed *A. thaliana* mutants lacking factors of the RDR6-SGS3 pathway behave like *dcl2-dcl4* mutant plants in terms of CMV accumulation[52–54], and geminivirus triggered virus-induced gene silencing is also impaired in these plants [55]. Primary vsiRNAs (i.e. those deriving from viral dsRNAs or dsRNA-like structures previously described) can direct cleavage of viral RNAs by AGO complexes (see later in the text) or can act as primers for RDR-dependent dsRNA synthesis. In the former case, RDRs can, in turn, use cleaved viral RNAs to synthesize long dsRNAs, which are diced into secondary vsiRNAs [56]. RDR functions are also required for amplifying a systemic antiviral silencing response to prepare defences in differentiated and meristematic plant tissues yet to be infected. For instance RDR6 activities exclude *Potato virus X* (genus *Potexvirus*, PVX) and other plant viruses from *N. benthamiana* meristems and ensure an efficient antiviral RNA silencing [57–59].

Viral dsRNAs or dsRNA-like structures are processed into specific size classes of vsiRNA duplexes by specific DCL proteins. The model plant *A. thaliana* encodes four Dicer-like proteins (DCLs); DCL1 is primarily involved in the genesis of 21 nt long miRNAs, DCL4, DCL2, and DCL3 typically produce sRNAs of 21, 22 and 24 nt, respectively.

The use of loss-of-function mutants in *A. thaliana* DCLs has shown that DCL2 and DCL4 play the primary roles in the generation of vsiRNAs in the case of (+) strand RNA viruses. Plants infected with (+) strand RNA viruses predominantly accumulate 21 nt vsiRNAs (with a few exceptions i.e. *Turnip crinkle virus*, see following discussion) made by DCL4, but not DCL1 [42,52,53,60,61]. DCL2-dependent 22 nt vsiRNAs constitute a small fraction of the total vsiRNA population in wild type plants, however loss of DCL4 results in a size shift of the vsiRNAs to DCL2-dependent 22 nt species. Moreover, the elimination of both DCL2 and DCL4 results in increased disease susceptibility, in particular to distinct mutant viruses defective in silencing suppressors (VSRs). DCL3 produces 24 nt-long vsiRNAs only in *dcl2–dcl4* double mutants, it thus appears to play a minor role in this process [42,52,53,61]. DCL1 is only indirectly implicated in vsiRNA generation, since a homeostatic control of all DCLs has been observed. *dcl1* hypomorphic *Arabidopsis* mutants consistently show up-regulation of antiviral activity against *Turnip crinkle virus* (genus *Carmovirus*, TCV) due to a significant increase in DCL4 expression [62]. Moreover, in TCV infection the VSR p38 impairs the AGO1/miR162-dependent down-regulation of DCL1 resulting in lower levels of DCL4 and DCL3 (Fig. 4) [63]. Accordingly, DCL2-dependent 22 nt vsiRNAs are predominantly produced during TCV infection [52,63]. In the case of plant viruses with DNA genomes, all four DCLs are involved in vsiRNA biogenesis. In contrast to RNA viruses, DCL3 always appears to be the active antiviral dicer and DCL1 seems to cooperate with other DCLs [46,64].

In plants, DRBs are cofactors of DCLs proteins. DRB1/HYL1 and DRB4 have been previously described as assisting DCL1 in miRNA biogenesis and DCL4 in ta-siRNA biogenesis respectively. Indications of the contribution of DRB4 to the generation of vsiRNAs come from studies on CaMV and its viral suppressor P6. P6 targets DRB4, impairing the activity of DCL4 and leading to reduced levels of DCL4-dependent 21 nt vsiRNAs, whereas DCL3-dependent 24 nt vsiRNAs are enhanced [65]. In the case of (+) strand RNA viruses, a consistent increase in viral RNA accumulation was observed in *drb*4 *Arabidopsis* mutants infected by a TCV derivative, although no evident reduction in vsiRNA accumulation was reported [62]. In contrast to DCL4, DCL2 and DCL3 do not require any of the known DRBs for production of vsiRNAs [66].

As with endogenous sRNAs, products of dicing of viral RNAs are duplexes of vsiRNAs, which undergo methylation of their 3′-terminal nucleotides at the 2′-hydroxyl group, mediated by HEN1 [12,13]. Blevins and colleagues provided evidence that HEN1 mediates the methylation of vsiRNAs, showing that *hen1 Arabidopsis* mutants accumulate less vsiRNAs from both RNA and DNA viruses [64].

3.2. vsiRNAs accumulation and VSRs

Given that the generation of vsiRNAs is a prerequisite for RNA silencing-based antiviral defence, it is tempting to speculate that some viral suppressors could inhibit this plant function. To date no VSRs have been demonstrated to target DCL proteins directly. However, several VSRs can act either upstream or downstream of dicing. *Pothos latent virus* p14 and TCV p38 (both viruses belonging to the family *Tombusviridae*) have the capacity to bind long dsRNA *in vitro* and to prevent dicing processes in a hairpin induced silencing assay [67,68]. The capacity of p14 and p38 to bind dsRNAs is size-independent but whether they bind short dsRNA-like structures within viral ssRNAs remains to be examined. More recently, the NSs protein of *Tomato spotted wild virus* (genus *Tospovirus*, TSWV) and of its relatives (plant viruses belonging to the *Bunyaviridae* family) was shown to possess affinity for long dsRNAs and be capable of inhibiting dicing *in vitro* and *in vivo* [69]. The V2 protein of the geminivirus *Tomato yellow leaf curl virus* (genus *Begomovirus*, TYLCV) interacts with the *A. thaliana* SGS3 or its homolog in tomato [70], which are required for RDR6-dependent generation of dsRNAs (see section on endogenous small-interfering RNAs).

The most widely used strategy of silencing suppression consists of the sequestration of vsiRNAs and thus functions downstream of the dicing process (reviewed in [71]). vsiRNA sequestration has been extensively illustrated in the case of the tombusvirus p19 protein. Functional p19 is organized in tail-to-tail homodimers, which measure the length of vsiRNA duplexes and specifically selects the central 19 bp long dsRNA duplex region [72,73]. Similarly, the 2b protein of *Tomato aspermy virus* (genus *Cucumovirus*, TAV) binds vsiRNAs when organised in homodimers but with a different crystal structure to p19 [74]. Instead, the *Beet yellows virus* (genus *Closterovirus*, BYV) p21 reveals an octameric ring architecture required for binding vsiRNAs [75]. Many siRNA-binding suppressors, including p126 of *Tobacco mosaic virus* p19 and the HcPro of *Tobacco etch virus* (TEV), have been shown to interfere with vsiRNA stabilization mediated by HEN1, likely facilitating their uridylation and subsequent degradation (Fig. 2A) [76–78].

Finally, the RNaseIII encoded by the *Sweet potato chlorotic stunt virus* (genus *Crinivirus*, SPCSV) exerts an endonuclease activity on 21 nt, 22 nt and 24 nt vsiRNAs, producing 14–15 nt products (Fig. 2B) [79].

3.3. Antiviral RISC effectors

DCL-dependent production of vsiRNAs is not sufficient to restrict viral infections and AGO proteins are clearly essential components of the core machinery of antiviral RNA silencing. Proteins of the AGO family (10 members in *Arabidopsis*, among which AGO1 is the best characterized) consist of a variable N-terminal domain and conserved C-terminal PAZ, MID and PIWI domains. While the MID and the PAZ domains bind the 5′ phosphate and the 3′ end of sRNAs respectively, the PIWI domain usually has RNaseH-like endonuclease activity that cleaves ssRNAs in the region complementary to the sRNA guide [80–83]. As described previously, many plant viruses have evolved suppressor proteins able to bind vsiRNAs. Indeed, in the *Cymbidium ringspot virus* (CymRSV)-*N. benthamiana* system fractionation analysis have shown that the large majority of vsiRNAs are free or are bound to low molecular weight protein complexes, likely consisting of vsiRNAs-bound to viral suppressors [84–86]. However, in line with observations for miRNAs, a minor fraction of vsiRNAs co-fractionates with larger complexes likely corresponding to free AGO1 and to an even larger RISC complex [84]. In addition, genetic inactivation of AGO1 without altering DCLs enhances plant susceptibility to the viral infections as measured through higher viral titres [62,87]. Moreover in *Arabidopsis* infected by CMV, TCV and *Turnip yellow mosaic virus* (genus *Tymovirus*, TYMV) AGO1 immunoprecipitates contain vsiRNAs [63,88]. Despite these findings, involvement of other AGOs cannot be excluded. Indeed, null *ago7* or ago2 *Arabidopsis* mutants accumulate higher level of TCV viral RNA than in wild type plants [62,89]. Furthermore, AGO2 and AGO5 immunoprecipitates have been shown to contain CMV vsiRNAs [89,90]. The loading of vsiRNAs into specific AGO complexes seems to exhibit similar patterns to those observed for miRNAs and other plant endogenous siRNAs; AGO1 preferentially incorporates RNAs bearing a 5′ terminal U, while AGO2 and AGO4 select those having a terminal A [91], and AGO5 those with a terminal C [90]. However, 5′-end dependent incorporation is not exclusive; despite a general preference, different sRNAs with the same 5′ terminal nucleotide can be sorted into different AGO proteins [91]. The significance of these observations for the assembly of an effective antiviral vsiRNA/AGO complexes remains to be clarified.

Experimental evidence indicates that RNA silencing-related viral inactivation is based on the endonucleolytic cleavage of viral RNAs [40,84] or satellite RNAs [92]. However, antiviral mechanisms based on translational arrest cannot be excluded (Fig. 2C). Indeed, the complexes composed of AGO2 and the incorporated CMV vsiRNAs [90] should not have the potential for viral RNA slicing since AGO2 lacks the DDH catalytic residues in its PIWI domain [80], although to date is still unclear the importance of a DDH residue in AGO cleavage activity [83].

Upon infection by virus with DNA genome (i.e. the geminiviruses *Cabbage leaf curl virus* — CaLCuV and *Beet curly top virus* — BCTV) AGO4 seems to contribute to the heterochromatinization of viral genomes. Indeed, *Arabidopsis* mutants of cytosine and histone methyltransferases, of methyl cycle enzymes and of other components of RdDM, including AGO4, are hyper-susceptible to viral infection [93]. Thus, *Arabidopsis* activates an antiviral transcriptional silencing strategy against such DNA viruses [93] (Fig. 2D).

The incorporation of vsiRNAs into AGO complexes does not guarantee their functionality in viral inactivation. For example, the level of accumulated vsiRNAs explaining the cleavage sites found within the CymRSV genome does not necessarily differ from that corresponding to non-targeted regions [84]. Besides the incapacity of vsiRNAs to be incorporated into AGO complexes, the context of the target site may play a role in determining the effectiveness of silencing. Recombinant *Plum pox virus* (genus *Potyvirus*, PPV) containing target sites of cellular miRNAs were targeted by AGO-miRNA with different efficiency depending on the location of the target site and – likely – on the mutation rate of the targeted region [94]. The context of target region may reduce the accessibility of the AGO complexes due to the presence of either stable secondary structures or to viral/host proteins bound to the viral RNA as observed in other systems [95,96].

3.4. Antiviral RISC and VSRs

The indication that AGO1 is a major, although not sole, determinant for RNA silencing-based viral RNA inactivation is further supported by the fact that AGO1 is targeted by VSRs (Fig. 2C). 2b of FNY-CMV (a severe

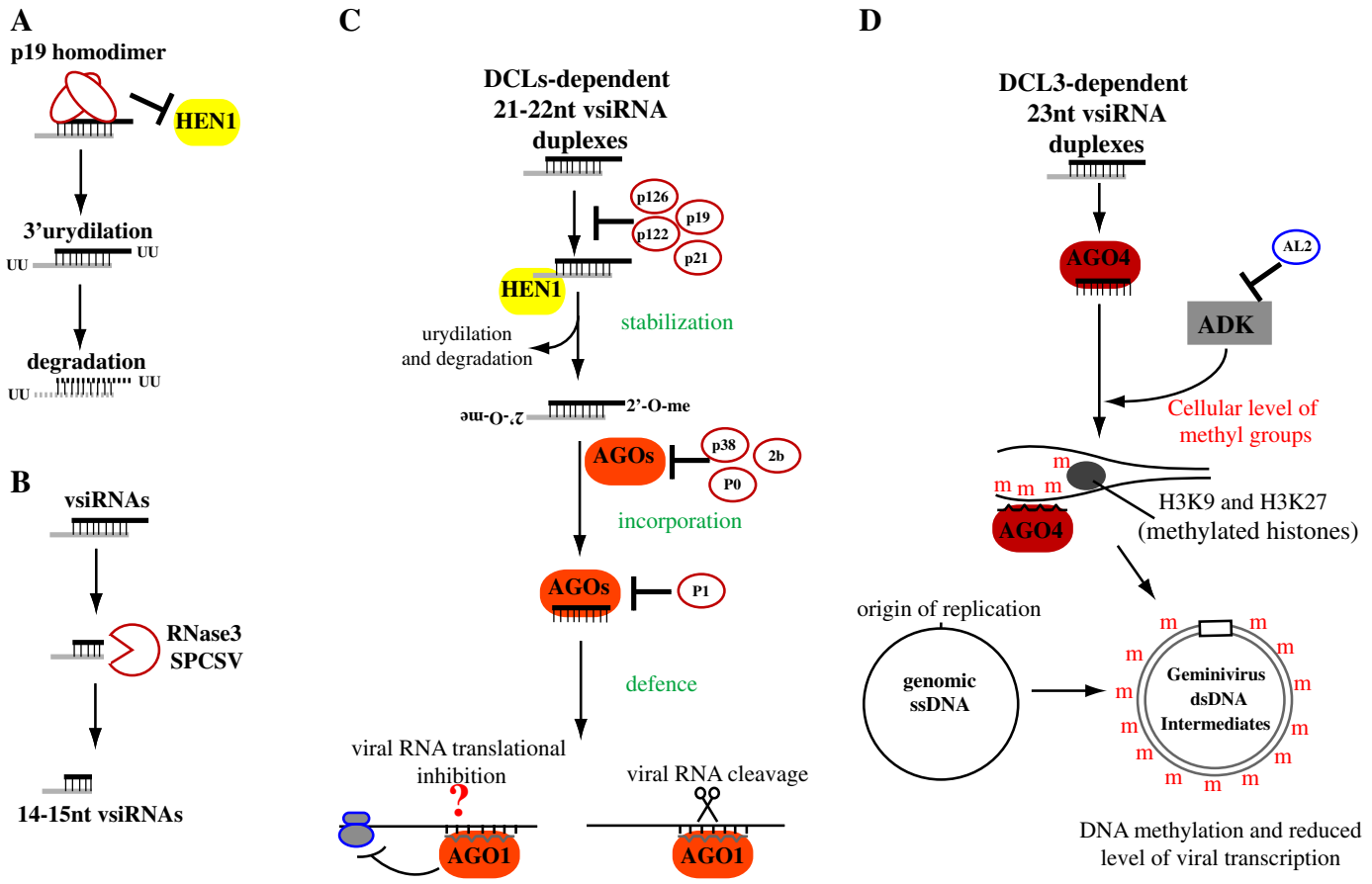

Fig. 2. Viral silencing suppressors (VSRs) and silencing strategies. A) p19 homodimers act as calliper that bind viral small RNA (vsiRNA) duplexes, preventing the action of HUA ENHANCHER 1 (HEN1) and resulting in their urydilation and degradation. B) RNaseIII encoded by the *Sweet potato chlorotic stunt virus* (SPCSV) is a VSR, which exerts an endonuclease activity on vsiRNAs resulting in 14–15 nt products. C) Antiviral RNA silencing pathway: vsiRNAs are stabilized by HEN1 and then incorporated into Argonaute1 (AGO1), the major antiviral slicer. Antiviral RNA silencing may also rely on translational arrest, although this has not yet been proven. VSRs (red circles) can act at different steps to suppress the antiviral RNA silencing pathway. D) Antiviral RNA-directed DNA methylation (RdDM): 23 nt vsiRNA from geminiviruses may be incorporated into AGO4 to target geminivirus dsDNA intermediates for cytosine and/or histone H3 methylation (at positions K9 and K27) resulting in a reduced level of viral transcripts. The geminivirus VSR AL2 (blue circle) may inhibit the ADK-dependent metyl cycle, thus suppressing RdDM.

strain of CMV) executes its suppressor function by interacting with AGO1 and by disturbing its slicer activity [88]. The same authors have shown that the primary interaction region on AGO1 is located in the PAZ and in part in the PIWI domains [88], which contain RNA-binding and RNaseH-like motifs [80], respectively. Moreover Zhang and colleagues have shown that 2b and AGO1 specifically associate with each other *in vivo* in transgenic *Arabidopsis* plants, in transient expression assays and in CMV-infected plants [88]. Whether the FNY 2b is able to perturb AGO1 *in vivo* before and/or after the incorporation of vsiRNAs is still not completely clear. The fact that both 2b of TAV (a CMV-related *Cucumovirus*, see previously) and 2b of CMV strain CM95R bind siRNAs [97], suggests that 2b of cucumoviruses has a dual mode of action, both preventing siRNA incorporation into AGO1 and perturbing its functions. The 29 kDa P0 protein of *Beet western yellows virus* (genus *Polerovirus*, BWYV) is required for virus infection [98] and does not exert any RNA-binding activity [99]. Instead, P0 possess the capacity to target AGO1 and other AGO paralogues containing a PAZ domain and the adjacent ND domain (i.e. AGO2, 4, 6 and 9) [100,101]. Moreover, P0 destabilizes the AGO1 protein before RISC assembly, thus preventing *de novo* formation of AGO1-complexes of high molecular weight *in vivo* [99]. P0 contains an F-box-like domain and has been shown to interact with components of the ubiquitination pathway [102], however P0-mediated AGO decay is proteasome-independent. Indeed the 26S proteasome inhibitor MG132 does not affect P0-mediated AGO1 ubiquitination and degradation [99,101]. More recently, the p25 protein encoded by *Potato virus X* (genus *Potexvirus*, PVX) was shown to interact and destabilize AGOs (except AGO5 and AGO9). Unlike the case of P0, the proteasome inhibitor MG132 restores the level of AGO1 in the presence of p25 suggesting that p25-mediated AGO1 destabilization is proteasome-dependent [103].

Sweet potato mild mottle virus (genus *Ipomovirus*, SPMMV) expresses the silencing suppressor protein P1, which, *in vivo*, targets both si/miRNA-loaded RISC. The suppression activity has been mapped to WG/GW motifs located at the N-terminal part of the P1 and acts through a direct interaction with AGO1 [104]. Notably, RISC assembly in plants and metazoans requires specialized AGO-binding proteins containing WG or GW residues (i.e. the plant PolV subunit NRPE1, the TAS3 component of RNA-induced transcriptional silencing in *Schizosaccharomyces pombe*, the human GW182 protein) [105–107]. Thus, P1 is thought to mimic host AGO1-binding proteins through its WG/GW motifs [104], although these interactor proteins have not been identified in plants. Similarly, the TCV p38 contains discrete GW residues, which were shown to be required for AGO1 binding *in planta*. Point mutations in p38 GW residues were sufficient to restore TCV virulence [63] demonstrating that p38 could have a dual mode of action, either sequestering siRNAs (see previously) or interacting with AGOs.

4. Alteration of endogenous small silencing RNAs and other regulatory pathways

4.1. Alteration of miRNA profiles

miRNAs regulate many biological processes ranging from basal maintenance of cellular functions to responses to environmental stresses. On the other hand, developmental anomalies are often associated with plant virus infection. This opens the questions of whether viral infections interfere with cellular miRNA functions and/or whether miRNAs are involved in responses to viral infection. Inhibition of miRNA pathways may be a strategy for viruses to influence host gene expression and create a favourable cellular environment for their proliferation. Indeed, TuMV specifically up-regulates miR158 and miR1885 in *Brassica rapa*, the putative target for miR1885 is predicted to be a member of TIR-NBS-LRR class of disease resistance proteins[108]. More emblematic evidence of how a virus can redirect host gene expression comes from the up-regulation of miR168, the miRNA known to negatively regulate Ago1 mRNA [109]. In several virus systems, miR168 was shown to be markedly over-accumulated during viral infection [110]. AGO1 is known to incorporate miRNAs and regulate several plant mRNAs. Moreover AGO1 incorporates viral siRNAs and is known to play a central role in antiviral silencing (see previously in the text). Thus, any viral strategy to down-regulate AGO1 expression would help the virus evade plant defences and at the same time, would perturb endogenous mRNA expression.

While various approaches (i.e. microarray, high throughput sequencing) have been used to reveal changes in miRNAs accumulation in virus-infected plants, it is not totally clear how viral infection determines these changes. As previously described, VSR-expressing plants often display similar developmental anomalies to those associated with miRNA pathway mutants. Transgenic approaches have been used to show that all VSRs having the capacity to bind sRNA duplexes (i.e. p19, HcPro, p21) can sequester miRNA/miRNA* duplexes and can affect the miRNAs pathway at an intermediate step. In particular, plants expressing such sRNA-binding VSRs show the effect of an inhibited turnover of the miRNA*, a higher accumulation level of the miRNA duplex as well as the arrest of the AGO1-miRNA assembly pathway and, in some circumstances, the up-regulation of the corresponding mRNA targets [111–113]. These effects are likely due to the miRNA-binding activity of the VSRs that prevent miRNA accumulation into AGO1 (Fig. 3A).

When expressed in transgenic *Arabidopsis*, TYMV p69 triggers a negative feedback regulation of DCL1 mRNA. This observation, likely explains the over-accumulation of all examined miRNAs, including miRNA156. In addition, the p69 transgenic plants exhibited a late flowering phenotype, which correlates with miR156-mediated down-regulation of SP3mRNA (a gene involved in early flower timing) [114]. The mechanism of action of p69 differs from that of previous mentioned viral sRNA-binding genes, since it can up-regulate the activity of miRNAs in inhibiting host gene expression.

The AC4 protein from *African cassava mosaic virus* (genus Begomovirus, ACMV) employs yet another mechanism and binds single-stranded miRNAs (instead of miRNA duplexes) *in vitro* and *in vivo* - inhibiting the miRNA-mediated negative regulation of gene expression [115]. Interestingly, these authors propose that AC4 acts downstream of RISC assembly using a still unknown mechanism (Fig. 3A).

Of course, the foregoing observations do not necessarily reproduce the effect of VSRs *in vivo*, since, during infection, viral protein expression is restricted in space and time to particular cell compartments within infected tissues. Moreover, there is evidence that VSRs are not always responsible for altering miRNAs profile; Bazzini and colleagues showed that plant viruses with weak or no post-transcriptional suppression activity (e.g. TMV *and Tomato mosaic virus*) can alter the profile of miRNAs to a greater extent than viruses with stronger suppression activity (e.g. TEV and *Potato virus Y*). Instead of TMV VSR (i.e. p126), TMV movement and coat proteins (MP and CP, respectively) are responsible for changes in miRNA levels and for provoking developmental anomalies in transgenic plants [116].

miRNA production could be affected at the transcriptional rather than (or in addition to) the post-transcriptional level (Fig. 3A). Indeed, a GUS reporter gene under the control of the miR164a promoter revealed a significant increase of GUS activity upon *Oilseed rape mosaic virus* (ORMV) infection and the level of miR164a primary transcripts was clearly augmented after ORMV infection [117]. While, in this system, induction of the pri-miR164a promoter was accompanied by increased accumulation of both pre-miR164a and miR164a, the viral infection caused a reduction of miR164a activity (measured by the increase of the mRNA target) [117]. This discrepancy is likely explained by the activity of the pre-readthrough replicase protein of tobamoviruses (a plant virus family to which ORMV belongs), a silencing suppressor whose activity is based on binding siRNAs and preventing their HEN1-mediated methylation — leading to accumulation of non-methylated miRNAs [76,118,119] (Fig. 3A). Virus-induced transcription of pri-miRNAs may be a widespread phenomenon, since a similar effect has been observed in the case of pre-miR168; in *A. thaliana*, TCV, CMV and *Ribbgrass mosaic virus* (RMV — another member of the genus *Tobamovirus*) infections are always associated with enhanced accumulation of the pre-miR168 [110]. The details of mechanisms by which a virus can stimulate pri-miRNA promoters are still obscure and deserve careful studies. It has been proposed that plant hormones may be candidate molecules mediating the connection between viral infections and induction of miRNA promoters. Indeed, Bazzini and colleagues have provided evidence that the pri-miRNA164a promoter is gibberellin-sensitive [117]. In addition, it is known that viral pathogens deliver effector molecules into the plant cell in order to manipulate hormone signalling components and to promote disease. In turn, changes in level of plant hormones often influence gene expression at transcriptional level [120].

Another core factor of the miRNA pathway is DCL1 (see earlier discussion). DCL1 mRNA is negatively regulated by miR162 [121]. Thus, both miR162 and miR168, negatively regulate the miRNA pathway itself by targeting DCL1 and AGO1 mRNAs, respectively (Fig. 4). Such feedback regulation of two core factors in the miRNA pathway is important to achieve an appropriate balance of miRNA steady-state levels, which depends on miRNA production by DCL1, and miRNA function mediated by AGO1. As discussed previously viral anti-AGO1 strategies alter both antiviral and miRNA functions in plants. For instance, CMV 2b, TCV p38, SPMMV P1, BWYV P0, impair the functionality of AGO1 [63,88,99–101,104] (Fig. 3A). In *A. thaliana*, the marked enhancement of DCL1 levels (in term of mRNA and protein accumulations) was associated with p38-dependent reduction in the activity of AGO1-dependent miR162. Similarly, the level of DCL1 mRNAs in most of transgenic *A. thaliana* plants expressing CMV 2b was up-regulated twofold to threefold with respect to wild type [88].

An alternative non-protein dependent strategy by which viruses can interfere with miRNA biogenesis was proposed for *Red clover necrotic mosaic virus* (genus *Dianthovirus*, RCNMV). Takeda and collaborators showed that RNA elements required for viral (−) strand synthesis can deplete DCL1 activity. Indeed, they observed that the level of miR171 was lowered in leaves where such viral RNAs were delivered [122].

4.2. Alteration of other endogenous siRNA profiles

The production of ta-siRNAs requires the miRNA-mediated cleavage of the TAS primary transcripts, the RDR6-mediated generation of the dsRNA and the endonuclease action of DCL4 (see before and Fig. 3B). Hence, any virus effector that target one of those steps would impair ta-siRNA production. Since most known ta-siRNAs are involved in developmental phase transitions such as leaf development and

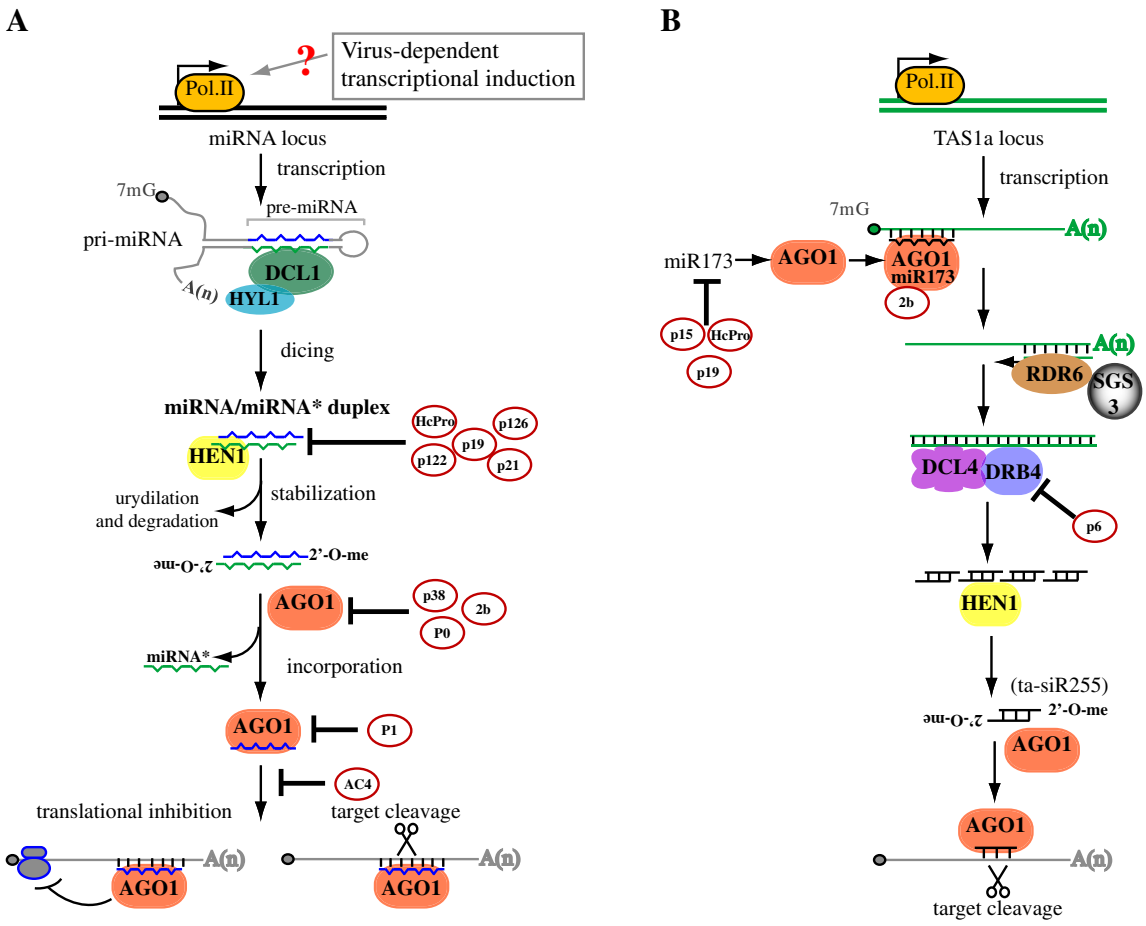

Fig. 3. Antiviral silencing suppressors (VSRs) and endogenous silencing pathways. A) micro (mi)RNAs are generated by transcription of miRNA genes by RNA polymerase II (RNA pol II). The primary RNA (pri-miRNA) possesses a stem loop structure, which is processed by Dicer1 (DCL1) with the contribution of HYPONASTIC LEAVES1 (HYL1) into a final 21nt miRNA/miRNA* duplex via the formation of miRNA precursor (pre-miRNA). miRNA/miRNA* duplexes are then stabilised by HEN1 and subsequently incorporated into RNA-induced silencing complex (RISC) containing AGO1. AGO1-miRNA complex represses gene expression through either mRNA cleavage or transgational repression. Various VSRs (red circles) can act at different steps to suppress the miRNA-mediated silencing pathway. B) *trans*-acting small interfering RNA 255 (ta-siR255), is generated by transcription of non-coding TAS1a gene by RNA polymerase II (RNA polII). The TAS1a transcript is cleaved by a RISC containing AGO1 and miR173. The resulting 3′ fragment acts as template for the formation of long double stranded (ds) RNA mediated by RDR6 and SGS3. These long dsRNAs are diced in a DRB4-dependent mannaer by DCL4 into phased 21 nt siRNAs, including ta-siR255. Various VSRs (red circles) can act at different steps to suppress the ta-siRNA pathway.

polarization [123], their inhibition by viral effectors may contribute to developmental aberrations observed in transgenic plants and, likely, in infected plants. Indeed, ta-siR255 (a siRNA derived from TAS1 loci) levels were decreased in transgenic plants expressing the CaMV P6, the FNY-CMV 2b, the *Tomato bushy stunt virus* (TBSV) p19, the TCV P38, the *Peanut clump virus* p15 and the TuMV HcPro. VSRs arresting the AGO1-miRNA assembly (i.e. p15, p19 and HcPro) would likely prevent the incorporation of miR173 into AGO1, thus impairing the formation of ta-siR255 upstream of the cleavage of the TAS1 transcript [56] (Fig. 3B). Conversely, CMV 2b decreases the accumulation of mature ta-siR255 (thus, increasing the corresponding mRNA target) by disturbing AGO1-directed cleavage of the TAS1 primary transcript [88] (Fig. 3B). On the other hand, CaMV P6 suppresses ta-siR255 accumulation, but not miR173 accumulation, by targeting DRB4, which is required for DCL4-dependent processing of the TAS1-dsRNA [65] (Fig. 3B).

24 nt siRNAs generated by a pathway involving DCL3, RDR2 and NRPD1 (a subunit of RNApolIV; see section on endogenous siRNAs) are incorporated into RNA-induced transcriptional silencing complexes (RITS) to mediate transcriptional gene silencing by RNA-directed DNA methylation (RdDM). RdDM is mainly involved in controlling the maintenance of epigenetically silent states at repeated loci, heterochomatin and transposable elements (TEs) [29,124]. However, more recently RdDM has emerged as a mechanism with important roles in the direct regulation of functional genes where TEs have been inserted in introns, in promoters or in the proximity of genes [125,126]. Furthermore, several genes were recently found to be methylated within transcribed regions or within their promoter regions [127]. Viral infections may induce transient modification of TGS leading to a hypo-methylated state of the genome that, in turn, can result in reactivation of retrotransposons [128] and in up-regulation of gene transcription [129]. The TMV resistance gene N of *Nicotiana glutinosa*, produces 2 mRNAs isoforms, denoted N_S and N_L. The N_S transcript encodes the full length N whereas the N_L transcript, which results from an alternative splicing of a 70 bp alternative exon, encodes a truncated N protein. The latter is required for full resistance to TMV [130,131]. Importantly, it has been shown that the alternative exon was generated through the insertion of a miniature inverted-repeat transposable element [132] and that the relative abundance of the N_L isoform is transiently increased during TMV infection [131]. Taken together, these data are consistent with interconnections between viral infection and components of the RdDM pathway. Indeed, DCL1-dependent repression of DCL3 expression (Fig. 4) (lacking a mechanistic explanation) has been proposed by Azevedo and colleagues as having a possible deep impact on RdDM [63].

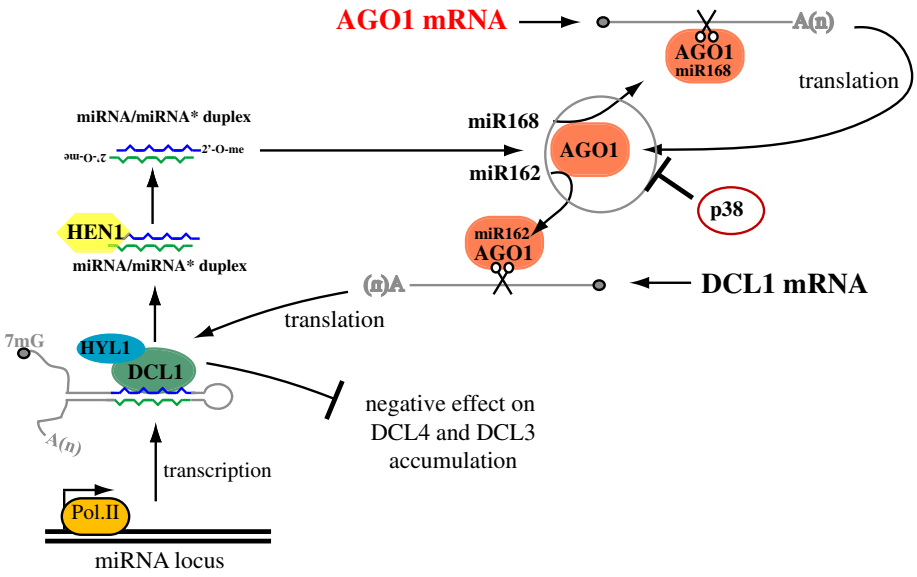

Fig. 4. Diagram of AGO1-dependent regulatory network of plant Dicers (DCLs). miR168 loaded into AGO1 down-regulates AGO1 mRNAs and regulates AGO1 accumulation. Conversely miR162 loaded into AGO1 down-regulates DCL1 mRNA and its translational product DCL1, which, in turn, manifests in reduced miRNA generation. The *Turnip crincke virus* (TCV) viral suppressor p38 (red circle) impairs the functionality of AGO1, including the miR162-dependent regulation of DCL1. This results in higher accumulation of DCL1 and, in turn, through an unidentified mechanism, a DCL1-dependent down-regulation of DCL3 and DCL4 protein levels.

4.3. Alteration of host gene expression

Geminiviruses are single-stranded DNA viruses that replicate in the host cell nucleus through dsDNA intermediates that assemble into minichromosomes [133]. Members of the genus *Begomovirus* (Family *Geminiviridae*) encode a transcriptional activator protein (AC2 or AL2) that is required for the expression of late viral genes [134]. It has been shown that AC2 can activate gene promoters, in part explaining the up-regulation of ca. 30 genes observed in transfected *Arabidopsis* protoplasts [135]. In transient transfection assays, AC2 was found to be a suppressor of post-transcriptional gene silencing; this activity might be explained by AC2-mediated induction of a cellular silencing suppressor [135]. However, AL2 can suppress TGS by inactivating adenosine kinase (ADK), an enzyme involved in the production of S-adenosyl methionine, which is a cofactor required in several cellular transmethylation events (i.e. histone methylation) [136,137]. Indeed, plant hosts employ viral chromatin methylation as a defence against geminiviruses and, in turn geminiviruses have evolved VSRs able to attenuate cytosine methylation [93] (Fig. 2D). Notably, transgenic *Arabidopsis* expressing AL2 and the related L2 protein from members of the *Curtovirus* genus (which belongs to the *Geminiviridae* family) show a genome-wide reduction in cytosine methylation [138], raising the possibility that reactivation of transposons and/or hypomethylation-dependent gene induction could be associated with geminivirus pathogenesis.

Several groups of mammalian DNA viruses produce viral miRNAs that regulate both viral and host gene expression [139]. An emerging field of investigation consists of addressing the possibility that vsiRNAs could target host functions in a sequence specific manner. Bioinformatics analysis aiming to look for homologies between vsiRNAs and host transcripts often suggest the possibility that vsiRNAs could inhibit host transcript expression and in the same case such a layer of virus/host interaction has been shown experimentally. For example, in CaMV infections, the 35S leader RNA releases one vsiRNA (vsiRCC1) that exhibits 20 nt of complementarity with the 5′UTR of the *Arabidopsis At1g76950* transcript (encoding a protein having a characteristic RCC1 domain). Semiquantitative RT-PCRs for the level of the mRNA and experiments with specific vsiRNA sensors revealed that the *Arabidopsis* transcript was indeed strongly downregulated in CaMV-infected plants and that it results from direct action of the vsiRCC1 [46]. The same authors revealed a list of host transcripts potentially targeted by vsiRNAs from the 35S leader of CaMV and involved in basic cell metabolism [46]. A systematic bioinformatics analysis of TMV-Cg vsiRNAs has revealed 4784 potential mRNAs targets in *Arabidopsis*; including a polyadenylation specificity factor (CPSF30, *At1g30460*) and an unknown protein similar to translocon-associated protein alpha (TRAP α) which gave positive 5′ RACE results supporting vsiRNA-driven cleavage [44]. The most emblematic case of vsiRNA-directed host mRNA down-regulation has been recently described to be directly linked with a specific virus-induced symptomatology. CMV Y satellite RNA (Y-sat) contains a 22-nt sequence complementary to the tobacco *ChlI* mRNA (encoding the tobacco magnesium protoporphyrin chelatase subunit I gene, a key component of the chlorophyll biosynthesis pathway, [140]). Shimura and colleagues have found that Y-sat produces a vsiRNA from the Y-sat/*ChlI* complementary region that specifically cleaves and downregulates *ChlI* mRNA, inducing a yellow mosaic of infected tobacco leaves [141,147] (Fig. 5).

5. Concluding remarks

Plant viruses alter endogenous RNA silencing pathways by at least two mechanisms: i) by producing their own sRNAs and ii) by altering endogenous sRNAs. Upon incorporation into AGOs these sRNAs contribute to virus-specific or non-specific plant defences by promoting mRNA regulation at transcriptional and post-transcriptional levels. VSRs are viral molecular tools, which possess the innate capacity to counteract the effect of sRNAs at various steps, and in turn they have been used as molecular tools to unravel specific RNA silencing pathways. The identification of novel VSRs with novel mechanisms of action will further assist in the process of dissecting virus/host interactions and plant responses to stresses. Such studies may in turn provide insights into gene regulatory processes in other eukaryotic organisms. The advent of high throughput technologies, including sRNA profiling and transcript profiling, is allowing unprecedented advances in the study of such networks and helping in the association of individual sRNA species with their regulatory targets [142,143]. RNA silencing, besides its important regulatory role in endogenous pathways, is an anti-microbial defence mechanism in crop plants, thus these studies

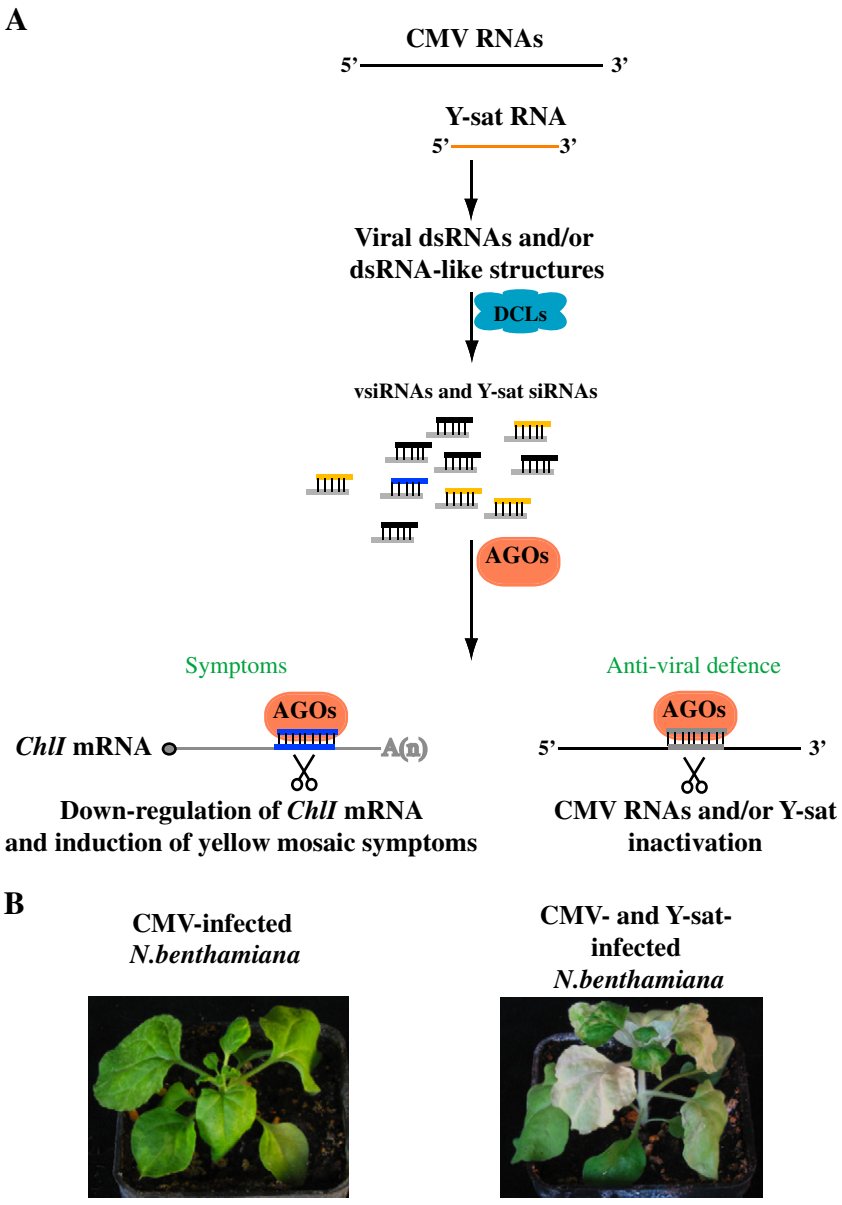

Fig. 5. Diagram of RNA silencing induced by CMV and Y-sat RNA. A) CMV genomic RNAs support the replication of the non-coding subviral RNA (Y satellite RNA, Y-sat). Both CMV and Y-sat RNAs generate vsiRNA (colored in black and yellow, respectively). Furthermore, Y-sat releases one siRNA (blue) complementary to a 22 nt long sequence within the *ChlI* mRNA. Upon incorporation into RISC complex containing AGOs protein, the Y-sat-derived siRNA cleaves *ChlI* mRNA and induces the yellow phenotype, whereas CMV-derived vsiRNA and other Y-sat siRNAs may be engaged in antiviral silencing. B) The yellow symptom induced by CMV and Y-sat (right panel) and not by the CMV alone (left panel) on *Nicotiana benthamiana* plants.

could potentialy lead to the development of effective strategies for controlling diseases.

In plants, heterologous genes can be expressed using either transgenic plants or plant viral vectors. Conversely, when viruses are designed to carry a portion of a host gene, processed siRNAs (i.e., vsiRNAs) can become an inducer of post-transcriptional gene silencing (PTGS). Furthermore, when viruses are designed to carry a portion of a promoter sequence, processed siRNAs can become an inducer of transcriptional gene silencing (TGS) leading to epigenetic modification of the host gene. To date, various plant viruses have been used as vectors to control host gene expression and observe subsequent phenotypic changes (e.g., color, shape and organ formation). Among them, PVX, TRV and CMV vectors have been used as a tool to induce TGS [59,144–146]. In particular, the CMV vector has also been shown to induce heritable gene silencing by targeting dsRNA to endogenous gene promoters in petunia and tomato [146]. As CMV and other viral vectors are not transmitted to seeds in many plant species, the epigenetically modified plants are inducer-free in the next generation, and the absence of any transgenes is a great advantage for practical use of such modified plants in agricultural contexts.

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