

Review

Viral RNA Silencing Suppression: The Enigma of Bunyavirus NSs Proteins

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Abstract: The *Bunyaviridae* is a family of arboviruses including both plant- and vertebrate-infecting representatives. The *Tospovirus* genus accommodates plant-infecting bunyaviruses, which not only replicate in their plant host, but also in their insect thrips vector during persistent propagative transmission. For this reason, they are generally assumed to encounter antiviral RNA silencing in plants and insects. Here we present an overview on how tospovirus nonstructural NSs protein counteracts antiviral RNA silencing in plants and what is known so far in insects. Like tospoviruses, members of the related vertebrate-infecting bunyaviruses classified in the genera *Orthobunyavirus*, *Hantavirus* and *Phlebovirus* also code for a NSs protein. However, for none of them RNA silencing suppressor activity has been unambiguously demonstrated in neither vertebrate host nor arthropod vector. The second part of this review will briefly describe the role of these NSs proteins in modulation of innate immune responses in mammals and elaborate on a hypothetical scenario to explain if and how NSs proteins from vertebrate-infecting bunyaviruses affect RNA silencing. If so, why this discovery has been hampered so far.

Keywords: RNAi; RNA silencing; innate immunity; bunyavirus; NSs; tospovirus; orthobunyavirus; RNA silencing suppression

1. Introduction—The Family of *Bunyaviridae*

The *Bunyaviridae*, with more than 350 identified species, is divided in five genera and contains several important viruses that cause major problems in human/animal health and agriculture production systems. All five genera of this family contain viruses pathogenic to either animals/humans (*Orthobunyavirus*, *Phlebovirus*, *Nairovirus* and *Hantavirus*) or plants (*Tospovirus*). Most bunyaviruses are arthropod-borne viruses (arboviruses), as they replicate in the arthropods by which they are transmitted (Figure 1). Hantaviruses present an exception, as they are rodent-borne and no arthropod vector has been identified so far.

Members of all five genera in the *Bunyaviridae* share several features. Bunyavirus particles are enveloped and generally spherical. Viral glycoproteins are embedded in the envelope membrane and presented as spikes on the outside. The core of virus particles contains the single-stranded (ss)RNA genome that is encapsidated by a nucleocapsid (N) protein and small amounts of the viral RNA-dependent RNA polymerase (RdRp, also denoted L protein). The bunyavirus RNA genome is tripartite and segments have either a negative or ambisense polarity (Figure 2). Genome organization strategies vary among members of different genera and may diversify even among members within a genus, as observed with orthobunyaviruses and phleboviruses. In general, though, the bunyavirus genome codes for four structural and up to two non-structural proteins. The L RNA is of complete negative polarity and contains a single open reading frame (ORF) on the viral complementary (vc)RNA that encodes the RdRp. With the exception of tospoviruses, the M RNA of all other bunyaviruses

is of negative polarity and contains one single ORF on the vc-strand coding for the precursor to the two glycoproteins (Gn and Gc), and in a few cases an additional non-structural protein NSm. The M RNA of tospoviruses contains an ambisense gene arrangement, and encodes a NSm on the viral (v)RNA strand and the glycoprotein precursor on the vcRNA. Tospovirus NSm protein facilitates the movement of viral ribonucleoproteins (RNPs) from cell-to-cell and presents an adaptation of this group of viruses to plants as a host. The S RNA segment is of negative polarity for members of the genera *Orthobunyavirus*, *Hantavirus* and *Nairovirus*, or ambisense for members of the genera *Phlebovirus* and *Tospovirus* [1–6]. The negative polarity S RNA encodes the major structural N protein on the vcRNA strand and, in certain members of orthobunyaviruses and hantaviruses, an additional non-structural protein (NSs) in an overlapping reading frame. For members of genera with ambisense S RNA, the NSs protein is encoded, separate from the N gene, by a second non-overlapping ORF on the vRNA strand (Figure 2).

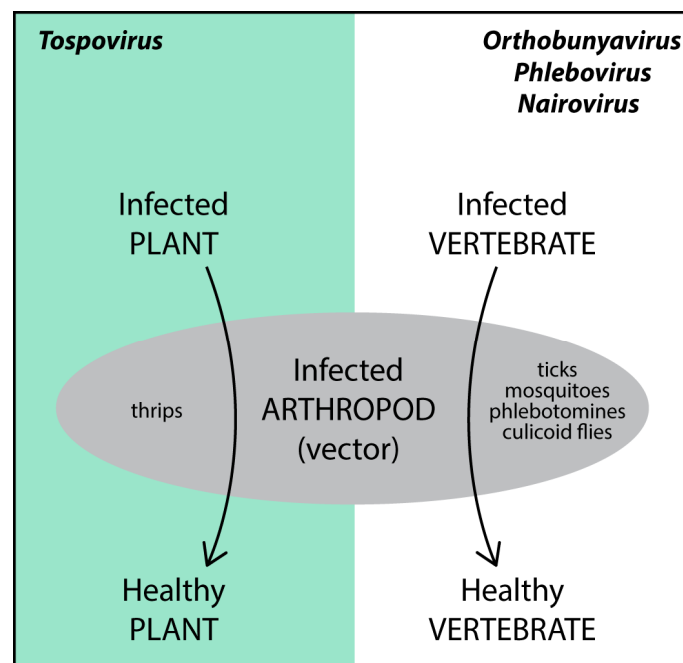


Figure 1. Bunyaviruses and their arthropod vectors.

During their infection cycle, all viruses encounter the host innate immune system as one of the first lines of host defense. In response to that, viruses have evolved various strategies to counteract the host innate immune system. In the case of bunyaviruses, the NSs protein is a knowingly important modulator of host innate immune responses, and a virulence factor [1–7]. In vertebrates, the interferon (IFN) pathway plays a major role in antiviral defense, and accordingly IFN-antagonist activity is found in the NSs of vertebrate-infecting bunyaviruses (reviewed by [7]). In plants and arthropods, where IFN pathway is not present, RNA silencing is an important part of antiviral innate immunity, and during infection of plants (by tospoviruses) and arthropods (by arthropod-borne bunyaviruses), members of the *Bunyaviridae* are targeted by antiviral RNA silencing. However, so far only the NSs protein of tospoviruses has been irrefutably demonstrated to have RNA silencing suppression (RSS also known as viral suppressors of silencing, VSRs) activity [8–10], which is relevant for the establishment of a successful infection of plant hosts. Concerning the arthropod vector, information related to the possible effect of NSs on antiviral RNA silencing during infection in arthropods remains scarce for both plant- and vertebrate-infecting bunyaviruses likewise. In this review we will present the state of the art on the modulation of host defense responses by bunyavirus NSs proteins, with emphasis on its effect on antiviral RNA silencing, to finally discuss the enigma surrounding NSs from bunyaviruses and its (possible) effect on arthropod and mammalian antiviral RNA silencing.

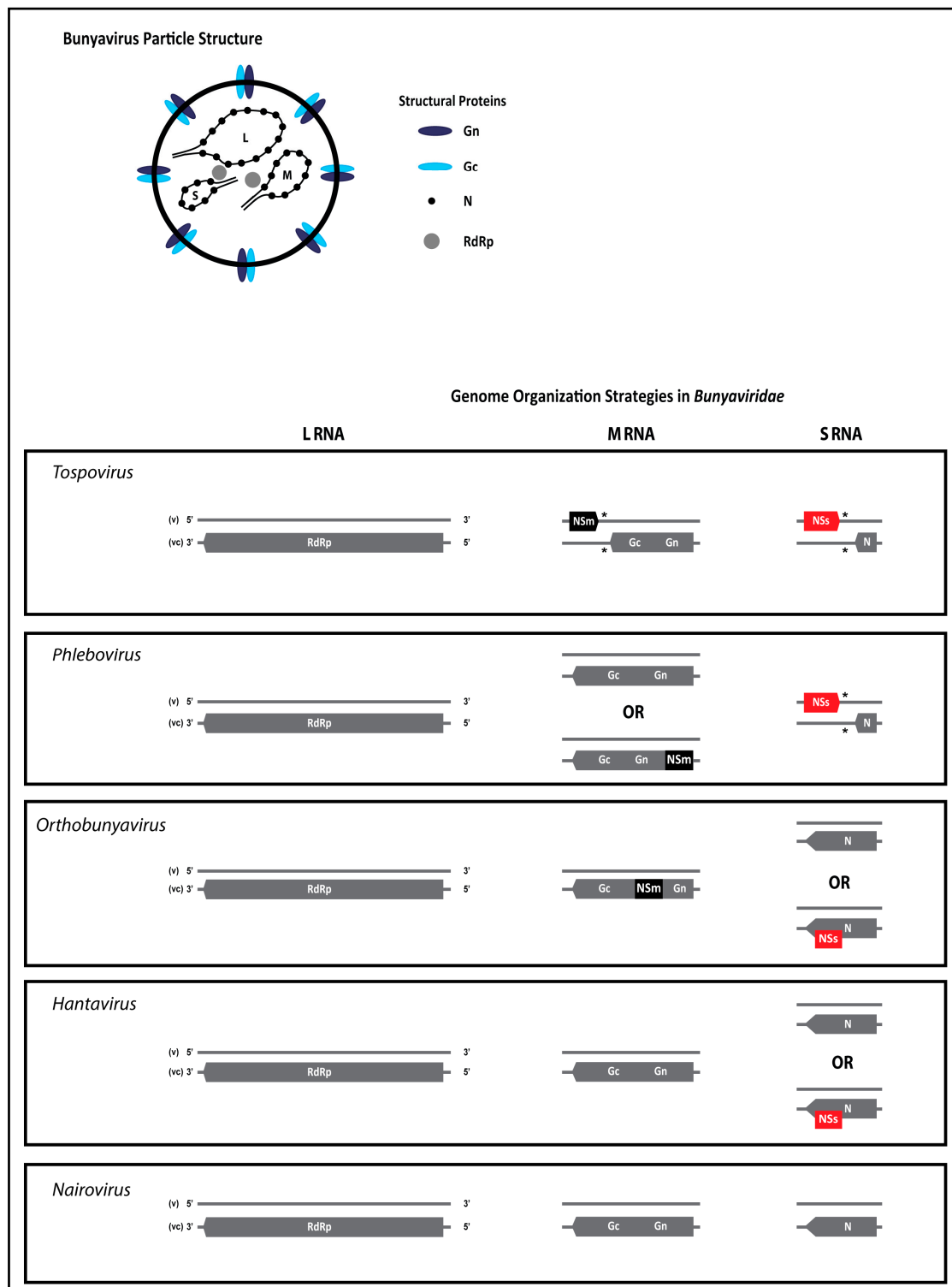


Figure 2. Particle structure and genome organization of bunyaviruses. L, M, and S RNA (large, medium, and small RNA segments, respectively), RdRp (viral RNA-dependent RNA polymerase), Gn and Gc (glycoproteins derived from the N-terminus and C-terminus of the precursor protein, respectively), NSm (non-structural protein of the M RNA), N (nucleocapsid protein), NSs (non-structural protein of the S RNA). (*) intergenic region (IGR).

2. Antiviral RNA Silencing

RNA silencing (also known as RNA interference, RNAi) is a gene regulatory mechanism conserved among eukaryotic organisms. In plants, fungi and invertebrates an antiviral RNA silencing pathway is triggered by double-stranded (ds)RNA structures (viral dsRNA intermediates, intramolecular hairpin structures in viral ssRNA, complementary viral transcripts) that arise during viral infections [11]. These viral dsRNA molecules are recognized and processed by Dicer enzymes (members from the RNase III family) into viral small interfering RNAs (vsiRNAs) approximately 21 nt in size [12,13]. From these, one strand is loaded in an argonaute (AGO) protein, the effector component of the RNA-induced silencing complex (RISC) [14,15]. The siRNA-programmed RISC surveils the cell for the presence of (viral) RNA target sequences with siRNA-sequence complementarity and next through AGO slicer activity cleaves the RNA target, initiating its degradation (Figure 3). Plants, fungi, worms, but not insects or mammals, contain RNA-dependent RNA polymerases (RDRs), which are essential components for amplification of the silencing signal [16,17]. As a result of amplification, secondary siRNAs are produced, leading to a more robust silencing response (Figure 3). Secondary siRNAs are important components of the systemic silencing response and travel short and long distances, in plants, respectively, through plasmodesmata (to neighbouring cells) and phloem (to distant parts of the plant). In the plant model *Arabidopsis thaliana*, the proteins involved in the amplification process include, among others, dicer-like proteins (DCLs) (processing also the de novo dsRNA in secondary siRNAs), cellular RDRs (synthesis of de novo dsRNA), and protein suppressor of gene silencing 3 (SGS3, a cofactor of RDR6) [18].

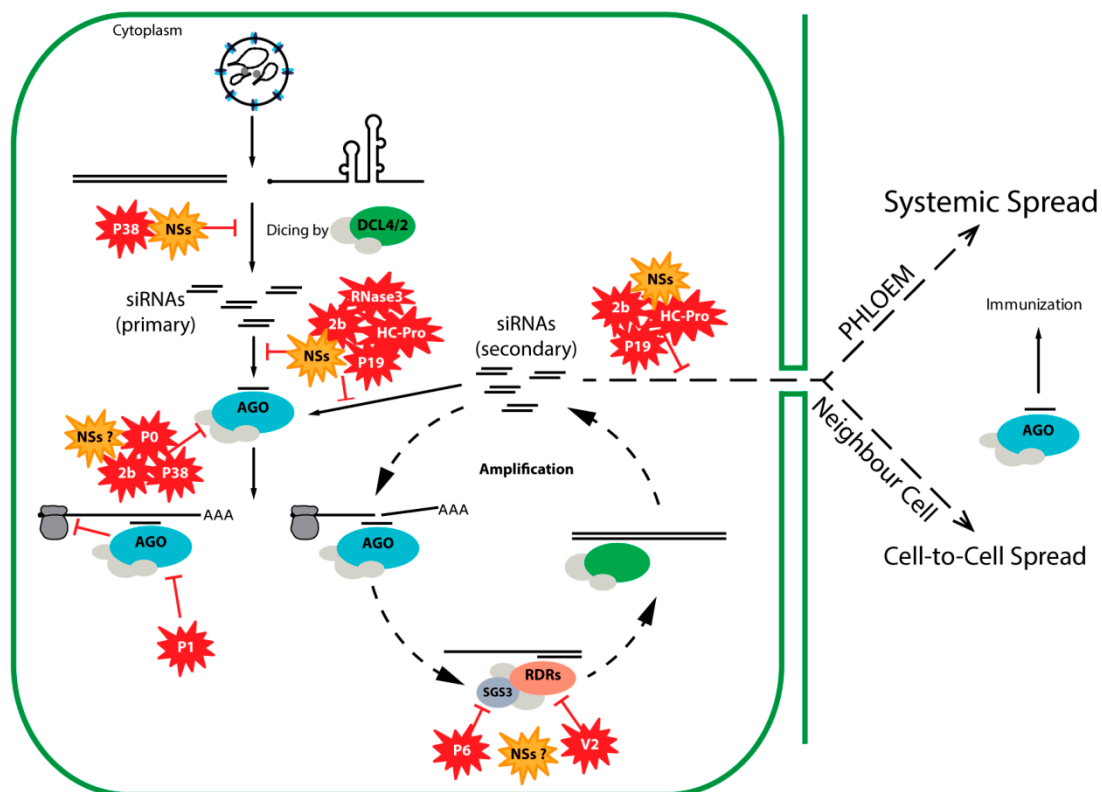


Figure 3. Schematic representation of plant antiviral RNA silencing and the counter-strategies used by several viral RSS proteins (indicated by the red symbols and, in the case of tospovirus NSs, orange symbols). AGO: argonaute protein; DCL: dicer-like protein; RDR: host-encoded RNA-dependent RNA polymerase; SGS3: protein suppressor of gene silencing 3; HC-Pro: helper component-proteinase; siRNAs: small interfering RNAs.

Although RNA silencing is a conserved eukaryotic mechanism, diversification occurred during evolution of the species, with the number of components varying among different organisms. *A. thaliana*, one of the best described (plant) models for RNA silencing, has four DCLs (DCL1–4), ten AGOs (AGO1–10) and six RDRs (RDR1–6). Some of these proteins have partially redundant roles, but specific combinations are known to be involved in the different plant RNA silencing pathways. For a more detailed review on this matter, readers are referred to [19]. While RNA silencing has been demonstrated to act antiviral in plants, fungi and invertebrates more than a decade ago, it was not until 2013 that studies involving analysis of RNA silencing in mammalian undifferentiated cells as well as the analysis of “in phase” production of siRNAs elegantly showed the presence of antiviral RNA silencing also in vertebrates [20,21].

3. Viral Strategies to Evade Plant Antiviral RNA Silencing Defense

In order to successfully infect hosts that contain an active antiviral RNA silencing defense system, viruses have evolved diverse strategies. Many of these have been identified during extensive studies on a wide range of plant viruses. Brome mosaic virus (BMV) replicates inside endoplasmic reticulum (ER)-derived spherules [22], therefore avoiding being perceived by the RNA silencing machinery. Other viruses, such as cauliflower mosaic virus (CaMV), during their infection produce decoy-RNA (generated from noncoding regions) that is targeted by Dicer, hence diverting the host RNA silencing machinery from targeting essential regions of the viral genome and transcripts [23].

A strategy that appears to be one of the most common among (plant) viruses is the expression of RSS proteins with the ability to suppress RNA silencing by targeting important steps of this antiviral pathway. These viral proteins are structurally and functionally diverse and employ a myriad of strategies to target key components of the RNA silencing pathway (Table 1) [18]. Due to the large number of RSS proteins already identified, it would be too extensive to describe each of them individually. Therefore, here only the most well known strategies used by viral RSS will be explained. More in depth reviews are available in literature [19,24].

A strategy used by many RSS is the binding and sequestration of dsRNA. Binding to dsRNA can be limited by the size of the molecule and some RSS proteins are able to bind only siRNAs. Size-selective binding to siRNAs has been shown for, e.g., P19 (carnation Italian ringspot virus, CIRV), P21 (beet yellows virus, BYV), γ B (barley stripe mosaic virus, BSMV) and HC-Pro (tobacco etch virus, TEV) [47,48] (Figure 3, Table 1). Other RSS, such as aureusvirus P14 (pothos latent virus, PoLV) and carmovirus P38 (turnip crinkle virus, TCV), are not limited by the size of the dsRNA molecule, binding dsRNA size-independently and hence being able to not only sequester siRNAs, but also to prevent or interfere with DCL cleavage of long dsRNA triggers (Figure 3).

Another strategy of interference involves the inhibition of HEN1-dependent methylation of siRNAs (in plants and flies this methylation is necessary for stabilization of siRNA). Interference with methylation has been shown for HC-Pro (TEV) and P19 (CIRV) [63]. A more unusual siRNA-targeting strategy is performed by RNaseIII enzymes of some viruses, e.g., RNase3 of sweet potato chlorotic stunt virus (SPCSV, genus *Crinivirus*), which contains endonuclease activity and cleaves siRNAs into inactive smaller products which are unable to activate RISC [45,46]. Several RSS achieve suppression of RNA silencing by preventing RISC assembly through targeting of argonaute proteins. Through GW-WG motifs (AGO-hooks) RSS proteins, such as P38 (TCV), are able to interact with and compromise argonaute activity [71]. Some RSS—P38 (TCV), P19 (CymRSV), HC-Pro (TEV), 2b (CMV)—modulate accumulation of miR168 (regulates AGO1 mRNA), consequently interfering with AGO1-related silencing [76]. Another strategy is targeting AGO1 for degradation, described for polioviral P0 [55]. While most RSS do not effectively target AGO1 once it is assembled into RISC, P1 (SPMMV) is able to inhibit programmed RISC, which it achieves by targeting AGO1 via GW-WG motifs present in its N-terminal region [62].

The amplification phase of RNA silencing is also targeted by several RSS. Interaction of V2 (TYLCV) with SGS3 (a cofactor of RDR6) was demonstrated to be essential for its RSS activity [25]. Nucleorhabdovirus P6 (RYSV) is an RSS that inhibits systemic (but not local) silencing and this is likely achieved by interaction with RDR6 and blocking of RDR6-mediated secondary siRNA synthesis [36].

Table 1. List of identified RNA silencing suppressors (RSS) from plant-infecting viruses, and the respective strategy used to suppress the RNA silencing machinery.

Genome	Family, Genus	Virus ¹	RSS	Strategy
ssDNA	<i>Geminiviridae</i> , <i>Begomovirus</i>	TYLCV	V2	• Suppresses local silencing, interacts with SGS3 [25–27]
ssDNA	<i>Geminiviridae</i> , <i>Begomovirus</i>	TYLCCNV	βC1	• Interacts with an endogenous suppressor of silencing (rgsCAM) to repress RDR6 expression and secondary siRNA production [28]
(–)ssRNA	<i>Unassigned</i> , <i>Tenuivirus</i>	RHBV	NS3	• Size-dependently binds siRNA and miRNA, suppresses silencing in plant and insect (drosophila) [8,29]
(–)ssRNA	<i>Unassigned</i> , <i>Tenuivirus</i>	RSV	NS3	• Size-independently binds dsRNA (minimum size of 9 bp) [30], suppression of local and systemic silencing [31]
(–)ssRNA	<i>Bunyaviridae</i> , <i>Tospovirus</i>	TSWV	NSs	• Binds dsRNA (size independently) [32] • Suppression of local and systemic silencing [8–10]
(–)ssRNA	<i>Bunyaviridae</i> , <i>Tospovirus</i>	GBNV	NSs	• Suppression of local silencing [33]
(–)ssRNA	<i>Bunyaviridae</i> , <i>Tospovirus</i>	GRSV	NSs	• Binds dsRNA (size independently) [32] • Suppression of local and systemic silencing [10]
(–)ssRNA	<i>Bunyaviridae</i> , <i>Tospovirus</i>	TYRV	NSs	• Binds dsRNA (size dependently) [32] • Suppression of local and systemic silencing [10]
(–)ssRNA	<i>Rhabdoviridae</i> , <i>Cytorhabdovirus</i>	LNyV	Phosphoprotein (P)	• Local and systemic silencing suppression (weak) in plants, but not in insects. Shown to not prevent siRNA accumulation [34] • Suppresses RISC-mediated cleavage and RNA silencing amplification, interacts with proteins AGO (1, 2, 4), RDR6 and SGS3 [35]
(–)ssRNA	<i>Rhabdoviridae</i> , <i>Nucleorhabdovirus</i>	RYSV	P6	• Interferes with production of secondary siRNAs likely through interaction with RDR6. Suppresses systemic silencing, but NOT local silencing. Initial analysis does not show binding to siRNA [36]
(+)ssRNA	<i>Bromoviridae</i> , <i>Cucumovirus</i>	CMV	2b	• Binds siRNA (and to a lesser extent long dsRNA) [37] • AGO1 and AGO4 interaction [38–41] • AGO1 repression (via miR168 upregulation) [42] • Interacts with RDR proteins [43]
(+)ssRNA	<i>Bromoviridae</i> , <i>Cucumovirus</i>	TAV	2b	• Binds siRNA (size selectively) [44]

Table 1. Cont.

Genome	Family, Genus	Virus ¹	RSS	Strategy
(+)ssRNA	<i>Closteroviridae</i> , <i>Crinivirus</i>	SPCSV	Rnase3	• Endonuclease activity cleaves siRNA resulting in silencing-inactive products [45,46]
(+)ssRNA	<i>Closteroviridae</i> , <i>Closterovirus</i>	BYV	p21	• Binds siRNA (size selectively) [47,48] • Interferes with the miRNA pathway [49,50]
(+)ssRNA	<i>Closteroviridae</i> , <i>Closterovirus</i>	CTV	p20, p23, CP	• p20 and p23 suppress local silencing [51] • p20 and CP suppress cell-to-cell spread of silencing [51] • p23 enhances viral accumulation and distribution in the plant host [52]
(+)ssRNA	<i>Luteoviridae</i> , <i>Enamovirus</i>	PEMV-1	P0	• Destabilization of AGO1 (mediated by an F-box-like domain), suppression of local and systemic silencing [53]
(+)ssRNA	<i>Luteoviridae</i> , <i>Polerovirus</i>	BWYV	P0	• Suppresses local but not systemic silencing [54] • Targets AGO proteins for degradation [55–57] through the autophagy pathway [58]
(+)ssRNA	<i>Luteoviridae</i> , <i>Polerovirus</i>	Other polero viruses:	P0	• CYDV, PLRV, CABYV: destabilization of AGO1 (mediated by F-box-like domain), with suppression of local but not systemic silencing (except PLRV P0, shown to suppress both) [53,56,59,60]. CLRDV and SCYLV: suppression of local but not systemic silencing [59,61]
(+)ssRNA	<i>Potyviridae</i> , <i>Ipomovirus</i>	SPMMV	P1	• Interacts with AGO1 component of RISC loaded with siRNA or miRNA. WG/GW motifs are essential for interaction with AGO1 and required for the silencing suppressor activity [62]
(+)ssRNA	<i>Potyviridae</i> , <i>Potyvirus</i>	TEV	HC-Pro	• Binds siRNA (size selectively) [47,48] • Inhibits 3' methylation of si/miRNA [63,64] • AGO1 repression via miR168 upregulation [42]
(+)ssRNA	<i>Potyviridae</i> , <i>Potyvirus</i>	TuMV	HC-Pro	• Interferes with biogenesis of primary siRNA (effect dependent on interaction with RAV2) [65] • Interferes with miRNA pathway [49,50,66]
(+)ssRNA	<i>Potyviridae</i> , <i>Potyvirus</i>	ZYMV	HC-Pro	• Binds siRNA, interacts with and inhibits HEN1 [67–69]
(+)ssRNA	<i>Tombusviridae</i> , <i>Aureusvirus</i>	PoLV	P14	• Binds ds(RNA) size independently [47,70]

Table 1. Cont.

Genome	Family, Genus	Virus ¹	RSS	Strategy
(+)ssRNA	<i>Tombusviridae</i> , <i>Carmovirus</i>	TCV	P38	<ul style="list-style-type: none"> • Binds dsRNA (size independently) [47] • Interferes with biogenesis of primary siRNA (effect dependent on interaction with RAV2) [65] • Targets (interacts with) AGO1 via GW-motifs [71] • AGO1 repression via miR168 upregulation [42] • Shifts DCL usage (enhances DCL1 levels, leading to consequential DCL3 and DCL4 downregulation) [71]
(+)ssRNA	<i>Tombusviridae</i> , <i>Tombusvirus</i>	CIRV	P19	<ul style="list-style-type: none"> • Binds siRNA size selectively [48,72,73] • Inhibits the 3' methylation of siRNAs [63] • AGO1 repression (via miR168 upregulation) [74]
(+)ssRNA	<i>Tombusviridae</i> , <i>Tombusvirus</i>	CymRSV	P19	<ul style="list-style-type: none"> • Binds siRNA size dependently [47,75] • AGO1 repression (via miR168 upregulation) [42,76]
(+)ssRNA	<i>Tombusviridae</i> , <i>Tombusvirus</i>	TBSV	P19	<ul style="list-style-type: none"> • Interferes with the miRNA pathway [49,50]
(+)ssRNA	<i>Virgaviridae</i> , <i>Hordeivirus</i>	BSMV	γB	<ul style="list-style-type: none"> • Binds siRNA size selectively [47]
(+)ssRNA	<i>Virgaviridae</i> , <i>Pecluvirus</i>	PCV	P15	<ul style="list-style-type: none"> • Binds siRNA size selectively [47]
(+)ssRNA	<i>Virgaviridae</i> , <i>Tobamovirus</i>	TMV	p122	<ul style="list-style-type: none"> • Binds siRNA and miRNA and interferes with HEN-1 mediated 3' methylation of small RNAs [77] • AGO1 repression (via miR168 upregulation) [42]
(+)ssRNA	<i>Virgaviridae</i> , <i>Tobamovirus</i>	TMV	p126	<ul style="list-style-type: none"> • Interferes with HEN-1 mediated 3' methylation of small RNAs [78] • Suppression of local and systemic silencing [79]
dsDNA(RT)	<i>Caulimoviridae</i> , <i>Caulimovirus</i>	CaMV	Decoy RNA	<ul style="list-style-type: none"> • Massive amounts of siRNAs are generated from a noncoding RNA (ncRNA) leader region, programming antiviral RNA silencing against this region and preventing effective targeting of the remaining genome [23]
dsDNA(RT)	<i>Caulimoviridae</i> , <i>Caulimovirus</i>	CaMV	P6	<ul style="list-style-type: none"> • Interaction with and inactivation of DRB4, and suppression of local silencing [80,81]

¹ BSMV (barley stripe mosaic virus), BWYV (beet western yellows virus), BYV (beet yellows virus), CABYV (cucurbit aphid-borne yellows virus), CaMV (cauliflower mosaic virus), CIRV (carnation Italian ringspot virus), CLRDV (cotton leaf roll dwarf virus), CMV (cucumber mosaic virus), CTV (citrus tristeza virus), CYDV (cereal yellow dwarf virus), CymRSV (cymbidium ringspot virus), GBNV (groundnut bud necrosis virus), GRSV (groundnut ringspot virus), LNYV (lettuce necrotic yellows virus), PCV (peanut clump virus), PEMV-1 (pea enation mosaic virus-1), PLRV (potato leafroll virus), PoLV (pithos latent virus), RHBV (rice hoja blanca virus), RSV (rice stripe virus), RYSV (rice yellow stunt virus), SCYLV (sugarcane yellow leaf virus), SPCSV (sweet potato chlorotic stunt virus), SPMMV (sweet potato mild mottle virus), TAV (tomato aspermy virus), TBSV (tomato bushy stunt virus), TCV (turnip crinkle virus), TEV (tobacco etch virus), TMV (tobacco mosaic virus), TSWV (tomato spotted wilt virus), TuMV (turnip mosaic virus), TYLCCNV (tomato yellow leaf curl China virus), TYLCV (tomato yellow leaf curl virus), TYRV (tomato yellow ring virus), ZYMV (zucchini yellow mosaic virus).

4. Arthropod RNA Silencing and Arboviruses

After the discovery of RNA silencing in *Caenorhabditis elegans* and plants, one of the first evidence of natural antiviral silencing in insect species came in 2002 during studies on flock house virus (FHV) infection of *Drosophila melanogaster* cells, in which FHV B2 protein was identified as a RSS [82]. The presence of this (gene regulation) mechanism has also been shown in other arthropods, including flies (e.g., fruit fly), mosquitoes, spiders and ticks [83,84]. *Drosophila* is one of the insect species where RNA silencing is probably best characterized, and although the mechanism is similar to the one of plants, it also shows important differences. *Drosophila* contains two Dicer proteins, three Argonaute proteins and to date no RDR has been identified in insects (reviewed in [85]). In the case of antiviral RNA silencing Dicer-2 recognizes and cleaves dsRNA structures (generated during virus replication) into siRNAs, from which one strand activates a RISC complex containing AGO2 to surveil the cell for ssRNA with sequence complementarity to the siRNA [14,86–88]. The pathway also includes other proteins such as Loquacious (Loqs, which increases affinity of Dicer-2 to dsRNA), R2D2 (aids siRNA loading in AGO2 and RISC assembly), component 3 promoter of RISC (C3PO, facilitates AGO2 endonucleolytic cleavage of the siRNA passenger strand) and HEN1 (stabilizes the guide strand by methylating its 3' terminal nucleotide) (a deeper review of arthropod RNA silencing can be found in [89–91]). Similar to plant-infecting viruses, several arthropod-infecting viruses have been shown to counteract arthropod antiviral RNA silencing by expressing RSS proteins that can target different steps of the RNA silencing machinery. B2 from *Drosophila*-infecting nodavirus FHV, one of the first RSS identified in arthropods, binds dsRNA size-independently and inhibits Dicer cleavage [92], but also directly interacts with the PAZ domain of *Drosophila* Dicer proteins [93]. B2 from Wuhan nodavirus (WhNV) has also been shown to bind dsRNA (small and long) (reviewed in [94]). Another example is protein 1A from cricket paralysis virus (CrPV), which suppresses RNA silencing by directly interacting with AGO2 [95], while 1A from *Drosophila* C virus (DCV) binds dsRNA and blocks Dicer-2 processing of dsRNA into siRNAs but does not bind siRNAs [96].

Arthropods are vectors for viruses (arboviruses) from several families (including *Flaviviridae*, *Togaviridae*, *Reoviridae* and *Bunyaviridae*). During persistent-propagative transmission, both plant- and animal-infecting arboviruses replicate in the arthropod vector [97,98], following a similar route of infection. The primary infection site is the midgut epithelium and in order to further disseminate in the arthropod organism and reach the salivary glands the virus must pass the midgut barrier, a determinant factor for vector competence (reviewed in [97–99]). Several studies indicate that RNA silencing is a relevant part of the midgut barrier and plays an important role in arbovirus infection and vector competence [100,101]. However, the antiviral role of midgut RNA silencing remains under debate and may also vary according to the arthropod species, as a recent investigation of alphavirus infection in *Anopheles gambiae* mosquitoes presents evidence that RNA silencing is active however it does not seem to have antiviral impact on the initial midgut infection, but only at later stages of infection [102]. Supporting the importance of antiviral RNA silencing in modulation of arbovirus infection, in arthropods where RNA silencing has been compromised or suppressed (e.g., by the action of a strong RNA silencing suppressor), arboviruses replicate to higher titers and the infection becomes pathogenic [103,104], which indicates that arthropod RNA silencing also modulates the equilibrium of an arbovirus persistent infection of the vector. Some strategies used by arboviruses to modulate antiviral RNA silencing include decoy strategies, e.g., the flavivirus small structured non-coding RNA from the viral 3'UTR referred to as subgenomic flavivirus RNA (sfRNA) [105] and the nucleic acid mediated decoy mechanisms of alphaviruses [106].

Members of the *Bunyaviridae* are arboviruses, and as such replicate in both the host (plant or vertebrate) as well as in the arthropod vector. While infecting arthropods, bunyaviruses are targeted by RNA silencing, as illustrated by production of virus-specific siRNAs (Table 2) [107–109]. It has not yet been demonstrated whether arthropod infection by bunyavirus requires viral expression of a suppressor of arthropod RNA silencing. For plant-infecting tospoviruses, the NSs protein suppresses RNA silencing in plants [8,9] and this protein is expected to also modulate antiviral RNA

silencing in thrips (although clear evidence for this is still needed). On the other hand, information on the effect of vertebrate-infecting bunyaviruses in either vertebrate or arthropod RNA silencing is scarce and limited to a few reports. Below, we review the current knowledge on the activity of NSs from bunyaviruses in the different environments where replication takes place.

Table 2. Reports on RNA silencing responses and production of virus-derived siRNAs during infection with members of *Bunyaviridae*.

Genus	Virus ¹	Organism	Remarks	Ref.
<i>Orthobunyavirus</i>	LACV	Insect (mosquito) <i>Aedes albopictus</i> (C6/36 cell line) <i>Aedes triseriatus</i> (MAT cell line)	Northern-blot analysis of S RNA-derived mRNAs reveals presence of small RNAs mapping to these regions	[110]
		Insect (mosquito and drosophila) <i>Aedes albopictus</i> (C6/36 cell line) <i>Drosophila melanogaster</i> (S2 cell line)	Deep sequencing analysis shows presence of virus-specific small RNAs mapping to all three genomic segments	[111]
	SBV	Insect (culicoides midges and mosquito) <i>Aedes aegypti</i> (Aag2 cell line) <i>Culicoides sonorensis</i> (KC cell line)	Deep sequencing analysis shows presence of virus-specific small RNAs mapping to all three genomic segments	[107]
<i>Phlebovirus</i>	RVFV	Insect (mosquito) <i>Aedes aegypti</i> (Aag2 cell line) <i>Aedes albopictus</i> (C6/36 cell line) <i>Aedes albopictus</i> (U4.4 cell line)	Deep sequencing analysis shows presence of virus-specific small RNAs mapping to all three genomic segments. Results indicate antiviral RNA silencing is related to modulation of bunyavirus persistent infection in arthropods.	[109]
<i>Tospovirus</i>	TSWV	Plant <i>Nicotiana benthamiana</i>	Northern-blot analysis shows presence of virus-specific small RNAs mapping to all three genomic segments and a closer analysis of the S RNA show small RNAs mapping to most of its sequence, but hardly to the IGR region.	[112]
	TSWV PolRSV	Plant <i>Nicotiana benthamiana</i> <i>Solanum lycopersicum</i>	Deep sequencing analysis shows presence of virus-specific small RNAs mapping to all three genomic segments, but hardly to the IGR regions (from both S and M segments).	[113–115]

¹ LACV (La Crosse virus), PolRSV (polygonum ringspot virus), RVFV (Rift Valley fever virus), SBV (Schmallenberg virus), TSWV (tomato spotted wilt virus).

5. Tospovirus NSs Counter-Defense against the siRNA Branch of the Antiviral RNA Silencing Pathway in Plants

A first glimpse on the importance of NSs for tospoviral infection in plants came in the early 1990s, when elevated levels of NSs expression were observed to correlate to a higher virulence of tomato spotted wilt virus (TSWV) isolates and more severe symptoms [116]. About a decade later, the ability of NSs to counteract the RNA silencing defense mechanism in plants was demonstrated [8,9]. Further studies on tospoviral NSs (particularly from TSWV) revealed that it is able to bind small and long dsRNA [32,117], suggesting that NSs interferes in two important steps: by binding long dsRNA it prevents these molecules from being recognized and processed into siRNAs by Dicer-like proteins (DCLs), while by sequestering siRNAs it prevents RISC from being activated and programmed (Figure 3).

More recently it was also shown that NSs of TSWV, as well as those of groundnut ringspot virus (GRSV) and tomato yellow ring virus (TYRV), suppresses long-distance systemic silencing [10], which is likely a consequence from interference in the biogenesis of the systemic signal (siRNAs). Interestingly, this same study also shows that suppression of local and systemic silencing can be uncoupled, as TSWV NSs mutants which lost the ability to suppress local silencing were still able to suppress systemic silencing [10]. These observations suggest that, besides sequestration of long and short dsRNA, NSs possibly also interferes in a systemic silencing exclusive step downstream the siRNA biogenesis (Figure 3). Suppression of systemic silencing without affecting local silencing has

been shown for other viral RSS proteins, such as nucleorhabdovirus P6, where it was suggested to block secondary siRNA production by interacting with RDR6 [36].

6. Tospovirus NSs Counter-Defense against the miRNA Branch of the Antiviral RNA Silencing Pathway in Plants

Next to the siRNA-mediated silencing, there is a branch of the RNA silencing pathway involving micro (mi)RNAs, which resemble siRNAs structurally and in size, but differ from those in their biogenesis, being processed from host-encoded transcripts that fold into imperfect stem-loop structures. The miRNA pathway plays a major role in regulation of eukaryotic endogenous gene expression, including expression of RNA silencing components. The best known example is the regulation of AGO1 expression by miR168 [118]. Interference with the miRNA pathway is also a viral strategy and several RSS proteins, such as tombusvirus P19 and cucumovirus 2b, are able to modulate miR168, causing a downregulation on AGO1 expression and this way affecting the antiviral RNA silencing response (Table 1).

Interference in the miRNA pathway has been described for TSWV NSs, shown to bind miRNAs *in vitro*, and to interfere with the miRNA pathway in planta [32]. In light of its affinity to long and small RNA duplex molecules, it is likely that TSWV interference in the miRNA pathway is accomplished at least partly through sequestration of miRNAs by NSs. Also for the GBNV NSs protein initial data indicate it interferes with the miRNA pathway [33].

7. TSWV NSs as Effector of Plant NLR-Mediated Intracellular Innate Immunity Response

Besides RNA silencing, another layer of the innate immunity system is represented by resistance genes and their R protein products. In plants the major class of dominant resistance (*R*) genes codes for the NB-LRR type, which are proteins consisting of three main domains. The N-terminal end is presented by a coiled-coil (CC) or Toll and interleukin-1 receptor (TIR) domain, followed by an internal nucleotide binding site (NBS) domain and a leucine-rich repeat (LRR) at the C-terminal end [119,120]. R proteins act as intracellular sensors of innate immunity and are highly pathogen specific. They are able to directly or indirectly perceive the presence of a pathogen by recognizing one of its effector proteins that often play a role in virulence and are referred to as avirulence factors (*Avr*). R proteins act like molecular switches that upon effector/*Avr* recognition trigger a resistance mechanism concomitant with a programmed cell death, leading to the appearance of small necrotic lesions (hypersensitive response, HR) at the site of pathogen entry. The establishment of HR prevents further dissemination of the pathogen throughout the plant host [121–124]. Some R proteins recognize viral RSS proteins, thereby acting as a plant counter-counter-defense against the viral counter-defense (RNA silencing suppression) against plant antiviral RNA silencing [125].

Recently, TSWV NSs protein was identified as the *Avr* for the single dominant *Tsw* resistance gene product, a protein that is also thought to belong to the class of NB-LRR genes [126]. In the constant battle between viruses and plants, viruses continuously keep on evolving and mutations on key amino acid residues of NSs lead to the appearance of so-called TSWV resistance-breaking (RB) isolates that do not trigger the *Tsw*-mediated HR response. Considering the role of NSs in counter defense against RNA silencing, mutations within this protein are likely fine-tuned to preserve (some) viral fitness and virulence, preventing virus clearance from the plant by antiviral RNA silencing. A study on engineered NSs mutants indicated that NSs RSS and *Avr* functions can be uncoupled. Furthermore, some of the resistance-breaking NSs mutants (both natural and engineered), mutated in predicted RNA-binding or putative Argonaute-binding domains, still suppress systemic silencing spread while having lost the ability to suppress local silencing [10,127]. Altogether, this supports the idea that the virus and its NSs protein can evolve to fine tune *Avr* and (local/systemic) RSS functions in order to lose (some of) its avirulence features while preserving a certain level of fitness [10,127].

8. Tospovirus NSs Counter Defense Against Antiviral RNA Silencing in Insects

Antiviral RNA silencing has already been demonstrated in several arthropods [96,128]. Also during an arbovirus infection of arthropod vectors the mechanism acts antiviral, more specifically in the midgut epithelium where RNA silencing seems to be an important part of the midgut barrier and influences viral dissemination to secondary tissues and vector competence [100]. In thrips, RNA silencing is functional and can be used as an investigative tool [129,130], however its role in antiviral defense has not been verified yet, and it remains to be investigated whether tospoviruses during propagative transmission by thrips are strongly targeted by antiviral RNA silencing and require a counter-defense by NSs.

In contrast to the situation in plants, less is known about the function of tospovirus NSs in the insect vector (thrips). Evidence shows that TSWV NSs is expressed in cells of thrips *Frankliniella occidentalis* (western flower thrips, WFT), especially in midgut epithelial cells (primary infection site), associated muscle tissue and cells in the salivary glands, from where tospoviruses are transmitted to the plant during thrips feeding [131–133]. Although to date little information is available on the molecular modus operandi of NSs in the context of thrips infection, recent reports shed light on this matter and slowly a clearer picture is emerging on the importance of NSs during an arthropod infection.

The role of NSs as a transmission determinant in thrips was recently demonstrated in experiments with NSs-defective TSWV isolates which lost RSS activity and became non-transmissible by thrips. A closer look indicated that these viruses were still acquired by thrips larvae upon feeding on infected plants, however virus accumulation in adult thrips was severely reduced, which likely explains why adult thrips do not support transmission of these NSs-defective isolates upon a next probing-feeding attempt [134]. In light of the antiviral RNA silencing role in the midgut epithelium and its influence in vector competence observed for other arbovirus-arthropod vector interactions [100], it is likely that the observed reduction in vector competence and virus titers are linked to the lack of RSS activity in these NSs-defective isolates.

Although not in thrips, silencing suppression function of tospovirus NSs has been investigated in arthropods including mosquitoes (Diptera), caterpillar (Lepidoptera) and ticks. Two studies showed that TSWV NSs is able to suppress luciferase silencing, when expressed in mosquito cell lines (U4.4—*Aedes albopictus*) [110] and in tick cells (ISE6—*Ixodes scapularis*) [135]. In another study, TSWV NSs expressed from a recombinant baculovirus enhanced the replication of this recombinant virus to higher titers in *Spodoptera frugiperda* insect (lepidopteran) cell lines [136] and increased its virulence in caterpillar insects in vivo [137]. These observations support that NSs is also able to suppress antiviral RNA silencing in arthropods.

There is indication that TSWV infection activates other thrips antiviral genes and defense responses—such as genes coding for antimicrobial peptides—proteins involved in pathogen recognition, receptors that activate innate immune response—such as Toll3—and members of signal transduction pathways activated by toll-like receptors [138]. Whether NSs has an effect in any of these other antiviral pathways in thrips is unknown.

9. NSs from Vertebrate-Infecting Bunyaviruses—Antagonizing Mammalian Innate Immunity

Vertebrate-infecting viruses must deal with the innate immunity in their mammalian hosts, including the well-known IFN-based defense [139,140]. NSs from several vertebrate-infecting bunyaviruses are well reported for their IFN-antagonist activity. For orthobunyavirus Bunyamwera virus (BUNV), La Crosse virus (LACV) and phlebovirus Rift Valley fever virus (RVFV), the NSs protein inhibits the type I IFN system by blocking RNA polymerase II transcription, consequently leading to a shutoff of the antiviral response genes [110,141–144]. RVFV NSs has additionally been shown to induce specific degradation of dsRNA-dependent protein kinase (PKR) [145], a process that occurs independently from the NSs-mediated blocking of host gene transcription [146]. IFN-antagonistic activity has also been identified in the NSs of tick-borne phleboviruses, e.g., the NSs of Uukuniemi

virus (UUKV) was shown to have weak IFN-antagonistic activity [147]. In the case of the tick-borne severe fever with thrombocytopenia syndrome virus (SFTSV), its NSs has been shown to form cytoplasmic inclusions involved in sequestration of host factors involved in RIG-I signaling as well as IFN signaling [148–151]. A more complete description of the diverse strategies employed by different phlebovirus NSs proteins to evade host IFN defense can be found in [152]. Several hantaviruses have been reported to contain, like orthobunyaviruses, an ORF overlapping the *N* gene and encoding a NSs protein with weak IFN-antagonistic properties [1]. For an extensive description on this subject readers are referred to excellent literature reviews [7,153]. In nairoviruses, a NSs protein has been identified only in Crimean-Congo hemorrhagic fever virus (CCHFV) and shown to induce apoptosis [154], however its effect on IFN pathway remains to be investigated.

Besides the IFN-induced innate immune responses, mammals contain additional layers of innate immunity that act against viruses, including antiviral RNA silencing [20,21]. Proteins from mammalian-infecting viruses have earlier been observed to possess RSS activity too, amongst which human immunodeficiency virus type 1 (HIV-1) trans-activator of transcription (Tat, Dicer interaction and inhibition), hepatitis C virus (HCV) core (Dicer interaction) and envelope E2 (AGO2 interaction), human influenza A NS1, Ebola virus VP35 and vaccinia virus E3L (all binding dsRNA), adenovirus VA (a non-coding RNA that folds into a stem loop structure and acts as a decoy for Dicer). Interestingly, all these very same viral proteins are known to act as IFN antagonists as well [155].

Thus far, only two papers have appeared on the identification of RSS activity in NSs proteins from vertebrate-infecting bunyaviruses in mammals, both with LACV NSs, but with contradictory outcomes. In one paper, RNA silencing was triggered by transfecting cells (human cell line 293T) with siRNAs, showing that in the additional presence of (transiently) over-expressed LACV NSs an apparent decrease of siRNA-triggered silencing was observed, which tempted the authors to suggest that LACV NSs exhibits RSS activity in mammals [156]. In another paper, researchers used LACV and recombinant LACVdelNSs viruses, and observed the outcome during infection in IFN-competent and IFN-deficient mammalian cell cultures (mouse embryo fibroblasts, MEFs) and mammalian animals (mice) (in vivo). In this case, however, LACV NSs did not provide an advantage to the virus [110].

10. NSs from Vertebrate-Infecting Bunyaviruses in Their Arthropod Vectors

Vertebrate-infecting bunyaviruses, with the exception of hantaviruses, are transmitted by arthropod vectors, including arachnids (ticks) and insects (mosquitoes, phlebotomines and culicoid flies) where, similarly to tospoviruses, they replicate and establish a persistent infection [157]. During infection of the arthropod vector, vertebrate-infecting bunyaviruses are targeted by antiviral RNA silencing, as indicated by the production of bunyavirus-specific small RNAs during bunyavirus infection (Table 2) [107,109,111]. The importance of RNA silencing in modulating bunyavirus replication and the establishment of a persistent infection in arthropods is supported by the fact that, at least for phlebovirus RVFV, persistency is only achieved in cells with active Dicer-2-based RNA silencing [109]. Similar investigations are needed to verify whether this is general to other *Bunyaviridae* genera.

Still, little is known regarding the role of NSs during propagative transmission of vertebrate-infecting bunyaviruses in the arthropod vector. Analysis of BUNV and a recombinant NSs-deletion BUNV indicates that NSs is required for efficient replication in cell lines (including mosquito *Aedes albopictus* cell line U4.4), and infection with NSs-deletion virus in mosquito *Aedes aegypti* revealed lower titers and delayed dissemination to salivary glands when compared to wild-type virus, suggesting that in the absence of NSs the virus has difficulties in overcoming cellular defenses in the midgut [158]. Considering the role of RNA silencing in the midgut barrier [101,159], it can be speculated that BUNV NSs counteracts the RNA silencing component of the midgut barrier. BUNV NSs was also observed to be non-essential in mosquito cell lines with impaired RNA silencing, further supporting that this proteins may have RSS activity [158]. NSs from orthobunyavirus LACV and phlebovirus RVFV have also been analyzed to some extent regarding their effect on RNA silencing in

arthropods (respectively, mosquito and tick cell lines), however in both cases suppression of silencing was not observed [110,135].

Altogether, available data indicate NSs is relevant during bunyavirus infection of arthropod vectors, however clear proof for presence (or absence) of RSS activity with NSs from vertebrate-infecting bunyaviruses in arthropods is still lacking.

11. The Enigma of NSs: Questions and Perspectives

While bunyaviruses are targeted by antiviral RNA silencing in plants, vertebrates and arthropods, currently only the NSs from plant-infecting tospoviruses has been clearly shown to contain RNA silencing suppressor activity. Considering the close ancestral relation of bunyaviruses, as well as many structural and functional similarities, the lack of clear proof on suppression of RNA silencing by the NSs from vertebrate-infecting bunyaviruses in both arthropods and vertebrates remains a matter for debate.

In light of this it is important to highlight that the two reported (contradictory) studies on the effect of orthobunyavirus LACV NSs in mammalian RNA silencing made use of different experimental set ups to induce silencing and to express NSs. The first study by Soldan et al. [156], in which synthetic siRNAs were transfected into mammalian cells (human cell line 293T) already expressing LACV NSs (from plasmid constructs transfected 24 h prior transfection of siRNAs), likely has used the best conditions to test LACV NSs for the ability to suppress RNA silencing due to a couple of reasons. Firstly, NSs was expressed a priori, being readily available at the time when siRNAs were transfected and allowing it to directly interfere with RNA silencing. This strategy has earlier been successfully used to demonstrate RSS activity with viral proteins [160,161]. Secondly, transfection of RNA duplex molecules smaller than 30 bp activates only the RNA silencing pathway, and not the interferon pathway [162,163]. As such, a transfection with siRNAs (21 nt) will effectively trigger RNA silencing only, and therefore results obtained with LACV NSs solely reflect interference on RNA silencing, not on IFN-induced defense responses. Altogether, this supports the observations made by Soldan et al. on the presence of RSS activity with LACV NSs. The downside of the study by Soldan et al. is that it involves a transient system and this does not reflect an authentic viral infection. In the second, contradictory study by Blakqori et al. [110], infections were performed with LACV recombinant viruses that either contained or lacked NSs. However, during this study it was only assumed that LACV NSs does not interfere with mammalian silencing because viruses had similar growth patterns and titers regardless of NSs, but the effect of NSs on the siRNA profile was not investigated. This second study used MEFs, instead of the human 293T cells used in the first study. Therefore, based on this result it would be too premature to discard the possibility of RSS activity in LACV NSs, nor with other bunyaviral NSs proteins. Future studies should also take into consideration the effect of NSs on small RNA profiles. Considering the recent success in verifying antiviral RNA silencing in mammals [20], future investigations on NSs from vertebrate-infecting bunyaviruses would also benefit from using a similar approach with undifferentiated cells, in which the production of virus-derived siRNAs is stronger and the IFN response is lacking or reduced [20,21].

Another reason why studies on this point for the vertebrate-infecting bunyaviruses so far have remained unresolved is that in mammalian cells the IFN-pathway is a major antiviral mechanism, while antiviral silencing is still being debated by some [164] and might be more secondary or limited to undifferentiated cells or certain cell types [20]. In addition, NSs from vertebrate-infecting bunyaviruses might not present a strong suppressor of RNA silencing [164]. Within the insect vector, evidence points to a role of RNA silencing in the midgut barrier where it influences vector competence [100,101] and modulates a persistent viral infection as supported by the observation that persistent viruses become pathogenic if an RSS active against insect RNA silencing is co-expressed [95,104]. Although speculative, in case bunyavirus NSs would present a strong suppressor of arthropod RNA silencing, it could thus disrupt the equilibrium of a persistent infection and turn it into a pathogenic one that might be fatal to the arthropod. A low level of RSS activity might thus be preferred and, although maybe more difficult to

proof experimentally, be sufficient to suppress RNA silencing in the midgut and support dissemination in the vector. Thus, whether vertebrate-infecting bunyaviruses truly need to counteract antiviral RNA silencing remains an issue that still requires further investigation and should not be ruled out. Based on the hypothesis that negative-sense RNA viruses may have their ancestry in arthropods [165], it is possible to speculate on the origin of NSs, which could have evolved as an adaptation to allow the ancestry insect-specific bunyavirus to cross and adapt to the plant or vertebrate secondary host. However, it is not possible to dismiss that NSs evolved initially in the ancestral arthropod host playing a role in modulation of arthropod antiviral RNA silencing. Further investigation of the several recently identified insect-specific bunyaviruses [166], some without a NSs ORF [167–169] while others seem to harbor a NSs (unknown function) [170], might shed light on the evolutionary history of bunyaviruses as well as on the role played by NSs.

In conclusion, NSs from bunyaviruses remains an enigmatic protein, being a virulence factor in different cellular environments. However, as reviewed above, its interference on antiviral RNA silencing is only clearly described for tospovirus infections in plants, while still being debated in arthropods and vertebrates. As new data are gathered on the relevance of RNA silencing during infection in mammals as well as during persistent infections in arthropods, this may eventually also contribute to a deeper understanding of how bunyaviruses possibly affect this antiviral defense mechanism. The advancement in deep-sequencing technologies and the use of undifferentiated cell lines may provide further tools to functionally analyze NSs during bunyavirus infection. Considering the importance of NSs for plant- and vertebrate-infecting bunyaviruses, understanding its role during infection and its modus operandi will remain a continuing challenge for many years to come.

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