Review

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Hit two birds with one stone: the multiple properties of (viral) RNA silencing suppressors

D'une pierre deux coups : les multiples caractéristiques des suppresseurs (viraux) de l'interférence par l'ARN

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Abstract. In any organism, gene expression regulation is provided by multiple factors to maintain a harmonious development of individuals. Discovered in the late 1990s, RNA interference (RNAi) completely remodelled the way in which gene expression regulations were initially apprehended. RNAi provides fine regulation at the cellular level and allows organisms to control their development, maintain their genomic integrity and fight against different stresses like viral infection. Exogenous or endogenous double-stranded RNAs initiate RNAi and are recognized and cleaved by Dicer protein in about twenty nucleotide duplexes small RNAs (sRNAs). One strand of the duplex is loaded into a ribonucleoproteic complex, named RISC (RNA induced silencing complex), composed of at least one ARGONAUTE protein and a sRNA. Therefore, the expression of any RNA possessing the complementary siRNA sequence will be specifically silenced either at the transcriptional or post-transcriptional level. RNAi plays a prominent role in the defence against viral infection and the last two decades of research have refined our knowledge of proteins involved in this pathway. Many viruses counteract the antiviral action of RNAi through the expression of factors (VSR, Viral suppressor of RNA silencing) that were first identified on virally infected plants. However, in mammals the antiviral role of RNAi remains controversial. Indeed, viral infections are controlled by the interferon response and the antiviral action of RNAi has not been clearly demonstrated *in vivo*. In this review, the main modes of defence suppression used by VSR and endogenous RNAi suppressors will be presented. Finally, the role of viral non-coding RNAs (ncRNAs) acting as suppressors of RNAi will be discussed.

Key words : VSR, silencing, PTGS, RNAi, ncRNA, siRNA

Résumé. La régulation de l'expression des gènes est assurée chez tous les organismes pour maintenir un développement harmonieux de l'individu. Découverte à la fin des années 1990, l'ARN interférence a complètement révolutionné la façon dont étaient conçues ces régulations. L'ARN double brin est la molécule initiatrice de cette voie et sera reconnu puis clivé par une protéine Dicer en duplex d'une vingtaine de nucléotides : les petits ARN interférents ou siARN (*small interfering* ARN). L'un des brins du duplex est alors incorporé dans un complexe ribonucléoprotéique, RISC (*RNA induced silencing complex*), avec une protéine ARGONAUTE. Dès lors, l'expression de tout ARN qui possède la séquence complémentaire au siARN sera réprimée au niveau transcriptionnel ou post-transcriptionnel. Cette spécificité de séquence assure une régulation fine au niveau cellulaire et permet aux organismes de contrôler leur développement, maintenir leur intégrité génomique et lutter contre différents stress. En cas d'infection virale, l'ARN interférence est particulièrement sollicitée puisque de nombreuses molécules d'ARN exogènes sont néo-synthétisées. Cette action antivirale associée à l'ARN interférence fut découverte chez les plantes à l'aube du XXI^{ème} siècle. Depuis, deux décennies de recherche

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ont permis d'affiner nos connaissances sur les multiples protéines impliquées dans cette voie et aussi de découvrir des protéines capables de supprimer l'ARN interférence (VSR, *Viral suppressor of RNA silencing*). Les premiers VSR ont été identifiés sur des plantes virosées et permettent aux virus de contourner la défense antivirale de la plante. Chez les mammifères, le rôle antiviral de l'ARN interférence reste discuté par la communauté scientifique. En effet, l'infection virale y est contrôlée par la réponse interféron et le rôle de l'ARN interférence n'a pas été formellement démontré *in vivo*. Les principaux modes de suppression usités par les VSR, qu'ils soient exprimés par des virus animaux ou végétaux, seront présentés dans cette revue et accompagnés par la description de suppresseurs endogènes. Enfin, ces différents modèles de suppression seront complétés et complexifiés par les ARN viraux non codants (ARNnc). La suppression du *silencing* n'est pas restreinte aux VSR de nature protéique et plusieurs exemples d'ARNnc viraux assurant cette même fonction seront présentés.

Mots clés: VSR, *Silencing*, PTGS, ARNi, ncARN, siARN

Introduction

RNAi or RNA silencing was initially observed on petunias in 1990 [1, 2], and was further characterized in 1998 by A. Fire and C. Mello in *Caenorhabditis elegans* [3]. This mechanism controls stress responses [4], development [5] and protects genome integrity from mobile elements such as transposons [6]. Found from unicellular [7] to human species [8], it is mediated by 21 to 30 nucleotides (nt) sequence specific long RNAs that inhibit gene expression at the transcriptional level (TGS: transcriptional gene silencing) [9, 10] and at the post-transcriptional level (PTGS: post-transcriptional gene silencing) [11]. Double-stranded RNA (dsRNA), the initiating molecule of RNAi [3], is produced by viral infections, or by RNA-dependent RNA polymerases (RDR), or transcription of inverted repeat or convergent regions [12, 13]. These dsRNAs are recognized and then cleaved by a type III endoribonuclease, Dicer [14], which generates duplexes of small RNAs (sRNAs). They are divided into two major classes: small interfering RNA (siRNA) and microRNA (miRNA). Although they diverge in their origins (endogenous and/or exogenous), biogenesis pathways and targets, their mode of action remains similar. These sRNAs are loaded into one ARGONAUTE (AGO) protein and unwinded to generate single-stranded RNAs (ssRNAs) [15]. One strand is displaced from AGO, the other one (guide strand) is retained to make the functional RISC complex. The guide strand recognizes by base complementary a targeted nucleotide sequence [16]. The effector RISC initiates either PTGS through translational inhibition or messenger RNAs (mRNAs) cleavage through the endonucleotidic activity (slicer) of AGOs proteins [17] or TGS by regulating DNA or histones methylation through proteins able to alter chromatin states [10]. Whether RNAi induces post-transcriptional or transcriptional silencing depends on the organism, the AGO protein and the nature of the small RNA loaded into AGO. In addition to preserving and regulating genome expression, RNA interference has an essential antiviral function in invertebrates [18] and in plants but remains controversial in mammals [19]. In 1999, one year after the publication of the Fire and Mello work, an antiviral action has been associated with RNA silencing in plants [20], but widely demonstrated at the beginning of the 21st century.

In plants, "Dicer" enzymes are named Dicer-like or DCL [21]. DCL1 generates miRNAs (*figure 1*) while DCL3 is responsible for the synthesis of endogenous 24 nt siRNAs involved in TGS [22, 23]. DCL4 is the main antiviral DCL and generates 21 nt siRNAs. In the absence of DCL4 activity, DCL2 protein has a redundant antiviral action and generates 22 nt siRNAs [24]. During RNA virus infection, double-stranded replication intermediates generated by the viral RDR and/or viral intramolecular fold-back RNA structures are DCL4 substrates, thus generating 21 nt viral siRNAs (vsiRNAs). They possess a phosphate group at the 5′ end and 2 nt overhang at the 3' hydroxyl end [25, 26] where they are protected from uridinylation and degradation by a methyl group added by the methyl-transferase HEN1 [27, 28].

In the model plant *Arabidopsis thaliana*, ten AGO proteins have been identified. The guide strand loaded into AGO is positioned at its 3' end in the PAZ domain [29, 30] while the 5' phosphate end is located in a pocket at the interface of MID/A and PIWI/B domains [31]. This folding is forming the PIWI domain which exhibits an RNase H-like structure and is responsible for target RNA cleavage [32]. Such slicing activity has been demonstrated only for AGO1, AGO2, AGO4 and AGO7 [33-36]. AGO1 and AGO2 are the major anti-viral AGO proteins against RNA viruses while AGO5, AGO7 and AGO10 have a minor role in antiviral defence

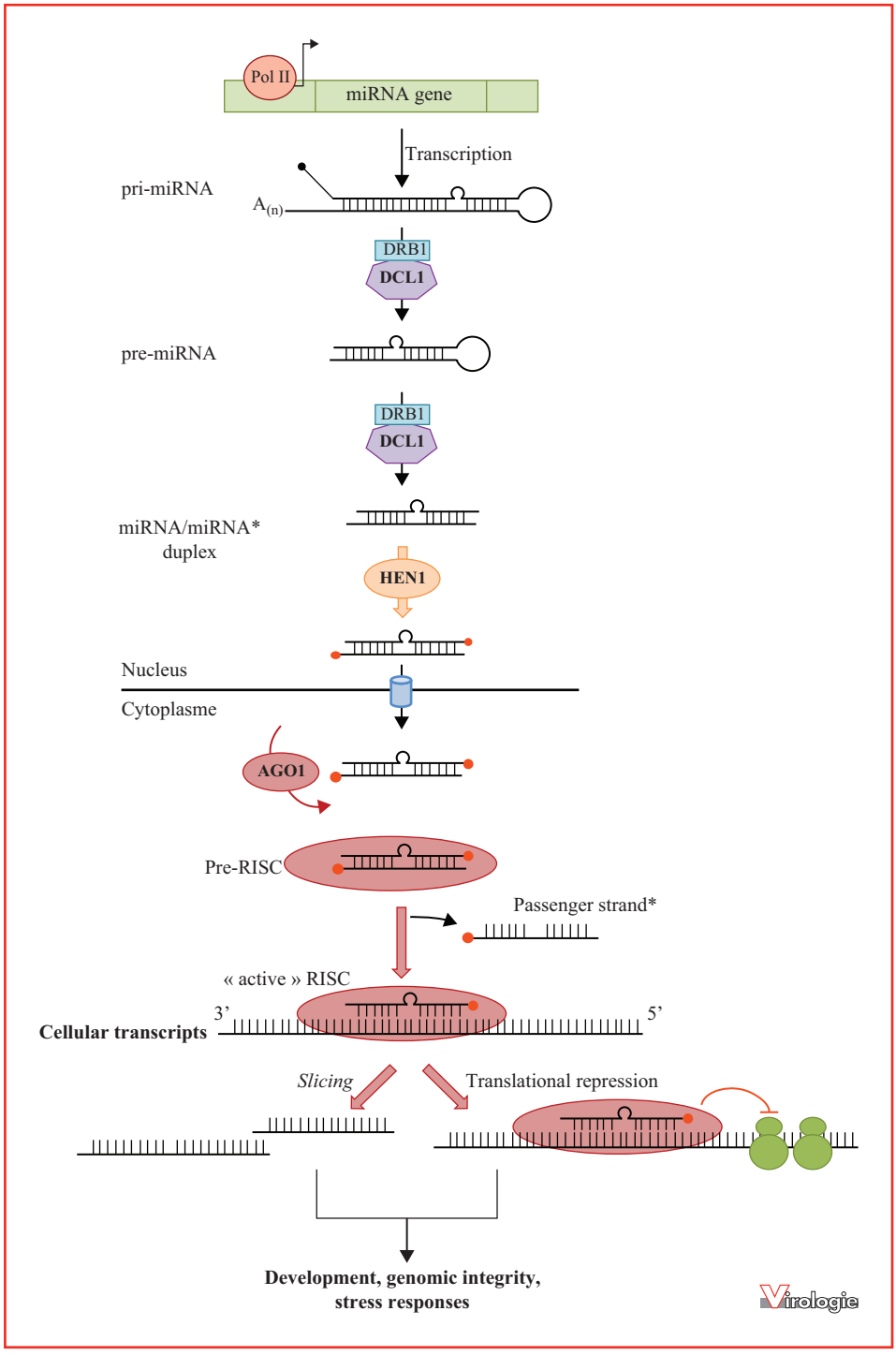

Figure 1. Simplified representation of plant miRNA biogenesis and modes of action. Pri-miRNAs are transcribed by RNA polymerase II, capped (black sphere) and polyadenylated ($A_{(n)}$). DCL1 and its cofactors, including DRB1 (double-stranded RNA binding 1, also referred to HYL1), ensure their processing into pre-miRNA. MiRNA duplexes are obtained following a second dicing step and methylated at their 3' end by HEN1 (orange sphere). The duplex is exported into the cytoplasm and loaded into AGO1 to form the pre-RISC complex. The passenger strand is removed and the "active" RISC complex ensures cell transcripts silencing by their cleavage or by inhibiting their translation through their base-pairing complementarity to the guide strand.

[34, 37-39]. Immunity against DNA viruses is mainly provided by AGO4 involved in TGS, where it regulates methylation levels of viral and cellular DNAs [40-42].

The formation of RISC has been a matter of debate for a long time, and two models were proposed: on the one hand, the RNA duplex is separated prior to guide strand loading into AGO, whereas on the other hand, the duplex in its double-stranded form is loaded into AGO and the selection of the guide strand, as well as the dissociation of both strands take place within the RISC. This latter model seems to be favoured following the discovery of small dsRNA loaded into human and *Drosophila* AGO proteins [43, 44]. Guide strand selection is not random but relies on duplexes thermodynamic stability: the strand having a lower stability at its 5' end is selected as a guide and the other, called "passenger", is eliminated [45, 46]. In *A. thaliana*, a base-dependent selective bias at the 5' end of the small RNA seems to favour the recruitment of AGO proteins: AGO1 tends to select strands with uracil whereas AGO2 and AGO4 select those with adenine and AGO5 has a preference for cytosine [47, 48]. This specificity has also been explained according to the structural resolution of the human AGO2 MID (middle) domain. Several studies have shown that selection of the guide strand in AGO was subjected to a strong bias according to the base present at the 5' end of miRNAs [49, 50]. A rigid loop of the MID domain ensures the specific recognition of adenosine monophosphate and uridine monophosphate, while its affinity for cytidine monophosphate and guanosine monophosphate is thirty times lower. In addition, when mutations are introduced in the loop domain, this specificity is no longer ensured and confirms the major role of the MID domain in this selective process [51]. When a perfect RNA duplex (case for siRNA) is loaded into AGO, the passenger strand can be cleaved and degraded [52-54]. In cleavage-deficient AGOs, the elimination process of the passenger strand remains poorly documented. Nevertheless, a mirror model has been proposed when the pre-RISC complex is loaded with an imperfect hybrid duplex miRNA-miRNA* [55].

In plants, viral RNAs are targeted by the guide strand through base complementarity and will be mostly cleaved by AGO1 or AGO2 to restrict viral infection. The cleavage products are used as a template by cellular RNA dependent RNA polymerases (RDRP or RDR) [56]. RDR6 is the major RNA polymerase involved in silencing amplification [57, 58] and, in association with its major cofactor SGS3 (suppressor of gene silencing 3) [59, 60], ensures *de novo* synthesis of dsRNAs processed by DCLs, which generate the so-called "secondary" vsiRNAs (*figure 2*, left and *figure 3*). This population of siRNAs can traffic through plasmodesmata to reach the plant vascular system and confers extensive antiviral immunity [61, 62].

Phytoviruses have evolved to express suppressors of RNA interference (VSR: viral suppressors of RNA silencing) to counteract this antiviral defence. These are principally proteins, although nucleic acid VSRs have been recently described. Virtually, all viruses express such counter-defence elements, but no sequence neither structure similarities have been established [63], suggesting that evolutionary convergence of such a viral function is likely. This convergence is enhanced by the various VSRs mechanisms already characterized, targeting multiple steps of the RNA interference pathway. This review will focus on presenting the different silencing suppression strategies employed by VSRs, without pretending to make their exhaustive list.

Discovery of VSRs

The tobacco etch virus (TEV) HC-Pro protein (helper component proteinase) was the first described VSR and confirmed the antiviral function of RNA silencing. Symptoms exacerbation of co-infected plants with potato virus Y (PVY) and potato virus X (PVX) was initially attributed to the potyviral HC-Pro expression, described as a broad-spectrum pathogenicity enhancer that increases heterologous virus replication. Transgenic expression of HC-Pro is sufficient to increase symptoms severity and accumulation of tobacco mosaic virus (TMV), cucumber mosaic virus (CMV) and PVX genomic RNAs [64]. In addition, HC-Pro is essential for viral long-distance movement [65]. Among the hypotheses about HC-Pro functions, it was suggested that the protein can inhibit a cellular defence mechanism [65]. Following RNA silencing discovery, this hypothesis was validated and HC-Pro became the first identified VSR [66]. After multiple studies, HC-Pro appeared as a multifunctional VSR able to target several steps of the RNA silencing pathway. HC-Pro limits RNA interference establishment by sequestering vsiRNAs and therefore limits RISC assembly [67, 68]. HC-Pro also decreases small RNAs methylation levels, by interacting on the one hand with HEN1 [69] and on the other hand with two key enzymes regulating the methionine biosynthesis cycle [70]. In addition, HC-Pro interacts with AGO1 at the ribosomal level and counteracts its translational inhibition function. Moreover, HC-Pro causes a decrease of AGO1 mRNA expression level by inducing the expression of miR168, a microRNA targeting AGO mRNA [71]. HC-Pro is not the only RNA silencing suppressor described in potyviruses. The VPg protein is also able to interact with SGS3, the cofactor of RDR6, to initiate its degradation by the proteasome and by autophagy. RDR6 subsequent degradation is observed

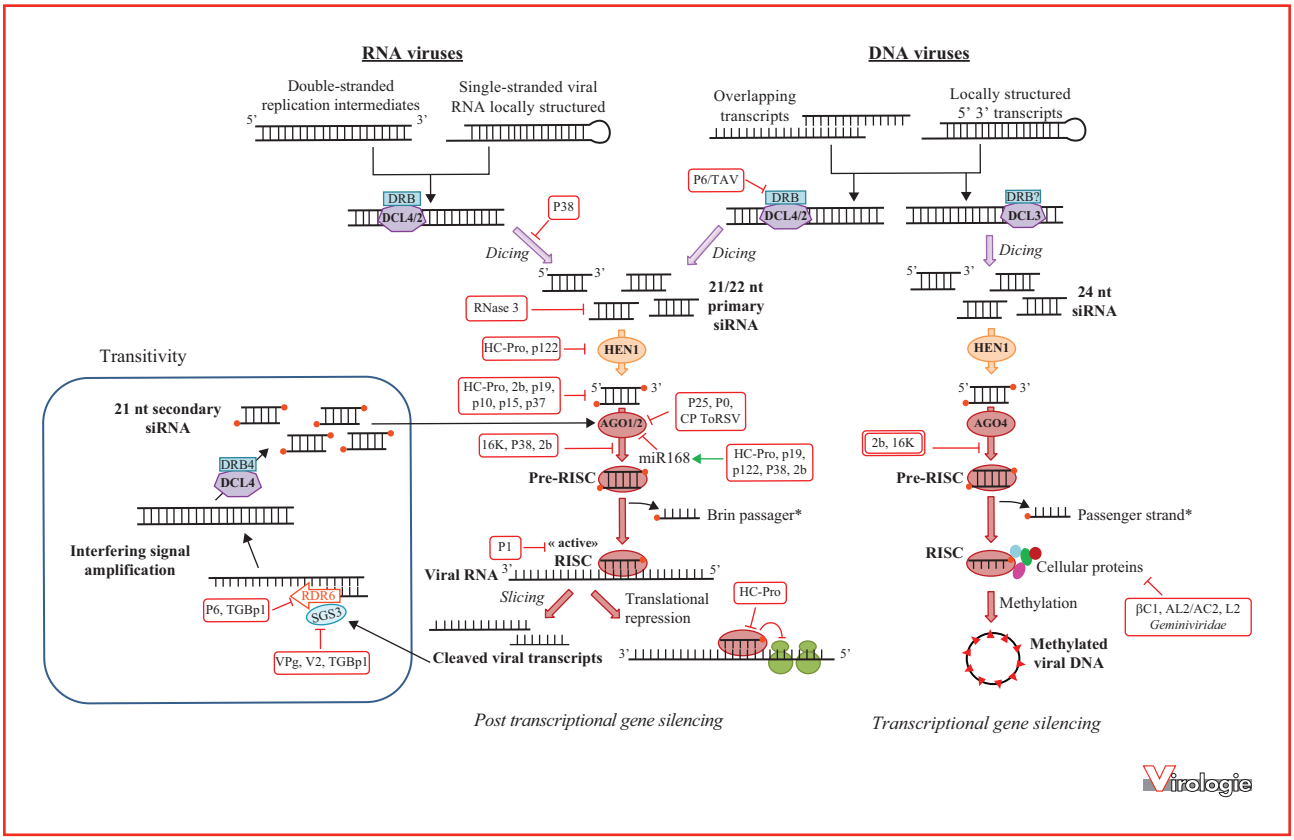

Figure 2. Graphical representation of antiviral defense provided by RNA interference and counter-defenses mediated by VSR. Replication intermediates (double-stranded RNA) and/or double-stranded structures of RNA viruses as well as overlapping and/or locally structured transcripts of DNA viruses are recognized and cleaved (dicing) by Dicer-like enzymes (DCL). DCLs are associated with their DRB (double-stranded RNA binding) cofactors to generate primary small interfering RNAs duplexes (siRNAs). A methyl group (orange sphere) is added to each 3' hydroxyl end of the duplex by HEN1 (Hua enhancer 1). SiRNAs are loaded into an ARGONAUTE protein (AGO) to form the pre-RISC complex (RNA induced silencing complex). One of the duplex strand, then named passenger strand, is eliminated to form the active RISC complex. When infected with RNA viruses, viral transcripts are targeted by base complementarity with the guide strand loaded in AGO and will be mainly cleaved by AGO slicing activity. Cellular RNA dependent RNA polymerases (RDRs) make use of cleavage products to amplify the interfering signal. Secondary siRNAs are generated by DCL4 and associate with AGO to form RISC complexes. Regarding DNA viruses, cellular proteins involved in DNA methylation associate with RISC to initiate the methylation of the viral genome (red triangles). VSRs are represented in red boxes. VSRs expressed by RNA viruses for which an action has been demonstrated in TGS are framed by a double line. *Geminiviridae* express VSRs that inhibit the activity of cellular proteins involved in methylation [192, 193].

and seems to be correlated to that of SGS3 [72]. This VPg-SGS3 interaction is described for four potyviruses that diverge in nucleotide sequence and host spectrum [72, 73].

The 2b protein of some cucumoviruses was among the first VSRs described in 1998 [74]. Co-localization and immunoprecipitation experiments revealed a nuclear and cytoplasmic interaction of 2b with AGO1 that correlated with a decrease in AGO1 slicing activity [75]. Similarly, the nuclear interaction between 2b and AGO4 restricts its endonucleolytic activity [40]. However, binding to these cellular factors is not the only suppression mechanism used by 2b protein. Its binding to small RNAs also appears critical for effective viral counter-defence [76]. A recent study modelling the 2b protein structure proposes a tetrameric complex in which each dimer interacts with a siRNA duplex [77].

If viral dsRNAs induce silencing, few viral counter-defence mechanisms are described to inhibit this initiation step. The double-stranded forms are mostly diced by cellular DCLs to form the vsiRNAs; and these are largely targeted by various VSRs in order to limit plant antiviral response. Nevertheless, the p6 (or TAV – Transactivator/viroplasmin) protein expressed by the cauliflower mosaic virus (CaMV) is a mul-

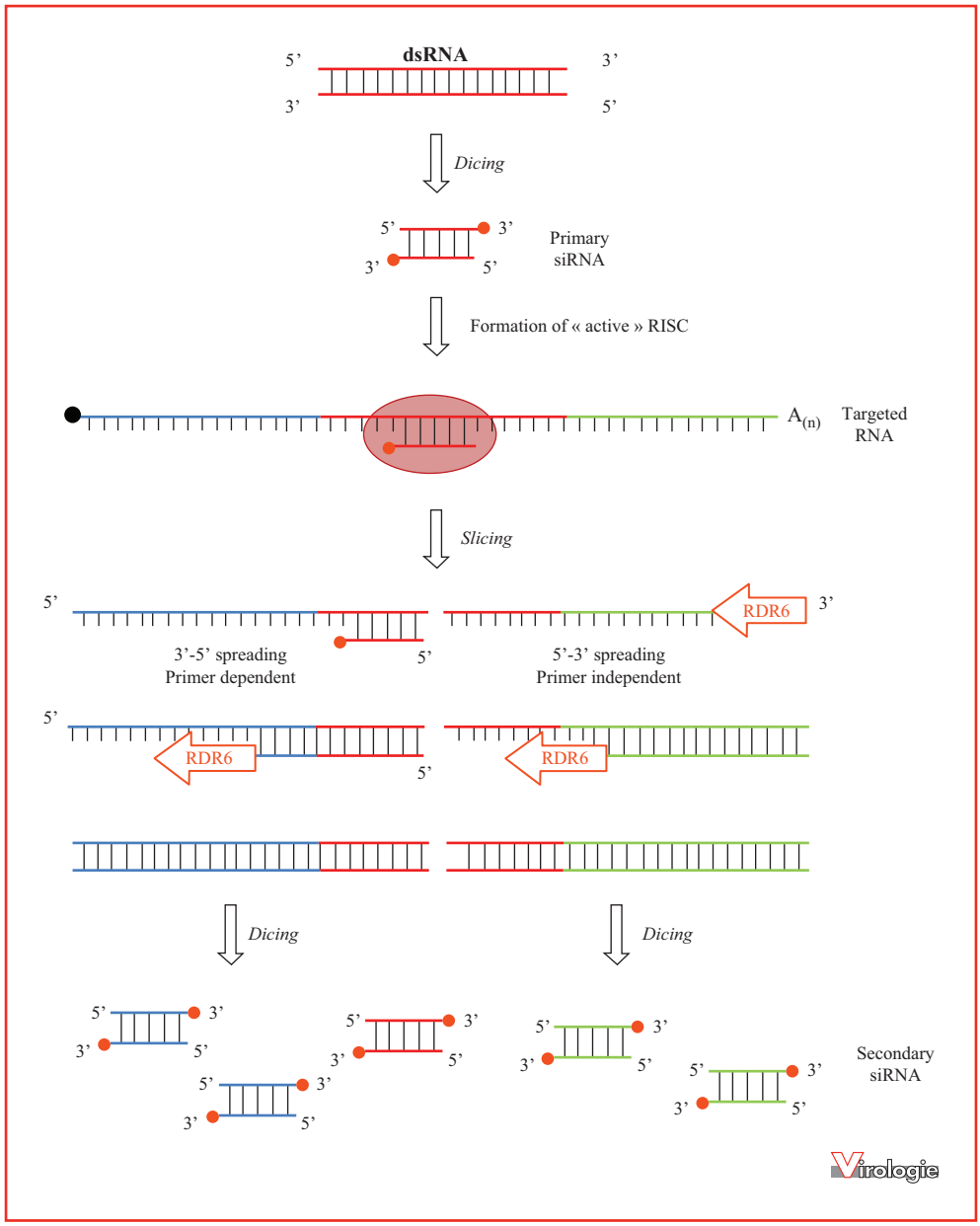

Figure 3. Transitivity mechanism representation. Following targeted RNA cleavage, primary siRNAs are used as primers by RNA-dependent RNA polymerase 6 (RDR6) to generate new dsRNA molecules (case for dependent primer spreading, left). RDR6 recruitment remains to be clarified in the independent primer spreading (right). Neo-synthetized dsRNA molecules are diced by DCLs to produce secondary siRNAs. This siRNA population is specific to the sequence targeted by primary siRNAs but also from its upstream and downstream regions, amplified by RDR6.
The black sphere represents the cap, the orange sphere the 3′ methyl group and A(n) the polyadenylated tail.

titask protein that also ensure a VSR activity [78]. TAV has a cytoplasmic and nuclear localization where it interacts with DRB4 (double-stranded RNA-binding protein 4), a cofactor of DCL4. This interaction limits but does not abolish DCL4 action since 21 nt vsiRNAs are still detected [79].

VSR: ubiquitous and polymorphic proteins of phytoviruses

Phytoviruses all appear to express VSR in order to counteract the antiviral defence provided by RNA interference. This first part will describe the vari-

ous strategies used by VSR that are presented in *figure 2*.

Proceed upstream of the silencing pathway: target viral siRNA

The vsiRNAs generated after Dicer cleavage can be targeted by VSRs that use various strategies (sequestration, modification and degradation) to limit their loading into RISC. Sweet potato chlorotic stunt virus (SPCSV) expresses RNase type III (RNase 3) which cleaves vsiRNA into 14 nt fragments that cannot be loaded into RISC, limiting the antiviral defence [80]. To date, this direct degradation of vsiRNA by a viral enzyme is the only example described for a phytovirus VSR.

A strategy commonly used by VSRs consists in sequestering vsiRNAs to limit and/or prevent their loading into RISC. The p19 protein of tombusvirus is one of the best characterized VSR: p19 specifically binds siRNA duplexes and does not bind single-stranded siRNAs or small dsRNAs with blunt ends nor long dsRNAs [81]. The sequestration specificity mediated by p19 has been explained thanks to crystallographic data. P19 homodimer acts as a caliper preferably recognizing 21 nt siRNAs and its affinity slightly decreases 5-fold and 6-fold when one base pair was removed or added respectively [82, 83]. Grapevine virus A (GVA) p10 protein is a VSR also limiting the systemic spread of the silencing signal by siRNA sequestration regardless of their sequence [84]. However, p10 VSR remains less characterized than p19.

The octameric and annular structural characterization of beet yellows virus (BYV) p21 protein has also clarified its function in silencing suppression. The octamer inner surface exposed is conserved and the positively charged amino acids can bind RNAs. However, unlike p19, binding is not specific to small dsRNAs since p21 is also able to bind ssRNAs. The affinity of p21 for ssRNA increases with the size of the nucleic acid. In addition, p21 also binds DNA but with a lower affinity [85]. Therefore, p21 seems to bind nucleic acids without a real specificity. Nevertheless, the co-immunoprecipitation of p21 transiently expressed in *Nicotiana benthamiana* revealed the presence of siRNAs. Electrophoretic mobility shift assay (EMSA) also confirmed p21 binding to miRNAs and siRNAs duplexes and not to their single-stranded form [67]. In addition, the formation of such complexes is not reproduced when using oligodesoxyribonucleotides, despite their strict sequence similarity to tested siRNAs [86]. These results are consistent with the higher affinity of p21 for RNAs, as previously described [85]. This ability to bind long RNAs rather than siRNAs could provide an additional layer of counter defence such as viral RNAs (replication intermediates and/or locally structures) are protected from DCL cleavage.

Other VSRs are also capable to bind siRNA duplexes. This is the case for the peanut clump virus (PCV) P15 protein which presents a unique feature in silencing suppression. The P15 protein has sufficient affinity to bind 22 nt siRNAs and inhibit their loading into the RISC. Its lower affinity for 21 nt siRNAs is counteracted by their targeting and sequestration within peroxisomes in a P15-siRNA complex [87]. This addressing relies on the PTS1 (peroxisome targeting signal 1) peptidic signal expressed at the C-terminus of the protein [88, 89]. If the PTS1 sequence is lost, 21 nt siRNAs are no longer optimally sequestered, traffic through plasmodesmata and "immunize" adjacent cells, thus limiting or blocking long-distance viral infection [87].

Limit the silencing signal amplification

Another suppression mechanism consists in inhibiting the silencing signal amplification ensured by RDR6 and its cofactors. This mechanism designated as transitivity (*figure 3*) exists in plants [56] and *C. elegans* [90] and is not found in other metazoans such as *Drosophila*. Rice yellow stunt virus (RYSV) P6 protein limits the silencing signal spreading and decreases secondary siRNAs accumulation. However, P6 has no effect on a local silencing established in transient plant expression system (by agroinfiltration) or in rice protoplasts. Its co-immunoprecipitation with RDR6, supported by their co-localization, suggests that P6 limits the action of RDR6 in the silencing signal amplification [91]. SGS3, the major cofactor of RDR6, is also targeted by several VSRs to limit transitivity. Tomato yellow leaf curl virus (TYLCV) V2 protein shares RNA binding properties identical to those of SGS3, including binding to dsRNAs having 5' overhangs. Indeed, a competition model has been proposed where V2 displaces SGS3 from its initial substrate [92]. The SGS3-RNA complex would then be compromised by V2, disrupting the cofactor function of SGS3 and RDR6 access to RNAs. Although the interaction between V2 and SGS3 has been demonstrated by yeast two hybrid and microscopy [93], it has not been detected in a co-purification assay using V2-GST-tagged variants [92]. A third mechanism limiting transitivity is described for TGBp1 protein of plantago asiatica mosaic virus (PlAMV). The TGBp1-mediated silencing suppression depends on its ability to form homo-oligomers that co-aggregate with RDR6 and its SGS3 cofactor [94].

Targeting AGO, the major effector protein of the RISC complex

Several VSRs target RISC whose AGO effector protein is responsible for translational inhibition or cleavage of targeted RNAs. Inducing AGO degradation is one of the strategies used to destabilize RISC.

Described in 2002 as a silencing suppressor [95], the polerovirus P0 protein contains an F-box domain necessary to bypass antiviral silencing and therefore to maintain viral pathogenicity. This F-box domain also ensures the interaction with the SKP1 (S-phase kinase related protein 1) ortholog protein in *A. thaliana* [96]. SKP1 belongs to the SCF complex (Skp, Cullin, F-box) which triggers proteins ubiquitination for proteasomal degradation. However, AGO1 degradation is maintained in presence of proteasomal inhibitor [97] and is in fact degraded through the autophagy pathway [98]. Nevertheless, the P0 protein does not seem to interact directly with AGO1 [99]. P0 does not provide viral counter-defence when AGO1 is pre-loaded with siRNA or miRNA and does not bind siRNAs *in vitro* [100]. The current model then proposes an interaction between P0 and a protein partner (to be identified) of RISC, preventing its assembly and leading to the subsequent degradation of AGO1.

Like P0, the tobacco rattle virus (TRV) 16K protein cannot inhibit pre-loaded RISC activity and does not bind small RNAs. 16K disrupts *de novo* formation of RISC and therefore limits targeted mRNA cleavage. These results are consistent with a decreased accumulation of siRNAs induced by 16K and could be explained by the interaction between 16K and AGO proteins. Indeed, protein-protein interactions between 16K and AGO1 or AGO4 have been demonstrated by BiFC (Bimolecular Fluorescence complementation) but only the interaction with AGO4 has been confirmed by co-immunoprecipitation. These data specify 16K actions on RISC but the relevance of AGO4 and/or AGO1 interactions remains to be elucidated [101].

The PVX P25 protein interacts with AGO1, AGO2, AGO3 and AGO4 but not with AGO5 and AGO9. These interactions have been detected in co-immunoprecipitation experiments following their transient co-expression in *N. benthamiana*. In this study, only AGO1 expression level decreases when co-infiltrated with P25 but is stabilized by MG132, a specific proteasome inhibitor. P25 thus seems to induce AGO1 destabilization through proteasomal degradation, although no ubiquitinated form of AGO1 could be detected [102]. However, direct interactions between P25 and AGO proteins mentioned above have not been demonstrated. An indirect interaction between P25 and AGO1 cannot be ruled out and may be explained by the absence of GW/WG motifs in P25, described as major interaction sites with AGO proteins [103].

These GW/WG motifs were first described in a human protein isolated from a serum, named GW182 because of its molecular weight and richness in glycine and tryptophan amino acids. Three paralogs of GW182 protein are expressed in vertebrates (TNRC6A/GW182, TNRC6B and TNRC6C) and one insect ortholog (GW182). Proteins belonging to the GW182 family are well characterized [104], in particular for their direct interaction with AGO proteins mediated by their GW/WG motifs. In *A. thaliana*, *C. elegans* and *Schizosaccharomyces pombe*, some GW/WG proteins are described. They also interact with AGOs and remain necessary to ensure a functional RNA interference pathway. However, due to a lack of some other domains conservation (*i.e.* other than GW/WG), these proteins do not belong to the GW182 family although their mode of action is similar.

Viruses have taken advantage of the relevance of such interactions by expressing VSRs that contain one or more GW/WG motif(s). These motifs provide an interaction with AGO proteins and allow VSRs to destabilize the formation and/or function of RISC. The P1 protein expressed by the sweet potato mild mottle virus (SPMMV) encodes three GW/WG motifs in its N-terminal domain. Immunoprecipitation and site-directed mutagenesis experiments have shown that they are necessary to maintain VSR function and AGO1 interaction. P1 inhibits AGO1 slicing activity only when it is loaded with miRNAs or vsiRNAs [105]. This ability to inhibit AGO1 function while belonging to an "active" RISC suggests that P1 could limit AGO1 interaction with its targeted RNA. Recent studies confirmed that P1 prevents the association of AGO1-sRNA complex to the target RNA [106]. This P1-mediated inhibition, however, remains to be characterized since various models are possible. Among them, two are favoured: (1) a non-competitive inhibition where the interaction of P1 with AGO1 can modify AGO1 structure thereby altering its binding to the targeted RNA; (2) a competitive inhibition where P1 interacts with AGO1 at the targeted RNA binding site. Interestingly, studies on human AGO2 and *Drosophila* AGO1 have shown that their interaction sites for GW182 proteins and for miRNAs are partially overlapping [31, 107]. If we transpose this to plants, the competitive model (2) seems most likely. Such a mechanism was subsequently discovered for other VSRs, as the turnip crinckle virus (TCV) P38 capsid protein. P38 possesses two GW/WG motifs at its N- and C- terminal ends. Both motifs are necessary to mediate AGO1 binding and silencing suppression and GW to GA mutations prevent TCV infection. Structure prediction of P38 as a dimer allowed to locate the C-terminal GW/WG motif at the protein surface that matches with a favourable AGO1 interaction site. However, such data could not be obtained for the N-terminal GW/WG domain. In a transgenic plant constitutively expressing an inverted repeated sequence (IR) (SUC:SUL plants), the coexpression of P38 (SUC:SUL x P38) resulted in a drastic reduction in siRNA-loaded AGO1 levels (siRNAs derived from the IR), while no major effect was observed on siRNA levels when P38 was brought in *trans* by viral infection (SUC:SUL + TCV). The P38 protein therefore seems to limit small RNA loading into AGO1,

but is not very effective in suppressing silencing of active RISC [108]. Although this interaction between P38 and AGO1 is necessary to ensure viral cycle progression, other mechanisms have also been described. *In vitro*, P38 is able to bind long dsRNAs regardless of their size as well as 21 nt siRNA duplexes [109-111]. In GFP and P38 transient co-expression experiments, GFP siRNAs are not detected and long GFP dsRNA are stabilised instead [109, 110]. According to these results, P38 could prevent DCLs access to dsRNAs, limiting their cleavage and subsequent biogenesis of vsiRNAs [110, 111]. A link was recently established between the P38-AGO1 interaction and dsRNA binding: mutation in the GW motif prevents AGO1 and RNAs binding [111]. P38 interaction with AGO1 could promote *in vivo* vsiRNA binding and sequestration. Thus, P38 could target two distinct steps in the silencing pathway, where its functional orientation could be finely regulated and coordinated during viral infection.

AGO1 stability is not affected by its interaction with P1 (SPMMV) or P38 (TCV) proteins. Conversely, the capsid protein CP and VSR of tomato ringspot virus (ToRSV) appears to induce a severe degradation of AGO1 by autophagy. This degradation relies on the interaction between CP and AGO1 *via* a GW/WG motif [112]. However, the study of pelargonium line pattern virus (PLPV) p37 protein has slightly complicated the relationship between a GW/WG motif, AGO1 interaction and silencing suppression. Although p37 possesses a GW/WG motif involved in its silencing suppression function, its VSR action is most likely explained by its binding to small RNAs. Indeed, a functional link between RNA binding and silencing suppression rather than an interaction with AGO1 has been established by studying p37 variants mutated in residues encompassing the GW motif [111].

Altogether, it appears that a broad spectrum of VSR proteins interact with AGO1, but that it is not always sufficient to confer a *bona fide* silencing suppressor function. Indeed, VSRs could at the same time affect other AGO proteins as described for the SPMMV P1 protein for which AGO2 binding is also reported. The biological significance of this interaction is not explained because no AGO2 inhibition has been observed in this context [106].

While no sequence homology is identified between VSRs, there is nevertheless a common counter-defence strategy developed by unrelated viruses. AGO1 homeostasis is partly regulated by miR168. In response to viral infection, AGO1 mRNA levels increase and miR168 expression is specifically induced by diverse VSRs such as crucifer-infecting Tobamovirus (crTMV) p122, cymbidium ringspot virus (CymRSV) p19, TCV p38, HC-Pro TEV or CMV 2b. This increase in miR168 accumulation causes a drastic reduction in AGO1 protein accumulation [71]. In addition to this conserved mechanism within distinct viral families, each VSRs studied also displays an additional way to suppress silencing (vsiRNA sequestration, interaction with AGO, *etc.*). Thus, the coupling between induction of miR168 and specific action of each VSR seems favourable to these viruses, targeting several steps or distinct effectors in the silencing pathway.

Taking advantage of endogenous suppressors

PTGS was initially associated with a plant defence mechanism that can be bypassed by VSRs-encoding viruses. In addition to these so-called VSRs, PTGS is also ensured by endogenous suppressors. Such gene products are necessary to downregulate RNAi. This is particularly essential in organisms where signal amplification and long distance movement of RNAi take place. Indeed, without regulating factors involved in these two mechanisms (transitivity and spreading), the whole organism could be affected by the presence of numerous RNAs used as templates for dsRNA production leading to deleterious silencing of endogenous genes. The function of these endogenous suppressors can be hijacked from their initial cellular task by viruses to enhance their suppression of RNA silencing. Such a situation is reminiscent of the discovery of *iap* (inhibitor of apoptosis) genes, whose prototype was discovered in baculoviruses and orthologs then identified from yeast to humans [113].

Nt-Rgs-CaM protein (regulator of gene silencing-calmodulin-like) of *Nicotiana tabacum* was the first endogenous suppressor characterized. It was identified in a yeast two hybrid screen using the HC-Pro VSR as a bait. Overexpression of Rgs-CaM induces tumors in transgenic tobacco lines, similarly as does HC-Pro. Its action in the suppression of PTGS was validated in transgenic *N. benthamiana* GFP lines where the efficiency of Rgs-CaM is comparable to that of HC-Pro for GFP messenger silencing. Moreover, the expression of HC-Pro *via* a transgene or a TEV viral infection induces the expression of Rgs-CaM. Thus, HC-Pro seems to activate the expression of Rgs-CaM to overcome the RNA silencing established during viral infection [114]. A recent study reported similar conclusions about the geminiviruses AL2 silencing suppressor. This VSR also induces the expression of *A. thaliana* rgs-CaM and their nuclear interaction was confirmed by BiFC. Rgs-CaM overexpression increases both the sensitivity to the tomato golden mosaic virus (TGMV-Geminivirus) and the accumulation of viral DNAs whereas knock-out lines for rgs-CaM are less susceptible to infection [115]. In the same year, βC1 VSR expressed by a DNA satellite (TYLCCNB-tomato yellow leaf curl China betasatellite) associated with a geminivirus (TYLCCNV-tomato yellow leaf curl China virus) was also shown to induce Nb-Rgs-CaM expression in *N. benthamiana*. βC1 and Nb-Rgs-CaM overexpression lead to a decrease in

RDR6 mRNA and secondary siRNA accumulation levels [116]. While the function of calmodulin-like proteins in silencing suppression was established, a mode of action has been recently proposed. An interaction between the calmodulin-like protein of *N. benthamiana* (NbCaM) and SGS3 has been detected in a yeast two hybrid assay and *in planta*. This interaction seems a prerequisite to induce SGS3 degradation by autophagy. Also, decreased expression of several factors involved in autophagy pathway inhibits NbCaM-mediated degradation of SGS3, and also TYLCCNV and its betasatellite (TYLCCNB) accumulation [117]. Thus, NbCaM suppresses silencing by inducing SGS3 degradation and several viruses take advantage of this mechanism by promoting the induction of this endogenous suppressor. However, a controversy persists on Nt-rgs-CaM endogenous silencing suppressor function. Indeed, interaction between several VSRs containing dsRNA binding domains and Nt-rgs-CaM has been demonstrated. By a similar mechanism described above, those VSRs appear to be degraded by autophagy like SGS3, effectively promoting antiviral defence of infected plants [118].

Further studies are needed to explain this dichotomy observed for calmodulin-like proteins functions in RNA interference pathway, where the nature of VSRs and viruses seem to play a significant role in the functional orientation of calmodulin-like proteins. A hypothesis is advanced by Nakahara *et al.* where Nt-rgs-CaM appears to interact with some VSRs *via* their negatively and positively charged residues respectively, thus destabilizing a pre-established interaction between VSR and vsiRNA [118].

The AtRLI2 (RNase L inhibitor 2) protein from *A. thaliana* is ortholog to a mammalian protein involved in the regulation of the interferon response [119] whose expression is induced by synthetic dsRNAs and viral infection in HeLa cells [120, 121]. By analogy, AtRLI2 is induced in plants where silencing has been established [119] and was identified as an endogenous silencing suppressor. Indeed, AtRLI2 overexpression causes a drastic reduction of siRNA accumulation [122]. This protein is widely conserved in eukaryotes and archaea. It regulates translation and/or ribosomes biogenesis and recycling in *Drosophila*, yeast and mammals but not in plants. Nevertheless, ABCE1 (ATP-binding cassette sub-family E member 1), the human ortholog of AtRLI2, retained its silencing suppressor function when expressed in *N. benthamiana*, *C. elegans* or mammalian cells. ABCE1 was the first human protein described as an endogenous silencing suppressor [123].

In plants, the degradation of RNAs associated with PTGS is mainly provided by AGO1. Its endonucleolytic activity cleaves the targeted RNA, complementary to the guide strand loaded in RISC. Following cleavage, two RNAs are released: one upstream of the cleavage site that contains the 5' end (5' fragment); the other downstream which contains the 3' end (3' fragment). The 3' fragment is degraded from 5' to 3' by the cytoplasmic exoribonuclease XRN4. The degradation of the 5' fragment involves two distinct pathways. If the targeted RNA is capped, DCP (decapping) proteins ensure the removal of the cap and allow XRN4 to perform the 5'-3' degradation. This 5' fragment has an unprotected 3' end following cleavage and allows access to terminal uridyltransferases (TUTase). HESO1 (HEN1 suppressor1) is the main TUTase involved in uridylation and degradation of siRNAs and miRNAs if they are not protected by the methyl group added by HEN1. It is possible that HESO1 could be recruited by RISC since AGO1 is co-immunoprecipitated by HESO1. Similarly, the exoribonucleases RICE1 and RICE2 (RISC-interacting clearing 3'-5' exoribonucleases) seem to be recruited at the 3' end by interacting with AGO1 and AGO10 and initiate the RNA 3'-5' degradation. The exosome associated with the SKI complex ensures the complete degradation of RNA [124]. For other targets, the 3' fragment degradation following cleavage by miRISC was reported to be decreased in an *A. thaliana xrn4* mutant. [125]. Moreover, a cellular RDRP-mediated silencing stimulation was observed in *xrn4* mutant background as well as an accumulation of cleavage products [126]. Furthermore, the identification of new endogenous silencing suppressors could be limited by the high efficiency of effectors belonging to the silencing pathway. Thus, in 2007, analysis of two AGO1 hypomorphic lines allowed the identification of XRN2, XRN3 and FRY1 as endogenous suppressors of RNA silencing [127]. Although silencing establishment is deficient in *ago1* lines, it is completely restored in *ago1/fry1* and *ago1/xrn4* double mutants, while it is only partial in *ago1/xrn2* and *ago1/xrn3*. Thus, AGO1's function is restored when FRY1, XRN2, XRN3 and XRN4 are lower expressed, demonstrating their roles as RNAi endogenous suppressors. The double mutant *xrn2/xrn3* exhibits over-accumulation of miRNA precursor loops generated during nuclear DCL processing, prior to nuclear export of miRNA/miRNA* duplexes. This phenotype is consistent with the nuclear localization of XRN2 and XRN3 [128]. The *fry1* mutant phenotype is similar to that of *xrn2/xrn3* and *xrn4* in terms of miRNA precursor accumulation in the nucleus and AGO1-generated 3' cleavage products accumulation in the cytosol respectively. To confirm the role of these endogenous suppressors, *xrn2*, *xrn3*, *xrn4* and *fry1* lines were infected with CMV, a cytoplasmic replicative virus. X*rn2* and *xrn3* lines behaved as a wild-type line with a viral infection reaching 66% to 75%. In contrast, *xrn4* and *fry1* plants showed a decreased accumulation of viral RNA and vsiRNA. This hyperresistance in mutant background confirmed the silencing suppression mediated by these two proteins [127].

In 2001, RRF-1, RRF-2 and RRF-3, three homologs to the RDRP EGO-1 [129] were studied in *C. elegans* in order to refine their role in the transitivity pathway [90]. Only *rrf-3* individuals were hypersensitive to RNA interference [130]. This sensitivity confirms the existence of a negative regulation which is mediated by RRF-3. Such a hypersensitivity phenotype was also described in *eri-1* nematode [131]. *In vitro*, ERI-1 (Enhanced RNA interference 1) specifically degrades siRNAs at the 3' overhang but not single-stranded and fully hybridized species. An exonuclease and a nucleic acid binding domains were identified in ERI-1 *in silico*. Thus, ERI-1 seems to limit RNA interference by binding and initiating the degradation of siRNA duplexes at their 3' end. Interestingly, the neuronal and gonadal-specific expression of ERI-1 may explain the lower efficiency of RNA interference in these tissues.

In *A. thaliana*, type III RNases are divided into two families: Dicer-like (DCL1 to DCL4) proteins that are widely studied for their functions in RNA interference; and RNase three-like (RTL1 to RTL5) proteins which are much less characterized. Although they all possess one RNase III domain (except RTL3 with two RNase III domains), only RTL1, RTL2 and RTL3 contain respectively 1, 2 and 3 dsRNA-binding domains (DRBs). RTL1 and RTL3 are poorly expressed in plants, while RTL2, RTL4 and RTL5 appear to be expressed in almost all plant tissues. Surprisingly, RTL1 mRNA accumulation level is twenty times higher during viral infection compared to mock control, while those of RTL2 and RTL3 remain unchanged. RTL1 expression is induced by four single-stranded RNA viruses that belong to distinct families (TCV; CMV; turnip vein clearing virus, TVCV and turnip yellow mosaic virus, TYMV). Therefore, RTL1 appears to be generally induced by a viral stress. When overexpressed, RTL1 induces a dramatic decrease in accumulation levels of three major classes of endogenous siRNAs (endogenous inverted repeat-derived-siRNA (endoIR-siRNA); polymerase IV-dependent siRNA (p4-siRNA) and transacting-siRNA (ta-siRNA)) produced by DCL2, DCL3 and DCL4 respectively, whereas the miRNAs biogenesis ensured by DCL1 is unaffected. In addition, GUS silencing induced by a 35S:GU-UG stem-loop construct was suppressed by RTL1 through inhibition of siRNAs accumulation. RTL-1 was then confirmed as an endogenous silencing suppressor [132]. Since RTL1 and DCL proteins display both RNase III and DRB domains, RTL1 could use identical substrates to those cleaved by DCLs, thus potentially limiting siRNA biogenesis by DCLs. *In vitro* and *in planta* cleavage experiments revealed RTL1 specificity for long dsRNA closed to a stem-loop structure. Its affinity for perfectly paired dsRNA had already been observed since RTL1 affected the level of DCL2, DCL3 and DCL4-matured precursors, but had no effect on rich-mismatches precursors (*i.e.* pri- and pre-miRNAs). The endoribonucleolytic activity of RTL1 generates cohesive ends on both cleaved strands and cleavage specificity depends on a consensus sequence of a perfectly paired duplex at the base of the stem loop structure [133]. Cleavage products generated by RTL1 appear to be completely degraded by cellular exoribonucleases. Thus, RTL1 seems to exert its antiviral function by initiating the degradation of viral replication intermediates and/or locally structured viral RNAs upstream of antiviral DCLs. This additional antiviral barrier is, however, suppressed by P38 (TCV), 2b (CMV) and HC-Pro VSRs which restore the accumulation of siRNAs [132]. VSRs, in addition to suppressing PTGS mediated by antiviral DCL, may also suppress RTL1 activity.

Animal viruses express VSR

In 2002, a silencing suppression function was associated to the B2 protein expressed by the Flock house virus (FHV) naturally infecting insects [134]. When transiently expressed in GFP silenced plants, B2 restores GFP fluorescence and reduces GFP siRNA accumulation levels. Transfection of FHV and FHV-ΔB2 in wild-type or AGO2 silenced *Drosophila* cell lines confirmed B2 as a suppressor of RNA silencing. In 2005, two B2 structures were obtained by distinct teams, one by nuclear magnetic resonance (NMR) [135], the other by co-crystallization with a 18 nt dsRNA [136]. B2 is a homodimer where each monomer is composed of three α-helix (α1, α2 and α3). The two structures are almost identical except for the orientation of one helix that was explained by a B2 conformational adaptation following its binding to the RNA in the crystallographic study. Positively charged residues are exposed at the dimer surface, effectively interacting with RNA backbone phosphate groups independently of its nucleotide sequence [136]. In contrast to p19, B2 does not provide specific recognition of 5' and 3' ends, also suggesting a size-independent RNA binding function. These structural data are in agreement with results published the same year that demonstrate B2 binding to siRNA duplexes and also to longer dsRNAs [137, 138]. B2 also seems to inhibit Dicer cleavage of long dsRNAs *in vivo* and *in vitro*, therefore limiting siRNA biogenesis [136, 137]. It thus appears that B2-mediated silencing suppression can take place at two levels: by masking long dsRNAs Dicer substrates and/or by sequestering siRNAs. In 2009, the interaction between the B2 C-terminal domain and the Dicer PAZ domain was demonstrated by yeast two hybrid and pull-down experiments [139]. Since the PAZ domain anchors one end of a dsRNA, B2 could then prevent dsRNA access to this Dicer pocket by a competitive

mechanism. In this scenario, B2 could in fact substitute for Dicer in dsRNA binding and would be consistent with its higher affinity for long dsRNAs compared to 21 nt duplexes [138].

Although mammals can rely on their innate and adaptive immune responses to restrict a viral infection, human viruses encode VSRs. The silencing suppressor function of influenza A virus NS1 protein was discovered and studied in the transient plant expression system [140, 141]. NS1 restores GFP expression in a reporter system and drastically reduces siRNAs accumulation levels targeting GFP mRNA, as for FHV B2 protein. The NS1-mediated silencing suppression is confirmed *in planta* by using the PVX viral vector, where NS1 exacerbates infection symptoms and increases viral pathogenicity. The function of NS1 has been partly elucidated *in vitro* where NS1 binds 21 nt siRNAs [140]. The co-crystallization of NS1 double-stranded RNA binding domain associated with a siRNA duplex confirmed previous results. Studying a protein in a different kingdom is not limited to NS1. A "trans-kingdom" analysis of rice hoja blanca virus (RHBV) NS3 protein was conducted on mammalian cells to carry out HIV-1 Tat viral protein functional complementation assays. NS3 is indeed capable of ensuring this functional complementation through its dsRNA binding domain and could then operate by sequestering small RNAs duplexes. This study confirmed the suppression of Tat-mediated silencing and proposed a Tat function similar to that described for NS3 [142]. A combination of these two "trans-kingdom" systems has been used to clarify Tat function's by comparing it to the p19 suppressor of Tombusviruses [143]. Their effects were both studied *in planta* by their transient expression in *N. benthamiana* protoplasts and also by their transfection into mammalian cell cultures. The authors shown that Tat performs silencing suppression downstream of dsRNA duplexes biogenesis, as p19 does. Tat and p19 promote HIV mRNAs translation and ensure an increase in virion production. Thus, cellular miRNAs mediated inhibition of viral RNA translation appears to be bypassed by HIV Tat VSR. This silencing suppression activity requires the functionality of Tat dsRNA binding domain. Similar work based in part on Tat functional complementation confirmed NS1 VSR function, and was also attributed to the VP35 of Ebola virus and the E3L protein (vaccinia virus) [144, 145].

The difficulty of studying RNA silencing in mammalian somatic cells resides in the existence of the antiviral interferon response. These two cellular pathways appear exclusive: if RNA silencing proteins are expressed and functional, the antiviral response is nevertheless ensured by the interferon pathway [146]. The relationship between antiviral immunity and RNA interference was demonstrated using mouse embryonic stem cells (ESCs) deficient in the interferon response. Infection of these non-differentiated cells with the encephalomyocarditis virus (EMCV) provoked the accumulation of vsiRNA. The nature of these small RNAs (size, 3' 2 nt overhang...) and *dicer* knockout lines confirmed their biogenesis *via* the RNA interference machinery. The antiviral effect of these vsiRNAs was not tested on EMCV replication but their association in AGO2 tends to validate their functionality to join an active RISC complex. The accumulation of vsiRNA decreased drastically when the cells were differentiated, which is in agreement with the functional prevalence of the interferon response in somatic cells. To evaluate the antiviral potential of vsiRNA, the Nodamura virus (NoV) was used. NoV expresses the B2 protein that inhibits Dicer activity like FHV B2 protein. NoVΔB2 maintained the accumulation of vsiRNAs in ESCs and in somatic cells and their Dicer-dependent biogenesis was abolished during a wild-type NoV infection [147]. In addition, the accumulation of NoVΔB2 virus was enhanced (i) in AGO2 mutated ESCs [147] (ii) and in new-born hamster kidney somatic cells that express the NoV B2 protein or the Ebola virus VSR VP35 [148]. Infection of suckling mice with NoV wild-type virus was lethal five days after inoculation, and all mice infected with NoVΔB2 virus survived. Viral clearance in NoVΔB2-infected mice was correlated with the accumulation of vsiRNA and with the loss of silencing suppression provided by the B2 protein [148]. These results are in agreement with an antiviral response mediated by RNA interference in mammals and studies conducted on the human enterovirus 71 (HEV71) confirmed the importance of this antiviral pathway [149]. VSR function was attributed to the non-structural protein 3A which limits vsiRNA biogenesis through long-double-stranded RNAs sequestration. Like NoV, a wild-type HEV71 virus induced an aberrant production of small RNAs characteristic of viral degradation products, whereas point mutations introduced into the 3A protein decreased its binding to double-stranded RNAs and caused the accumulation of vsiRNA. These vsiRNAs were generated by Dicer from viral replication intermediates, they co-immunoprecipitated with AGO and ensured the degradation of RNA expressing viral sequences. The accumulation of these vsiRNAs was correlated with viral clearance and a drastic decrease in pathogenicity. These experiments, conducted both in mammalian somatic cells and in mice, also showed that these data were independent of the interferon pathway, demonstrating the true role of RNA interference in antiviral defence in mammals. While several studies established the role of RNA silencing as antiviral defence in mammals, this recent attribution is not yet unanimous and offers multiple perspectives. Table 1 shows some examples of animal viral suppressors.

When viral non-coding RNAs get involved

The field of "non-coding" continue to fascinate since the discovery of miRNAs at the end of the twentieth century [150]. Nowadays, more than 38,500 miRNAs are referenced, all species combined (http://www.mirbase.org/index.shtml, accessed 2nd august, 2019). The family of small non-coding RNAs associated with gene silencing is now extended to siRNA (small interfering) as well as to piRNA (piwi interacting). These latter are however restricted to animal's gonadal tissues, Dicer-independent and specific to transposable elements sequences [151]. Long non-coding RNAs (lncRNA – size greater than 200 nucleotides and up to tens of kilobases) also have major regulatory functions. LncRNAs are involved in majority of cellular processes and consequently in multiple pathologies since many lncRNAs affect epigenetic regulation, cell cycle control or immune responses [152].

Some viruses, in addition to their VSR protein, also express non-coding RNA acting as an additional VSR. Viral miRNA regulation was demonstrated for the first time in 2004 by studying Epstein-Barr virus (EBV) [153]. EBV fine-tunes its viral cycle through the expression of 44 virally-encoded miRNAs whose cellular targets are multiple. These viral miRNAs control for instance immune system escape, viral latency maintenance and apoptosis inhibition. They also regulate the expression of viral proteins such as DNA polymerase (BALF5), latent membrane proteins (LMP1 and LMP2) involved in lymphocytes transformation and immortalization and BFLF2 that is required for nucleocapsids cytoplasmic translocation. By targeting their mRNA, viral miRNAs seem to restrict the lytic cycle in favour of maintaining viral latency and to promote immune system bypass [154]. The expression of such viral miRNAs has been identified in several other *Herpesviridae* viruses [155] and also in baculoviruses [156]. In addition, two non-coding transcripts are also expressed by EBV: EBER1 and EBER2 (EBV-encoded small RNA) of 167 and 172 nt respectively. Their interactions with PKR (protein kinase R), TLR3 (toll-like receptor 3) and RIG-1 proteins (retinoic acid-inducible gene I) modulate the immune system to promote viral pathogenicity and host cells transformation [157]. The expression of non-coding RNA targeting PKR is reminiscent of the adenovirus situation.

Human adenoviruses (AdVs) are dsDNA viruses of about 36 kilobases. They all express a 160 nt-long non-coding transcript, VA RNAI (virus-associated RNA). A second non-coding RNA, VA RNAII, is expressed by 80% of AdVs and they are both transcribed by RNA polymerase III from intragenic regions. These RNAs are highly structured and VA RNAI has three distinct domains: an apical stem, a central domain and a terminal stem. These VA RNAs are exported from the nucleus by the exportin 5 (Exp5) which provides cytoplasmic translocation of dsRNA binding domain (dsRBD) proteins, small RNAs such as transfer RNAs and miRNA precursors, and Dicer mRNA [158]. Exp5 ensures the export of RNAs having a short 3' overhang, a 5' paired end as well as a minimum 15 nt stem. Interestingly, this structure is present at the VA RNAI terminal stem end and directly interacts with Exp5. Synthesized up to 10^8 copies per cell, VA RNAI seem to saturate Exp5 thus entering into direct competition for pre-miRNAs and Dicer mRNAs exports. The interaction between Dicer mRNAs and Exp5 is therefore drastically reduced, resulting in a lower accumulation of cytoplasmic Dicer proteins. VA RNAI limit pre-miRNAs export as well as their cytoplasmic maturation by decreasing the amount of Dicer proteins available. In the cytoplasm, VA RNAs limit RNA interference by saturating the remaining Dicer. Like miRNAs, VA RNAI and VA RNAII have many mismatches rendering them perfect mimicry substrates. Although less abundant than VA RNAI, VA RNAII are preferentially cleaved by Dicer and are loaded into RISC, referred to "mivaRNAs" (VA RNA-derived miRNAs). Several targeted RNAs, complementary to mivaRNAs, have been identified without establishing a direct link between their silencing and a benefit for the virus, except for the cullin 4A. The post-transcriptional suppression of cullin 4A by mivaRNAII stimulates the signaling pathway of JNK (Jun N-terminal kinase) in favour of viral replication [159]. Nevertheless, the nucleotide sequence of VA RNA is poorly conserved within adenoviruses, and the cleavage efficiency and cleavage number achieved by Dicer as well as guide strand selection in AGO2 seem to be specific to each viral serotype. All of these elements diminish the possibility to obtain mivaRNAs that could be considered as "consensus" by ensuring common genes knockdown. VA RNAs may be considered as simple competitors that saturate Dicer and cause a global deregulation in miRNA population. Further work is needed to elucidate the function of these mivaRNAs whose Dicer biogenesis is well conserved in all adenoviruses and confirm the importance for AdVs to produce such pro-viral substrates [158]. In mammals, however, the interferon response remains the major antiviral pathway [160] where protein kinase R (PKR) is activated by dsRNA. VA RNAI is also capable of binding PKR to prevent its dimerization, thereby limiting activation of this antiviral pathway by competing with viral dsRNAs [161, 162].

The identification of VSRs in flaviviruses was not very successful [163] until the discovery of the NS4B non-structural protein of dengue virus [164]. This silencing suppression function was *a contrario* assigned to the structural CP protein of the yellow fever virus [165]. Although known examples of flaviviral VSRs proteins are scarce,

Table 1 Summary of viral animal suppressors.

Family	Genus/Species	VSR	Cell types/ organisms where VSR function has been demonstrated	References
Orthomyxoviridae	Influenza A virus (IAV)[i]	NS1[a]	- Insects cells - Plants: *N. benthamiana* - Mammals cells	[140, 145, 194, 195]
Poxviridae	Vaccinia virus[i]	E3L[a]	- Insects cells - Mammals cells	[144, 145]
Filoviridae	Ebola virus[i]	VP35[a]	- Mammals cells - Plants: *N. benthamiana*	[144, 196]]
Retroviridae		Tat[a]	- Mammals cells - Plant cells (protoplasts)	[143, 197, 198]
	Human immunodeficiency virus I (HIV-1)[i]	TAR[b]	- Mammals cells	[199]
		RRE[b]	- Mammals cells	[200]
Coronaviridae	Severe Acute Respiratory Syndrome (SARS)[i]	Nucleocapsid N[a]	- Mammals cells	[201]
Adenoviridae	Adenovirus[i]	VA RNA[b]	- Mammals cells	[159, 202]
Picornaviridae	Human enterovirus 71[i]	3A[a]	- Mammals cells - Mice	[149]
Flaviviridae	Hepatitis C virus (HCV)[i]	Core[a]	- Mammals cells	[203-205]
	Yellow fever virus[i]	Capside C[a]	- Mosquito vector	[206]
	Flavivirus[i]	sfRNA[b]	- Mosquito vector - Insects cells - Mammals cells	[163, 168]
Nodaviridae	Nodamura Virus[i] Nodamura Virus[ii]	B2[a]	- Insects cells - Mammals cells - Mice	[137, 145, 147, 148, 207]
	Flock house virus (FHV)[ii]		- Plants: *N. benthamiana* - Insects cells - Insects: Drosophila	[134, 136, 139]
Dicistroviridae	Drosophila C virus (DCV)[ii]	1A[a]	- Insects cells - Insects: Drosophila	[208]
	Cricket paralysis virus (CrPV)[ii]			[209]
Birnaviridae	Culex Y virus (CYV)[ii]	VP3[a]	- Insects cells - Insects: Drosophila	[210]
	Drosophila X virus (DXV)[ii]			

The distinction between mammalian[i] and insect[ii] viruses is indicated in the "Genus/Species" column. The nature (Proteic[a] and nucleic[b]) of the VSR is indicated.

flaviviruses all ensure the expression of a non-coding RNA (subgenomic flavivirus RNA, sfRNA), collinear with the 3' UTR, essential for viral pathogenicity [166]. It was initially named subgenomic flaviviral RNA and its biogenesis was then associated with an incomplete degradation of genomic viral RNA by XRN1, a cellular exoribonuclease involved in the regulation of RNA catabolism [166]. Several studies have identified RNA structures that stall XRN1 and induce the subsequent accumulation of sfRNA (*figure 4A*) [167]. The function of sfRNAs was associated with silencing suppression by studying the West Nile virus (WNV), whereas none non-structural viral proteins tested possess a VSR activity [163]. SfRNA-mediated silencing suppression is effective in siRNA and miRNA pathways, in insects and mammalian cells and these results have been successfully transposed to the dengue virus. These data were supplemented by studying the Kunjin virus (KUNV) which confirms the involvement of sfRNA in silencing suppression in infectious context in the mosquito vector *Culex quinquefasciatus* [168]. These studies also reveal a specific co-immunoprecipitation of sfRNA 3' UTR region with Dicer and AGO2 in KUNV infected cells. In that way, WNV sfRNA are able to inhibit *in vitro* Dicer-mediated cleavage of long dsRNA by a competitive mechanism since sfRNA-specific cleavage products are accumulated [163]. In fact, like VA RNAs, sfRNAs could mimic viral genomes by saturating Dicer and AGO2 to limit anti-viral cellular defence.

A similar mechanism is described for BNYVV (beet necrotic yellow vein virus). The genome of this multipartite plant virus is composed of four to five positive stranded RNA segments. Each of them is capped, polyadenylated and encapsidated in individual helical particles. RNA1 and RNA2 are sufficient to ensure viral replication on model plants such as *N. benthamiana*. However, on natural hosts (*i.e.* of the *Beta* genus), RNA4 and RNA3 are required for viral transmission and systemic movement respectively. The viral long-distance movement is not dependent on RNA3 *per se*, but rather depends on the accumulation of a non-coding viral RNA derived from RNA3 [169, 170], initially designated as RNA3sub [171]. The "core" sequence (about 250 nt) is encoded in the vicinity of RNA3sub promoter sequence and is involved in viral long-distance movement [172]. The study of the "core" sequence allowed the identification of the 20 nt "coremin" motif conserved in all Benyviruses [173] and known as "Box1" within Cucumoviruses. CMV subgenomic RNA5 accumulation is dependent on this "Box1" sequence fold-backed as a stem-loop [174]. For BNYVV, the "coremin" sequence also remains essential for RNA3sub accumulation and ensures viral long-distance movement on *Beta* species [169]. Furthermore, the RNA3sub production and accumulation out of the viral context and *in vitro* defines its RNA3sub as a non-coding RNA (ncRNA3), derived from an incomplete degradation of RNA3 [169]. The production of this ncRNA3 is attributed to the cellular protein XRN4 of *A. thaliana* that degrades RNA3 from 5' to 3' to reach the "coremin" sequence where XRN4 appears to be blocked by a structural feature (*figure 4B*) [175]. BNYVV viral RNA1 and RNA2 are sufficient to ensure long-distance infection and viral movement on *N. benthamiana* model plant, where RNA1 expresses proteins necessary for viral replication, whereas structural, movement and silencing proteins are expressed from RNA2. The p14 protein suppresses silencing by limiting the production of secondary siRNAs through the transitivity mechanism and is essential to ensure viral systemic spreading [176]. However, the hypomorphic BA2 version of p14 VSR is unable to provide viral long-distance movement but is partially complemented by ncRNA3. Indeed, nearly 40% of plants infected with RNA1 and RNA2-BA2 present a systemic infection when RNA3 is supplemented in the inoculum. This complementation is only observed with the wild-type RNA3 and not at all with a RNA3 unable to ensure ncRNA3 accumulation. Thus, ncRNA3 provides a functional complementation to a p14 mutant deficient in its VSR function [170].

Conclusions and outlooks

RNA interference ensures essential functions in genes expression regulation in many organisms by maintaining their harmonious development and fighting against different biotic stresses. Although this review focuses on viral infections, RNA interference is also essential for regulating gene expression upon bacterial infections [177] and during abiotic stresses [178].

One of the main features of RNA interference is to reduce expression levels in a sequence-specific manner. Following this mechanism, it is then possible to target any RNA (partially) complementary to the guide strand loaded in RISC. This pathway ensures limitless potential and a total diversification of RNA populations to be silenced. Phytoviruses have to fight this machinery and, to overcome this selection pressure, they express a protein and/or an RNA able to bypass this antiviral silencing. Although VSRs appear to target a limited set of RNAi factors (AGO, DCL, RDR, sRNAs), each VSR uses a unique mechanism to counteract RNAi. Diversity of these mechanisms seems to exceed the number of targets available. The functional versatility of VSRs complicates their study and their characterization is challenging. Indeed, multiple VSRs were described but the mode of action for only a few of them was determined thanks to structural studies (*e.g.* p19 and p21). Endogenous silencing suppressors should also be considered. They are essential for RNA interference regulation and VSRs have taken advantage of their suppressor functions. Their expres-

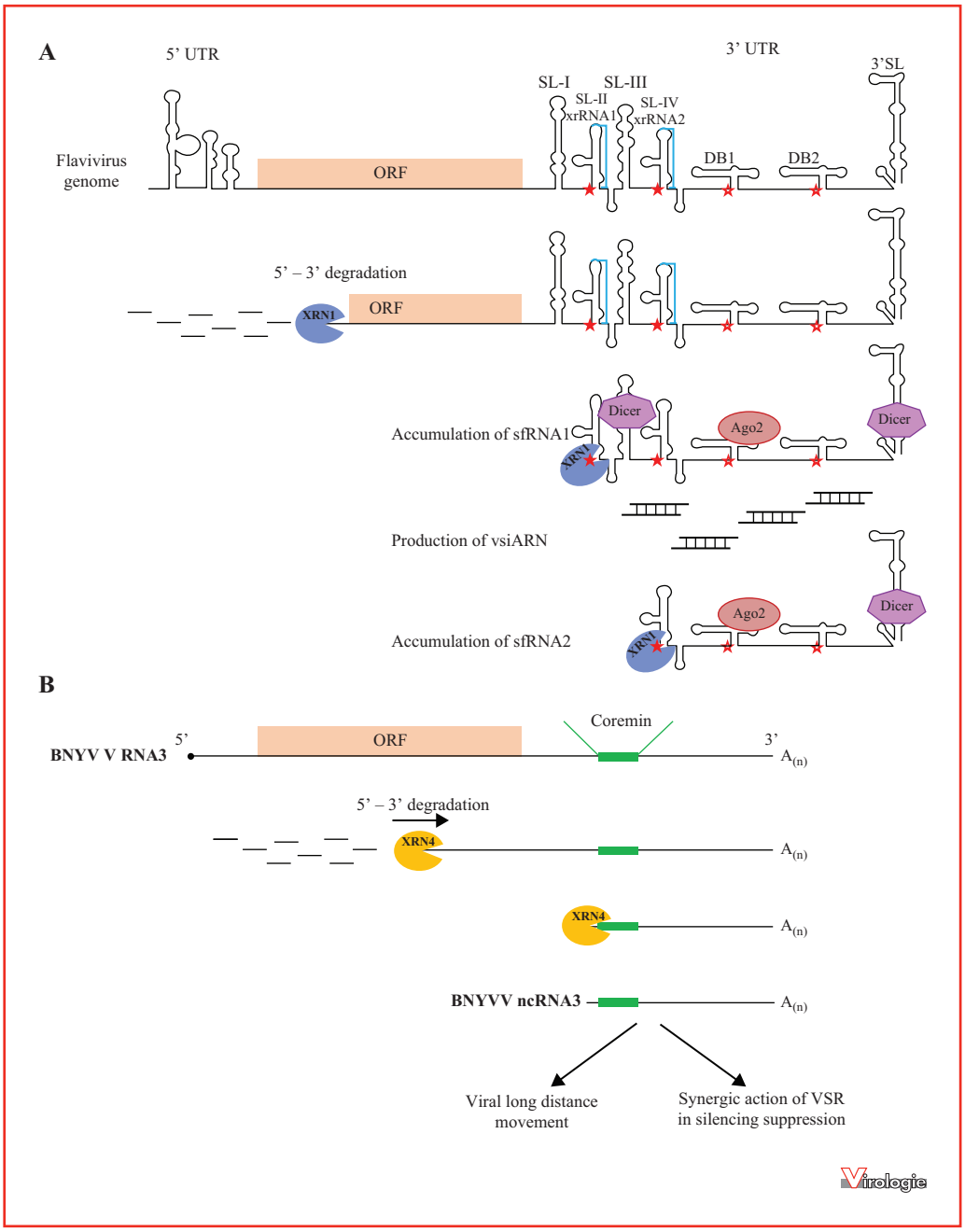

Figure 4. Biogenesis models of sfRNA flaviviruses and ncRNA3 BNYVV. A) Top, schematic representation of West Nile virus (WNV) genome. XRN1 spreading on RNA is inhibited (full red stars) by the formation of pseudoknots (shown in blue) at SL-II and SL-IV positions leading to subsequent sfRNA1 and sfRNA2 accumulation. Almost all flaviviruses ensure sfRNA1 and sfRNA2 expression and additional species, sfRNA3 and sfRNA4 (empty red stars), may accumulate depending on flaviviruses and host studied (human, mosquito, cell cultures). SfRNAs seem to be a decoy for Dicer and Ago2 and viral siRNAs (vsiRNAs) accumulate. SL-II and SL-IV structures are also described as xrRNA1 and xrRNA2 (Xrn1-resistant RNA). SL, stem-loop; DB, dumbbell; ORF, open reading frame; UTR, untranslated region. The black sphere corresponds to the cap. For review see [211]. Image adapted from [212]. **B)** A population of BNYVV genomic RNA3 is degraded from 5' to 3' by XRN4 exoribonuclease activity. About 1230 nucleotides are thus eliminated, including the open reading frame (ORF). The progression of XRN4 is blocked by the coremin sequence and non-coding RNA3 (ncRNA3) accumulate. A structural element containing the coremin sequence seems to induce XRN4 stalling, but this structure remains to be determined. A comparable degradation mechanism is described for BNYVV RNA5 [169].

sion is often induced by viral infection and they enhance VSRs counter-defence at the expense of infected plants.

Although VSRs studies were restricted to proteins, interest for viral RNA acting as VSR has recently increased. It is not surprising to encounter nucleic acids dedicated to suppressing RNA interference, this mechanism itself being initiated and governed by RNA. One regulatory mechanism limits endogenous silencing and consists in diverting miRNAs from their initial targets by "miRNA sponges". RNA having multiple desired miRNA binding sites are overexpressed to promote the formation of miRISC complexes on these miRNA sponges. The miRNAs are thus sequestrated on these "sponges" and their initial function is diverted. This regulatory mechanism is particularly relevant for human health but the design of "sponges" remains complicated and the off-target risk must be controlled [179, 180]. The first endogenous miRNA "sponge" was found in plants [181] and later in prokaryotes [182]. Not surprisingly, viruses have managed to take advantage of this new regulatory mechanism, like the herpesvirus saimiri and murine cytomegalovirus [183, 184]. Expression of viral non-coding RNA has been demonstrated for several plant and animal viruses. In contrast to VSRs proteins, functional conservation appears for some non-coding RNAs, where they can serve as a decoy in the silencing machinery. Their common feature consists in saturating the main antiviral proteins of the RNA interference pathway (Dicer-DCL, AGO *etc.*). A competition mechanism is currently preferred, where DCL and AGO are saturated with a viral non-coding RNA decoy, preventing them from being effective at their antiviral function. Few examples are known where these non-coding viral RNAs are integrating an active RISC complex to inhibit the expression of a specific gene. This saturation process, however, is not generalized to all viral ncRNAs since some viruses, such as EBV, express a panoply of miRNA disrupting the cell cycle in favour of viral infection without presenting such a competitive mechanism.

Finally, RNA interference seems closely related to several RNA degradation mechanisms named RQC (RNA quality control). Three major RNA monitoring pathways are Nonsense-mediated decay (NMD), Non-stop decay (NSD) and No-go decay (NGD). They respectively ensure the degradation of RNAs containing premature termination codons (PTC), missing stop codon or with stalled ribosomes. RNA viruses display several strategies to encode and express their proteins within a genome that has to be as short as possible: overlapping genes, subgenomic RNAs and readthrough. Such a compaction in the viral genetic information allows the formation of ideal substrates for the RQC machinery such as micro-ORFs, non-capped and non-polyadenylated RNAs, long 3'UTR sequences and stop codons recognized as PTC by NMD factors [185]. In plants [186] and animals [187], these viral RNAs are recognized as aberrant by the NMD machinery and subject to degradation. Nevertheless, various strategies are used by viruses to circumvent the deleterious effects of NMD on viral amplification. For example, the resistance of Rous sarcoma virus (RSV) to NMD is conferred by a RNA structure downstream of a stop codon. This structure rich in pyrimidines ensures the recruitment of PTBP1 protein (polypyrimidine tract binding protein 1) instead of NMD proteins [188]. Another strategy deployed by human T-cell lymphotropic virus type 1 (HTLV-1) consists in binding the main proteins involved in NMD, thus preventing them from performing their function [189]. Whatever the strategy employed, viral RNAs are stabilized by NMD inhibition.

Initially, a dichotomy was admitted between RQC and RNA interference where aberrant endogenous RNAs were targeted by the RQC pathway while regulation of endogenous and exogenous genes was provided by RNA interference. Nevertheless, multiple proteins involved in RQC pathways prevent RNA interference formation. RQC-associated proteins mutations stimulate RNA interference and induce RNA degradation *via* the silencing pathway. In this review, an example was given for exoribonucleases XRN2, XRN3 and XRN4 but this principle is now extended to other RQC factors: proteins associated with the exosome and its cofactors, proteins involved in deadenylation and decapping processes *etc.* [190]. Although these endogenous silencing suppressors have been identified, one question remained unsolved in RNA interference downregulation: how can we explain that transitivity regulates antiviral response and transgene expression while endogenous RNAs (mostly regulated by miRNA) are not subjected to this amplification process. Again, interlinking between RQC and RNA interference is undeniable. In fact, SKI2 protein is associated with the exosome to ensure RNAs 3'-5' degradation in RQC and also ensures the degradation of 5' cleavage products released after RISC complex slicing in the miRNAs pathway. SKI2 prevents miRNA-triggered transitivity activation by inhibiting the biogenesis of secondary siRNAs whereas they accumulate in *ski2* plants following RDR6 amplification [191]. The regulation of endogenous genes expression is thus preferentially subject to RQC, while their management by the silencing machinery would be deleterious.

RNA interference represents an outstanding regulation platform for genes expression control, finely tuned by multiple proteins and nucleic acid factors. Its relationships with various cellular pathways (RNA quality control, RNA editing *etc.*) provides hierarchical but also coordinated regulation processes.

Although it remains a very complex system, RNA interference is easily circumvented during viral infection. Viruses appear as impressive disrupters able to avoid this antiviral mechanism and have even evolved to take advantage from it. However, there are still many perspectives to elucidate

mechanisms used by viruses and mediated by their VSR and associated non-coding RNAs to bypass immune defence.

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