

Viral counterdefense on RNA silencing

**Analysis of RNA silencing suppressors from arthropod-borne
negative strand RNA plant viruses**

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Thesis

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Abstract

This thesis describes that RNA silencing suppressor (RSS) proteins encoded by negative-stranded RNA plant viruses are able to interfere with different RNA silencing pathways in a variety of organisms by interacting with double stranded (ds)RNA molecules. These RSS proteins are able to counteract the antiviral RNA silencing response in their plant host and insect vector, and even in mammalian cells, that are non-hosts for these viruses. Whereas Rice hoja blanca virus NS3 has been shown to bind size specific dsRNA, most tospovirus NSs proteins, in contrast, bind dsRNA size-independently and thus are able to interfere at two different stages in the RNA silencing pathway: RNA-induced silencing complex assembly and in Dicer cleavage. In addition to the interaction with the antiviral small interfering (si)RNA pathway both RSS proteins were able to interfere with the micro (mi)RNA pathway, which is important for host gene regulation. This is probably due to the structural similarities among the dsRNA molecules (siRNA and miRNA/miRNA* duplexes). Computer prediction supports the idea that the miRNA pathway (or at least certain miRNA/miRNA* duplexes) could potentially act as antiviral response in insects and plants, as recently reported for mammals. Furthermore, the ability of NS3 to trans-complement HIV-1 for the proposed HIV-1 RSS, the Tat protein, and restore virus production at wild type virus levels, supports the recent observation that at least certain mammalian-infecting viruses are restricted by small dsRNA molecules and highlights the use of well characterized plant RSS proteins as tool to study viral counter defense in a variety of eukaryotic systems.

Chapter 1

General Introduction

The discovery of RNA silencing as the plant's innate immune system against viruses

The plant's innate immune system against viruses is very different from that against fungi and bacteria. The basal antiviral defense relies on the recognition and sequence-specific breakdown of (double-stranded, ds) viral RNA rather than targeting the pathogen's proteins. This recently disclosed defense system, which is generally referred to as antiviral RNA silencing or RNA interference (RNAi) will be discussed as well as the strategy how viruses may counteract this innate defense system.

Unlike fungal and bacterial pathogens, viruses are exclusively intracellular parasites, multiplying in either the cytoplasm or the nucleus of host cells. As a consequence, the interplay between host defense systems and the virus is a strictly intracellular event. Since only the mid 90's plant molecular biologists and virologists have become aware that the plant possesses a sequence-specific RNA breakdown mechanism, often referred to as post-transcriptional gene silencing (PTGS) or RNA silencing, and that this mechanism acts as the major innate immune system against viruses. The discovery of this defense system occurred accidentally, by encountering unexpected results during attempts to obtain virus-resistant plants through genetic engineering approaches. In the 80's several groups were investigating whether transgenic forms of virus resistance could be obtained according to the concept of "pathogen-derived resistance" (PDR). This concept was first described by Grumet et al. (1987), who proposed the possibility to exploit pathogen-derived genes as a means to obtain resistance in a variety of host-parasite systems. It was suggested that deliberate expression of such genes in altered form, level or developmental stage, could interfere with pathogen replication resulting in specific host resistance. Among possible targets for PDR-mediated virus resistance, the most broadly exploited viral genes were those coding for the coat protein (CP), replicase and movement protein (Baulcombe, 1996, Powell et al., 1990, Prins & Goldbach, 1996). Following the demonstration that expression of a viral CP confers a level of resistance to the pathogen (Powell et al., 1989), it was observed in control experiments that non-translatable CP transgenes conferred similar levels of resistance as the functional gene. For years this phenomenon was referred to as RNA-mediated resistance, and only in 1993 William Dougherty and co-workers (Lindbo et al., 1993) linked this phenomenon to "co-suppression" in plants and "quelling" in fungi (Cogoni & Macino, 1999a, Cogoni & Macino, 1999b) which involve sequence-specific degradation of transcripts from both transgenes and their homologous endogenous counterparts. In turn, co-suppression was discovered when transgenic petunia plants with additional copies of endogenous genes involved in flower pigmentation, became completely white due to a dramatic decrease in expression level of the respective genes (Napoli et al., 1990, van der Krol et al., 1990). Identification of (induced) RNA silencing as the principle mechanism of transgenic resistance to viruses has – in retrospective – been a major break-through. Rapidly, multiple publications appeared providing evidence that RNA silencing is a naturally occurring, ancient mechanism having a major function in regulating gene expression, transposon behavior, and viral infections (Carthew & Sontheimer, 2009). Moreover, RNA silencing occurs not only in plants and fungi, but has later

been found also in invertebrate (Fire et al., 1998) and vertebrate animals, including humans (Carthew & Sontheimer, 2009, Elbashir et al., 2001, Hammond et al., 2000, Zamore et al., 2000).

A crucial discovery was the finding of short, virus-derived dsRNA molecules in infected host plants, explaining the sequence specificity of the RNA breakdown mechanism (Hamilton & Baulcombe, 1999). These short dsRNA species are commonly referred to as small interfering RNAs (siRNAs).

Next to the discovery of virus-specific siRNAs, it was demonstrated that plants that are deficient in essential RNA silencing genes, show enhanced viral pathogenicity (Dalmay et al., 2001, Morel et al., 2002, Mourrain et al., 2000). These findings, and the fact that all tested plant viruses encode proteins that interfere with, and suppress the RNA silencing pathway, supported the idea that RNA silencing acts as innate antiviral defense system in plants. The viral proteins antagonizing RNA silencing, often already known as “virulence factors”, are commonly referred to as RNA silencing suppressor (RSS) proteins (Brigneti et al., 1998, Kasschau & Carrington, 1998).

Current views of RNA silencing as antiviral mechanism in plants

With increasing insights, it was found that RNA silencing is not one single RNA breakdown pathway but encompasses several pathways among two major ones, the siRNA and the microRNA (miRNA) pathway. The former includes the antiviral defense branch of the system, while the miRNA pathway is primarily involved in regulating (host) gene expression. Figure 1-1 presents a simplified scheme of the RNA silencing pathways in the plant (most data have been obtained from *Arabidopsis*). As visualized in the scheme, RNA silencing starts with the recognition of long dsRNA by a type III endonuclease, called Dicer-like protein (DCL) in plants (the term Dicer was coined for a similar enzyme in the fruit fly *Drosophila melanogaster* (Bernstein et al., 2001)). It will be obvious that in particular RNA viruses are excellent targets to provoke (antiviral) RNA silencing: they replicate through (partially) dsRNA intermediates, while also the single-stranded (ss) genome contains extensive secondary structure. For viruses with a DNA genome, like the caulimo- and geminiviruses, the viral transcripts are the targets for RNA silencing, induced by secondary structures (e.g. the 35S RNA transcript of cauliflower mosaic virus, CaMV) and/or by overlapping sense-antisense transcripts (Chellappan et al., 2004, Du et al., 2007, Moissiard & Voinnet, 2006, Molnar et al., 2005, Sharp & Zamore, 2000).

The siRNA pathway

The siRNA pathway represents the antiviral branch of RNA silencing and this process takes place entirely in the cytoplasm (Covey et al., 1997, Ratcliff et al., 1997) (Figure 1-1). It is known that plants encode different DCLs. In *Arabidopsis* DCL-4 is the most important one in the antiviral siRNA pathway against positive stranded RNA viruses while DCL-3 is needed for long-distance silencing and all 4 DCLs (DCL-1 to -4) are required for the antiviral defense

against DNA viruses like Cauliflower mosaic virus (CaMV) (Moissiard & Voinnet, 2006). When DCL-4 is inactivated, its function is partly replaced by DCL-2 (Gascioli et al., 2005).

DCL-4 cleaves the viral dsRNA target molecules into small viral specific dsRNA molecules (siRNA) of 21-26 nucleotides (nt) in length with 2 nt overhangs at their 3' ends (Dunoyer et al., 2005, Gascioli et al., 2005, Hamilton et al., 2002). After cleavage by DCL, the 21 nt siRNAs are incorporated into the RNA induced silencing complex (RISC), which harbors a member of the Argonaut (AGO) protein family, a key molecule of RISC (Hall, 2005). After unwinding and degradation of the passenger siRNA strand (or siRNA*), the guide siRNA strand is used to identify complementary single stranded viral RNA sequences. After duplex formation between the guide siRNA strand and viral ssRNA, RISC (more specifically the AGO protein) facilitates target cleavage of the viral ssRNA molecule, resulting in sequence-specific RNA degradation of the viral RNA (Tomari & Zamore, 2005). A special feature of the silencing pathway in plants, fungi and *C.elegans* is the possibility to amplify the silencing signal, in order to extend silencing along the target gene, using a host encoded RNA-dependent RNA polymerase (hRdRp). The hRdRp is able to produce new dsRNA molecules in an either primer-dependent or -independent manner; those can again enter the siRNA pathway, resulting in secondary siRNA molecules (Baulcombe, 2004, Cogoni & Macino, 1999a, Sijen et al., 2001, Vaistij et al., 2002) (Fig. 1-1).

RNA silencing is not only induced within the infected cell; plants are able to pre-program not yet infected cells by spreading the silencing signal beyond the site of initiation. This feature is called systemic silencing and can be divided in short-distance spread (10-15 cells) and phloem-dependent long-distance transport. Recent results in *Arabidopsis* have indicated that DCL-4 is essential for the short distance silencing, indicating 21 nt siRNA as mobile silencing signal. More experiments using transgenic plants and chemically designed siRNA molecules, indicates that the DCL-4 produced 21 nt siRNA molecules move to the surrounding cells in a complex without Argonaut and that the host encoded RNA dependent RNA polymerase 6 (RDR6) is not needed. More experiments are needed to answer the question if the siRNAs move as duplex or single stranded RNA and if the movement occurs via plasmodesmata (Dunoyer et al. 2010).

The long-distance silencing is suggested to rely on the activity of DCL-3, producing 24 nt siRNA molecules (Voinnet, 2005b, Yoo et al., 2004); although recent results point to the DCL-4 produced 21 nt siRNAs (Dunoyer et al. 2010). Further analysis is required to resolve the precise mechanism for both short-distance and long-distance systemic silencing.

The miRNA pathway

The miRNA pathway has no primary function in antiviral defense; it rather represents a gene expression regulation mechanism, shared with animals, to down-regulate genes.

Comparison of the siRNA and miRNA pathway (Figure 1-1) reveals a high degree of similarity. Both start with the processing of longer dsRNA substrates into small dsRNA species, of which the guide strands are incorporated into RISC (often denoted siRISC or

miRISC, respectively) and searching for complementary ssRNA molecules. A fundamental difference is that whilst the siRNA pathway occurs entirely in the cytoplasm, the miRNA pathway starts in the nucleus. The miRNAs are endogenous RNA species, encoded by host genes. Chromosomal miRNA genes are transcribed mostly by RNA polymerase II to deliver primary miRNAs (pri-miRNAs) that are folded into partly double stranded stem-loop structures and become a substrate for DCL-1 to generate precursor miRNAs (pre-miRNAs). Cleavage of pre-miRNAs, again performed by DCL-1, generates mature 21-22 nt miRNA/ miRNA* which, unlike siRNAs, are not completely double-stranded (Bartel, 2004, Voinnet, 2009). The miRNA/miRNA* duplexes are then exported from the nucleus by the nuclear export receptor HASTY (the *Arabidopsis* ortholog of the insect/mammalian *EXPORTIN5/ MSN5*). In the cytoplasm, the miRNA/miRNA*s are incorporated into RISC, unwound and used as guide to find perfectly or partly complementary ssRNA sequences, resulting in degradation and/or translational inhibition of target mRNAs. These target mRNAs often encode transcription factors that, in turn, are in charge of regulating multiple genes (Chen, 2005). Most miRNAs are expressed in a tissue-specific manner and some are able to down-regulate the expression of key RNA-silencing proteins, like DCL and AGO. The complementary miRNA target sequences in the host mRNAs can be present in the coding sequence, in the 3' untranslated region (UTR) or even in the 5' UTR (Voinnet, 2009).

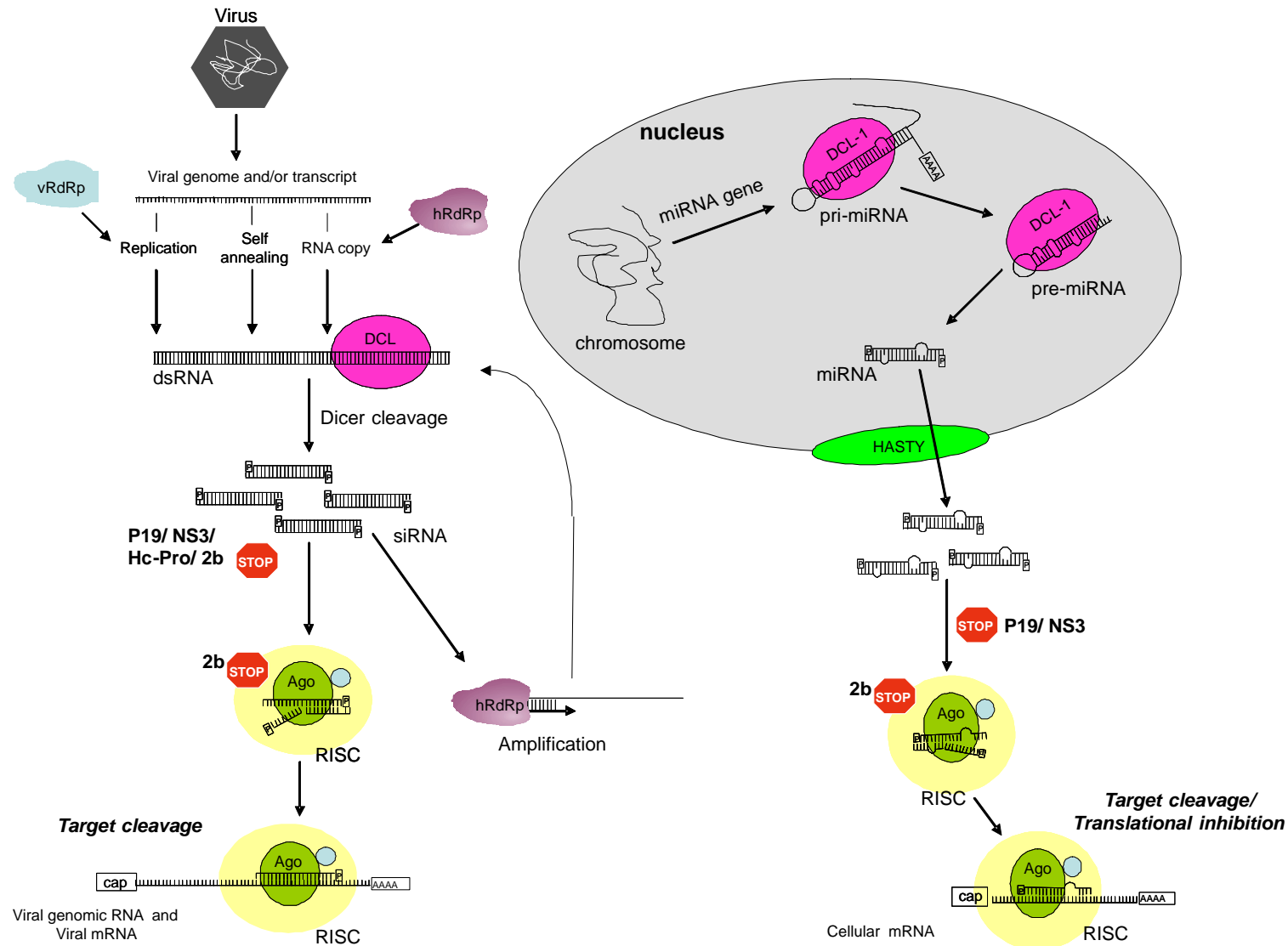


Figure 1-1: Schematic representation of the siRNA and miRNA pathway in plants and the inhibitory action (indicated “stop”) by some selected viral RSS proteins (tombusviral P19, ariusviral P14, potyviral HC-Pro and cucumoviral 2b). RISC, RNA induced silencing complex; DCL, Dicer-like protein; Ago, Argonaut protein; vRdRp, viral RNA-dependent RNA polymerase; hRdRp, host-encoded RNA-dependent RNA-polymerase; RSS, RNA silencing suppressor.

Other RNA silencing mechanisms

Next to the antiviral siRNA and the miRNA pathway, several other pathways have been found, either especially in the plant or in other organisms, like *C.elegans* and zebrafish.

In plants, the transacting siRNAs (ta-siRNA) and the natural siRNAs or cis-acting siRNAs are two additional endogenous siRNA molecules.

Transacting-siRNAs derive from TAS-gene transcripts, which are initially targeted by a miRNA molecule (Allen et al., 2005) and subsequently cleaved, followed by transformation of one of the resulting two strands into dsRNA by RDR6 and SGS3 (suppressor of gene silencing 3) and cleavage by DCL4 (Peragine et al., 2004, Yoshikawa et al., 2005). These RDR6-derived 21 nt ta-siRNAs guide the cleavage of their target RNA (Vazquez et al., 2004) in a complex with AGO1 (Baumberger & Baulcombe, 2005).

The natural siRNAs (nat-siRNAs) on the other hand derive from pairs of natural cis-antisense transcripts, cleaved by DCL2 into 24 nt nat-siRNAs. The resulting cleavage of their transcript and guide sequence can again generate, this time, 21 nt nat-siRNAs by DCL1. The nat-siRNAs are believed to be activated by abiotic or biotic stress and function as plant adaptive protection mechanism (Borsani et al., 2005, Katiyar-Agarwal & Jin, 2007).

Besides post transcriptional gene silencing, siRNAs also play a key role in transcriptional gene silencing (TGS). This process is active in the nucleus and involves heterochromatic siRNAs that often derive from repeat elements (24 nt repeat associated; ra-siRNA) like transposons or centromeric repeats and are dependent on DCL3, RDR2 (Chan et al., 2005) and most times DNA-dependent RNA polymerase IV (Pol IV) (Herr, 2005, Onodera et al., 2005). Incorporation of these in complex with AGO4 and other proteins, enables this complex to direct methyltransferases to homologous DNA loci, resulting in RNA-directed DNA methylation (RdDM) or histone modification (Kanno et al., 2005, Pontier et al., 2005). This activity, probably, mainly serves to protect the genome against damage caused by transposons (Xie et al., 2004, Zilberman et al., 2004).

Next to plants, endogenous siRNAs have been well characterized in *C. elegans*, shown to be dependent on the activity of the host encoded RdRp (Ruby et al., 2006, Sijen et al., 2007). Although lacking a RdRp, endogenous encoded siRNAs (endo-siRNAs) have recently been found in mouse (Tam et al., 2008, Watanabe et al., 2008b) and *Drosophila* somatic cells, (Chung et al., 2008, Czech et al., 2008, Ghildiyal et al., 2008, Kawamura et al., 2008, Okamura et al., 2008a, Okamura et al., 2008b), corresponding to transposons, structured cellular transcripts and overlapping transcripts. Most endo-siRNAs map to transposons, of which the long terminal repeat (LTR) retrotransposons are predominant compared to the non-LTR retrotransposons and DNA transposons. The endo-siRNAs distributed evenly throughout the entire transposon, lacking any hot-spot, demonstrated by mapping of the endo-siRNAs to the transposons (Tam et al., 2008, Watanabe et al., 2008a, Watanabe et al., 2008b, Yang & Kazazian, 2006). Experiments have determined Dicer-2 and AGO-2 as key components of the endo-siRNA pathway in *Drosophila* (Ghildiyal et al., 2008, Kawamura et al., 2008), as also observed for the antiviral siRNA pathway.

Most of the RNA silencing pathways are Dicer dependent, however in germlines Dicer independent small dsRNAs have been found, silencing selfish DNA elements (e.g. transposons) and maintaining the germline DNA integrity. These 24-30 nt small dsRNA molecules, referred to as PIWI interacting RNA (piRNA), silence transposons by directing DNA methylation and are associated with PIWI-class Argonaut proteins, only expressed in germline cells. Recent studies suggest that both endo-siRNAs and piRNA act in germline cells, with piRNAs being the predominant pathway (reviewed by Aravin & Hannon, 2008).

RNA silencing components

To get better insights into the dynamics and the preposition of the (key)-compounds of the different RNA silencing pathways, *in vitro* systems were developed. One of the first and most successful was the preparation and use of extracts from cultured *Drosophila* cells or their embryos. Only recently some crystal structures of some of the key-components with their target molecules (like Argonaut with a siRNA molecule or *Giardia* Dicer) have been described (Wang et al., 2008a, Wang et al., 2008b, Yuan et al., 2006).

Dicer and partners

The Dicer protein was first isolated from *Drosophila* and identified as a member of the Type III RNase (Bernstein et al., 2001, Hammond et al., 2000). RNase III-like enzymes can be divided into three classes (Figure 1-2) (Lamontagne et al., 2001), but all share a homologous ribonuclease domain (known as RNase III domain) and produce duplex RNA fragments with a characteristic terminal-end structure that consists of a 5'-phosphate group and two nucleotide overhang at the 3'-end (Ji, 2008, Zhang et al., 2004). Members of class 1 RNase only contain a single RNase III domain and a joined dsRNA-binding domain (dsRBD). Members of class 2 and 3, represented by Drosha and Dicer enzymes, respectively, contain two RNase III domains whereas most of the class 3 Dicers additionally contain a N-terminal RNA helicase domain and a PIWI/Argonaut/Zilli (PAZ) domain, features that are shared with the Argonaut proteins.

Class 1

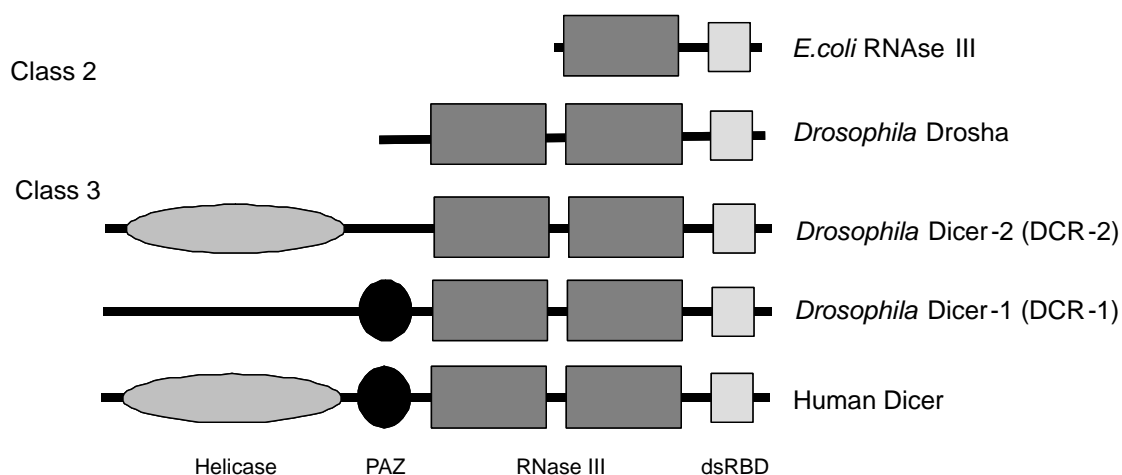


Figure 1-2: Schematic overview and domain structures of representatives of the three classes of RNase III like enzymes (Aliyari & Ding, 2009). DCR, Dicer.

Meanwhile, Dicer proteins or Dicer-like proteins (DCLs) have been identified in almost all eukaryotes, although large differences are observed in the levels of Dicer among organisms. Whereas *Drosophila* encodes Dicer-1 and Dicer-2, *Arabidopsis* encodes 4 different DCL proteins which are at least partly linked to the miRNA or (antiviral) siRNA pathway (Baulcombe, 2005, Lee et al., 2004c, Tomari & Zamore, 2005). In contrast, *C.elegans* and mammals only encode 1 Dicer which acts in the siRNA and the miRNA pathway.

Although Dicer theoretically cleaves any dsRNA molecule into small dsRNA, the recognition of various dsRNA substrates and size of produced molecules differs. In *Arabidopsis* for example, DCL-1 processes the primary miRNA (pri-miRNA) into miRNA/miRNA* duplexes. DCL-2, -3 and -4 produce siRNA molecules sized 22 nt, 24 nt and 21 nt, respectively (Henderson et al., 2006, Qi et al., 2005). The proposed model for *Giardia* Dicer in which its two RNase III domains form an intra-molecular dimer connected to the PAZ domain, which anchors the end of the dsRNA, explains this size specificity. The size of siRNA molecules produced is thereby determined by the distance between the RNase III domains and the PAZ domain (MacRae & Doudna, 2007, MacRae et al., 2007).

In invertebrates and vertebrates, the biogenesis of miRNA relies on another RNase III –like enzyme, called Drosha (Lee et al., 2003). The maturation of, for example insect miRNAs, occurs in a step wise manner, slightly different from the miRNA pathway in plants. In *Drosophila* precursor (pre)-miRNAs, resulting from Drosha cleavage of pri-miRNA (Han et al., 2004, Lee et al., 2004b), are exported from the nucleus to the cytoplasm by exportin 5 (Lund & Dahlberg, 2006). There, Dicer-1 cleavage into the 22 nts miRNA/miRNA* occurs (Kim, 2005, Lee et al., 2004c). While Dicer-1 requires ATP for its activity, Dicer 2 preferentially acts in an ATP-dependent manner (Jiang et al., 2005, Liu et al., 2003). Uploading of miRNA/miRNA* into RISC and subsequent target cleavage or translational inhibition occurs in a similar fashion as previously described for plants. Whereas Drosha requires a binding partner, for functional activity, called Pasha (DGCR8 in humans), a protein that contains tandem dsRBDs (Denli et al., 2004, Han et al., 2006, Landthaler et al., 2004), Dicer proteins also have binding partners containing tandem dsRBD, like Loquacious (Loq) in *Drosophila* (Forstemann et al., 2005), hyponastic leaves 1 (HYL-1) in *Arabidopsis* (Kurihara et al., 2006) and TAR RNA-binding protein (TRBP) in humans (Haase et al., 2005), but these are expendable for Dicer activity.

Argonaut proteins

The silencing induced complex incorporates all type of small dsRNA molecules to trigger sequence specific gene silencing. One of the key proteins in these complexes is Argonaut (AGO) that interacts with siRNAs, miRNAs and piRNAs (Hock & Meister, 2008). Like Dicer, AGO contains a PAZ domain that binds RNA in a sequence-independent manner and recognizes the ss siRNA at the 3'-end (Lingel & Izaurralde, 2004, Lingel et al., 2003). Next to

the PAZ domain, a MID domain binds the uploaded small RNA via its 5' - phosphate-group in concert with the C-terminal RNase H-like PIWI domain (Hutvagner & Simard, 2008). The guide RNA strand uploaded and bound by AGO allows the complex to be directed to ss RNA target sequences with complementarities to the RNA guide strand. Different silencing events may occur depending on the formed complex and degree of base pairing (Okamura et al., 2004). In case of extensive base pairing between target RNA and RNA guide strand and subject the incorporated AGO protein harbors slicer activity, target cleavage (slicing) occurs at the central position by the RNase H like activity of AGO. In *Drosophila* all AGO proteins contain slicer activity but AGO1 primarily binds miRNA/miRNA*s, being a weak slicer (Miyoshi et al., 2005, Rand et al., 2005). Among the four human AGO proteins, three do not exhibit slicer activity (Meister et al., 2004).

In case of partial complementarities of the RNA guide strand to its target, as observed for nearly all animal miRNAs, mostly translational inhibition is induced. The translationally arrested RNA targets sequester in the majority in cytoplasmic bodies, called Processing bodies (P-bodies), most probably induced by the interaction between the AGO protein and i.e. GW182 proteins. GW182 proteins interact with essential proteins of the translation machinery, like PABPC1, and are therefore thought to cause translational inhibition (reviewed by Eulalio et al., 2008).

Bound guide strands can also direct the silencing induced complex to nascent transcripts where subsequent DNA or histone modification leads to transcriptional gene silencing (TGS) (Baumberger & Baulcombe, 2005, Qi et al., 2006).

Most organisms encode more than one AGO protein and their interaction with different proteins leads to the formation of several RISC with distinct functions. An antiviral role of AGO proteins has first been reported in 2002 for AGO1 in *A.thaliana* and AGO2 in *Drosophila* (Li et al., 2002; Morel et al., 2002). Whereas in *A.thaliana* AGO1 is involved in the miRNA pathway, AGO1 mutants are also hypersensitive to CMV infections and CMV-specific siRNAs are found in a complex with AGO1 (Morel et al., 2002). Other plant AGO proteins involved in antiviral RNA silencing, have not been identified yet, most likely due to the possibility to replace each other (like the DCLs) and/or virus-specificity like in the case of CMV.

During a Flock house virus (FHV) infection of *Drosophila* viral specific siRNAs are mostly, if not all, loaded into AGO2 (van Rij et al. 2006; Aliyari et al. 2008). The observed rescue of a B2 (RNA silencing suppressor, RSS) deficient FHV infection in AGO2 silenced *Drosophila* supports the antiviral role of AGO2 (Li et al., 2002).

Is antiviral RNAi restricted to plants and insects?

After the ground-breaking work of Andrew Fire and Craig Mello (Fire et al., 1998) on their RNA silencing discovery in nematodes, increasing evidence indicated that RNA silencing is an ancient gene regulation mechanism occurring in almost every eukaryotic organism, from algae and plants to insects and humans (Sontheimer & Carthew, 2005, Tomari & Zamore, 2005, Voinnet, 2005a). The mechanism shared between all these organisms is the miRNA

pathway, whereas the antiviral siRNA pathway may be shared only among plants and invertebrates and probably not by mammals. For insects, the existence of a separate antiviral siRNA branch within RNA silencing has been well established, and insect-infecting viruses, in turn, have been shown to encode RNA silencing suppressor (RSS) proteins (e.g. protein B2 of FHV, (Li et al., 2002)) to combat this antiviral response. In infected mammalian cells, virus-derived siRNAs have so far not convincingly been detected in high concentrations (Parameswaran et al., 2010, Pfeffer et al., 2004), but antiviral RNA can be readily induced upon transfection with dsRNA (hairpin RNA) containing viral sequences (Haasnoot et al., 2007, Lopez-Fraga et al., 2008, Marques & Carthew, 2007). Whilst a separate antiviral siRNA branch may be absent in mammals there is increasing evidence that also mammalian e.g. human viruses encounter antiviral RNA silencing and this may exclusively occur through the miRNA pathway, strengthening the idea of human viruses encoding RSS proteins too (Berkhout & Jeang, 2007, Grassmann & Jeang, 2008, Murakami et al., 2009, Triboulet & Benkirane, 2007).

Viral suppressors of RNA silencing

During a compatible interaction between a virus and its host plant, infected plant tissues contain significant amounts of virus-derived siRNAs, indicating that the invading virus is actively targeted by the antiviral silencing machinery. Plant viruses would not exist if they had not generated an efficient strategy to counteract this antiviral RNA silencing. Indeed, they do so by encoding RSS proteins that are able to suppress RNA silencing (Table 1-1).

Table 1-1: List of identified RNA silencing suppressor (RSS) proteins, their activity to suppress the siRNA pathway and their other known functions

Virus sort	Virus genus	Virus	RNAi suppressor	Activity	Other function	Reference
+ ssRNA			<i>Plant-virus</i>			
	Aureusvirus	Pothos latent virus	P14	dsRNA binding (long & short)		1
	Carmoviruses	Turnip crinkle virus	p38 (CP)		Coat protein	2-4
	Cucumovirus	Cucumber mosaic virus	2b	Ago binding, dsRNA binding (siRNA)	Movement	5-9
	Closterovirus	Beet yellow virus, Citrus tristeza virus	P21, (P20, P23, CP)	dsRNA binding (siRNA)	Replication enhancer	2,10-12
	Comoviruses	Cowpea mosaic virus	S protein (small CP)		Coat protein	13
	Crinivirus	Sweet potato chlorotic stunt virus	P22 (RNase 3)	RNaseIII		14(Kreuze et al., 2005)
	Dianthovirus	Red clover necrotic mosaic virus	P88, p27	DCL interaction	Replication	15
	Hordeivirus	Barley Stripe mosaic virus	γ b (Cysteine-rich protein)	dsRNA binding (siRNA)	Replication enhancer, movement, virulence	2, 16
	Pecluvirus	Peanut clump virus	P15 (Cysteine-rich protein)	dsRNA binding (siRNA)	Movement	2
	Poleovirus	Beet western yellows virus,	P0	Ago1 (degrade Ago1, direct interaction)		17
	Potex virus	Potato virus X	P25		Helicase and Movement protein	18
	Potyvirus	Tabacco etch potyvirus,	P1/Hc-Pro	dsRNA binding (siRNA and miRNA), Preventing RISC assembly	Movement, polyprotein processing, aphid transmission	2, 5, 10, 19-21
	Sobemovirus	Rice yellow mottle virus	P1	dsRNA binding (siRNA)	Movement	22
	Tombus virus	Carnation italian ringspot virus	P19	dsRNA binding (siRNA and miRNA)		22-24
	Tobamovirus	Tobacco mosaic virus	P126 (part of replication complex)		Replication	22, 25
	Tobravirus	Tobacco rattle virus	16K	Cysteine rich protein		26
	Tymovirus	Turnip yellow mosaic virus	P69, P7b, P50 (MP)		Movement protein	27
	Vitivirus	Grapevine virus A	P10			28
- ssRNA	Tospovirus	Tomato spotted wilt virus	NSs	dsRNA binding		29, 30

Virus sort	Virus genus	Virus	RNAi suppressor	Activity	Other function	Reference
dsRNA	Tenuivirus	Rice Hoja blanca virus	NS3	dsRNA binding (siRNA&miRNA)		29, 30
	Phytoreovirus	Rice dwarf virus	Pns10			32
	Begomovirus	Tomato leaf curl virus	C2	DNA binding, NLS		22, 33, 34
DNA	Begomovirus	African cassava mosaic virus	AC4	miRNA binding	Transcriptional activator	35-37
	Curtovirus	Beet curly top virus	L2	Protein binding		38
<i>Vertebrates/ invertebrate virus</i>						
+ ssRNA	Nodavirus	Flock house virus & NoV	B2	dsRNA binding		39, 40
	Hepacivirus	Hepatitis C virus	Core protein			41
ss RNA	Lentivirus	HIV-1	tat		Transcription, inhibitor of PKR	42
	Spumavirus	PVF-1	Tas			43
- ss RNA	Ebolavirus	Ebola virus	VP35	dsRNA binding	Interferon antagonist (PKR)	44
	Orthomyxovirus	Influenza A virus	NS1	dsRNA binding	Interferon antagonist (PKR), Poly (A) binding, inhibitor of mRNA export	45-48
	Orthobunyavirus	La Crosse virus	NSs		Interferon antagonist	49, 50
dsRNA	Orthoreovirus		Σ3	dsRNA binding		51
dsDNA	Adenovirus	Adenovirus	VA1 RNAs	Dicer binding, exportin 5 saturation	Translation, PKR inhibitor	52, 53
	Poxvirus	Vaccinia virus	E3L	dsRNA binding	interferon inhibitor	47, 54

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Among the first viral RSS proteins identified was HC-Pro of potyviruses, a multifunctional protein involved in aphid-mediated virus transmission, genome amplification, polyprotein processing and viral long-distance movement (Anandalakshmi et al., 1998, Beclin et al., 1998, Brigneti et al., 1998, Kasschau & Carrington, 1998). This protein was already known as “virulence factor”, because of a causal linkage between HC-Pro expression and severity of disease symptoms (Atreya et al., 1992, Atreya & Pirone, 1993). The latter is now readily explained in view of its function to suppress the host’s antiviral RNA silencing. The RSS and proteolytic activity are independent, separable properties within HC-Pro, in contrast to genome amplification and long-distance movement functions, which seem to be related to the RSS activity (Kasschau & Carrington, 2001). At least part of these activities can be explained by the affinity of HC-Pro for siRNAs and its interference with their methylation, reducing siRNA stability (Ebhardt et al., 2005, Lakatos et al., 2006, Li et al., 2005). Transgenic suppressor assays in *Arabidopsis* or *Nicotiana* spp. Identified an increasing number of plant viral RSS proteins (Li & Ding, 2006, Roth et al., 2004). These include positive-, negative- and dsRNA viruses as well as the geminiviruses, which have an ssDNA genome. Most viruses encode only one RSS protein which acts on a single step in the siRNA pathway, resulting in partial suppression (Li & Ding, 2006). The situation for geminiviruses is more complicated though, as among their different viral species the RSS activity appears to reside in different proteins (Bisaro, 2006, Voinnet et al., 1999). The closterovirus citrus tristeza virus is also unique in coding for three proteins involved in RSS action (Lu et al., 2004, Satyanarayana et al., 2002).

RNA silencing is an ancient cellular mechanism shared by most living organisms (Diaz-Pendon & Ding, 2008, Li & Ding, 2006). It is obvious that viruses and antiviral RNA silencing will have co-evolved over a very long period, and hence viral RSS proteins are expected to form one or more clusters of similar proteins containing conserved sequence motifs. This is, surprisingly, not the case: viral RSS proteins are extremely variable among viruses and differ in their genomic position, molecular size and amino acid sequence. The best example is presented by the situation within the family *Tombusviridae* in which, depending on the species, the RSS function resides in the viral coat protein (CP), the viral polymerase, or in a separate viral protein (Figure 1-3, see also Merai et al., 2006, Merai et al., 2005, Takeda et al., 2005). The general picture that emerges after comparison of different plant viruses is that their encoded RSS activity is often part of a multifunctional protein.

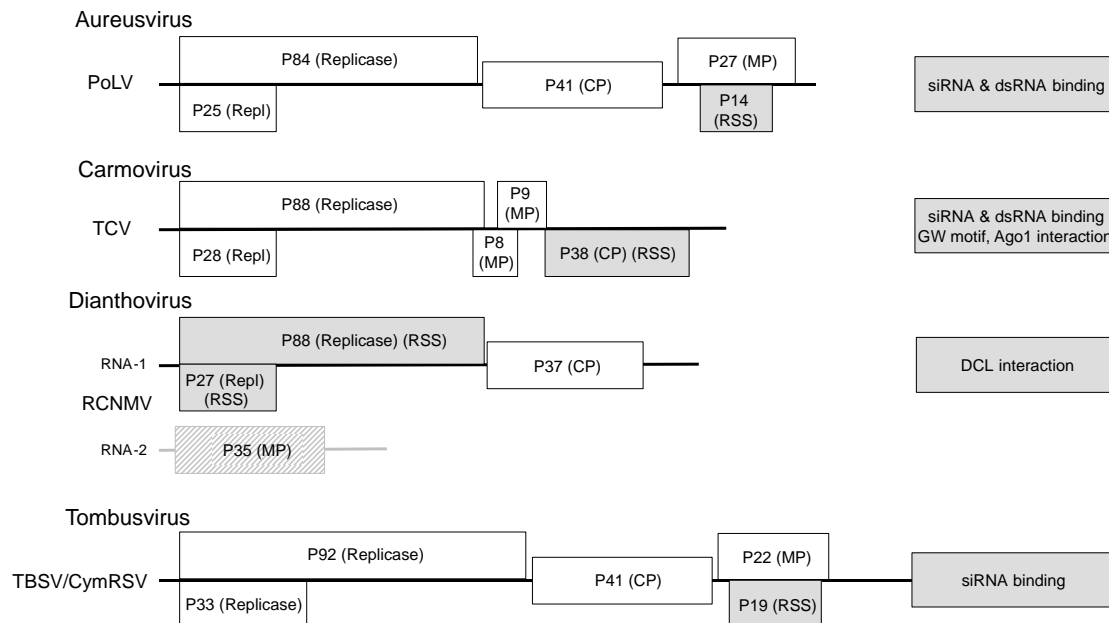


Figure 1-3: Schematic representation of the genome organization of 4 different species belonging to the *Tombusviridae*. Open reading frames (ORFs) are indicated as open bars. Grey ORFs represent the identified RNA silencing suppressor (RSS) proteins and list their additional functional activity. PoLV, Pothos latent virus (genus *Aureusvirus*); TCV, Turnip crinkle virus (genus *Carmovirus*); RCNMV, Red clover necrotic mosaic virus (genus *Dianthovirus*); CymRSV, Cymbidium ringspot virus (genus *Tombusvirus*); CP, coat protein; MP, movement protein; Repl, replicase; DCL, Dicer like protein. (Drawing after Takeda et al., 2005).

Viral RSS proteins not only have different motifs and sequences, also their mode of action differs. Some RSS proteins act by sequestering dsRNA molecules, either size-specifically like tombusviral P19 (exclusively binding siRNAs) or non-specifically like aureusviral P14 (also binding longer dsRNAs) (Lakatos et al., 2006, Merai et al., 2006, Merai et al., 2005). Others bind protein factors of the RNA silencing pathway, like cucumber mosaic virus (CMV) protein 2b. While most RSS proteins interfere with only a single step in the RNA silencing pathway some are able to block at different points, e.g. CMV protein 2b which is able to both sequester siRNAs and to interact with AGO within the RISC complex (Goto et al., 2007, Zhang et al., 2006).

RSS proteins encoded by different virus families often share no homology at the amino acid sequence level, even if they have a similar mode of action (Lakatos et al., 2006, Merai et al., 2006). So far not a single sequence motif characteristic for (a subclass of) RSS proteins has been identified. This is surprising as RNA silencing is generally regarded to be an ancient mechanism. One explanation for this could be that the long-lasting evolutionary interplay with the plant's antiviral RNA silencing mechanism has driven viruses to continuously change and adapt their suppressor protein sequences (and their coding sequences) to keep ahead of the host defense system. The RNA silencing's selective pressure would then act as a major evolutionary driving force resulting in extreme speciation. This would also explain the overwhelming excess of RNA virus species *versus* DNA virus species within the plant kingdom. A similar situation may have occurred in bacteria where the great majority of phages have DNA genomes, targets for the (DNA-based) restriction/modification system in bacteria.

Another explanation might be that RSS genes have been introduced into viral genomes through multiple independent evolutionary events. An argument in favor of this alternative is the observation that RSS genes often overlap with another viral gene, including in some cases the polymerase gene. In evolutionary terms it is believed that overlapping genes are created by overprinting, meaning that an existing coding sequence is translated in a different reading frame (Ding et al., 1995, Keese & Gibbs, 1992). According to this scenario, the lack of sequence homology between different RSS proteins would be explained by multiple independent introductions into viral genomes. A third explanation is the idea of convergence, in which viral proteins with different functions have independently evolved to encompass for RSS activity.

Next to plant viral encoded RSS proteins, insect viruses have also been found to encode RSS proteins (Table 1-1). The first one identified was the B2 protein of FHV (Li et al., 2002). This RSS acts as dimer by binding dsRNA, both long and short, and is able to inhibit Dicer cleavage *in vitro* (Chao et al., 2005, Lingel & Sattler, 2005). *Drosophila* infection with a B2-deficient FHV results in the generation of siRNAs that predominantly originate from both + and – strands in the 5'-terminal region of the RNA genome. The interaction of B2 with the viral replicase additionally supports the idea that B2 exerts its RSS activity by binding to viral dsRNA, produced as replicative intermediates, and avoids their cleavage by Dicer (Aliyari & Ding, 2009). Next to “true” insect viruses, like FHV, *Drosophila* C virus (DCV) and Cricket paralysis virus (CrPV), viruses infecting both insects and mammals encode RSS proteins too (Nayak et al., 2010, van Rij et al., 2006). The Nodamura virus (NoV), for example, encodes the B2 protein, which acts similar to the FHV B2 RSS protein in both insect and mammalian cells (Li et al., 2004, Sullivan & Ganem, 2005).

More recently, a number of RSS proteins have been identified in mammalian viruses, e.g. NS1 of Influenza A virus, VP35 of Ebola virus, Tat of Human immunodeficiency virus type 1 (HIV-1), Core and E2 of Hepatitis C virus (HCV) and the non-coding VA RNAs of adenovirus (Bennasser et al., 2005, Bucher et al., 2004, Haasnoot et al., 2007, Ji et al., 2008, Li et al., 2004, Lu & Cullen, 2004, Wang et al., 2006b). However, for most of them, their activity as RSS is still under debate, due to contradictory results, and their functional relevance being disputed (Kok & Jin, 2006, Lin & Cullen, 2007). Most of these RSS proteins encoded by mammalian infecting viruses are also known to antagonize the interferon pathway through their ability to bind long dsRNA, resulting in difficulties to discriminate between their interferon antagonistic function and possible RSS activity.

Possible interactions between viruses and the miRNA pathway

During a viral infection process the viral RSS protein also, besides the antiviral siRNA pathway, interferes with the miRNA pathway. Since both pathways share similar key molecules, nearly all tested RSS proteins with affinity to siRNA molecules equally bind miRNA/miRNA* duplexes *in vitro*, as shown for e.g. tombusviral P19 (Dunoyer et al., 2004, Silhavy et al., 2002). Transgenic *Arabidopsis* plants expressing viral RSS proteins, like

tombusviral P19, potyviral Hc-Pro and CMV 2b (Chapman et al., 2004, Dunoyer et al., 2004) show drastic effects on phenotype, reminiscent of virus disease symptoms, and support the idea of RSS interference on the miRNA pathway *in vivo*. However, transgenic flies constitutively expressing several RSS proteins did not show similar observations (Berry et al., 2009).

Whereas viral siRNAs from mammalian infecting viruses (Pfeffer et al., 2004) have so far only been detected in low concentrations for specific viruses (Parameswaran et al. 2010), and the functional relevance of RSS proteins for these viruses is debated (Lin & Cullen, 2007), mammalian-infecting DNA viruses replicating in the nucleus are shown to encode their own miRNAs. The first one reported is from the Epstein-Barr (EBV) gamma-herpes virus (Pfeffer et al., 2004), but meanwhile up to 23 have been identified for this virus and other viruses of the herpes family, a.o. simian polyomaviruses and human adenovirus (Cai & Cullen, 2006, Cai et al., 2006, Grundhoff et al., 2006). Probably, the miRNA biogenesis in mammals recognizes the viral nuclear transcripts from these viruses and process them. Although not much is known on the function of these miRNAs they are proposed to target and regulate viral gene expression and host gene expression, similar to the viral siRNAs of CaMV, another nuclear-replicating circular dsDNA plant virus (Shivaprasad et al., 2008).

Host encoded miRNAs may act antiviral by either directly targeting the viral genome/transcript or indirectly. Indirect antiviral activity could result in e.g. regulating the expression of host proteins essential for virus infection or antiviral host response. For several mammalian encoded miRNAs an antiviral effect has been demonstrated (reviewed by Umbach & Cullen, 2009). The possibility of mammalian viruses to counter defend this by a RSS remains to be investigated.

Viral immune evasion by membrane associated replication complexes

Next to RNA silencing suppression, viruses have also evolved other strategies to evade the antiviral innate immune system, i.e. RNA silencing. A common strategy found by all positive ss RNA viruses (mammalian, insect and plant infecting) is the replication in membrane associated vesicles. These membranes can arise from different host organelles, dependent on the investigated virus. Most RNA replication complexes have been associated with endoplasmatic reticulum membranes, as observed for picorna-, flavi-, arteri- and bromo-viruses. However, mitochondrial (nodavirus), endosomal and lysosomal (togavirus) as well as peroxisomal and chloroplastal (tombusvirus) membranes are used, too. For some viruses, specific non-structural proteins have been identified, targeting the RNA replication complex to the membranes of specific organelles, acting either as polytopic or monotopically integral by their hydrophobic domains. Some of these proteins induce membrane invaginations or vesicles in transient expression systems, even in the absence of viral replication or other viral proteins. Replicating in membrane associated vesicles does not only create a protective environment for the viral RNA molecules against host defense mechanism, but also results in a more efficient replication by increasing the local concentration of necessary viral and/or host

proteins (reviewed by Salonen et al., 2005). Double stranded RNA viruses, like rotavirus, use a similar strategy. Their transcription occurs in the double-layered viral particle and synthesis of new negative strand RNA molecules takes place during entering of synthesized plus strand RNA molecules into the newly assembled virion core (Carter, 2009). Negative strand RNA viruses on the other hand are believed to protect their RNA by packaging into ribonucleoprotein (RNP) complexes (Weber et al., 2006).

RNA silencing suppressor proteins of insect transmitted negative strand RNA plant viruses

Plant viruses also replicating in their insect vectors, such as the rhabdo-, tospo- and tenuiviruses (Falk & Tsai, 1998, Jackson et al., 2005, Wijkamp et al., 1993), need to counteract RNA silencing in two very distinct types of organisms. It is to be expected that this may be achieved by specifying an RSS protein which blocks a step in the RNA silencing mechanism that is conserved between plants and insects.

The Rice hoja blanca virus (RHBV) belongs to the genus *tenuivirus* infects rice and is transmitted by leaf hoppers (Ramirez et al., 1993, Ramirez et al., 1992). Its genome consists of 4 RNA segments (Figure 1-4) (Ramirez et al., 1992). Whereas RNA1 is of entire negative polarity and encodes the RNA dependent RNA polymerase (RdRp). RNA2 to 4 are ambisense and each contains 2 non-overlapping ORFs on opposite strands (Ramirez et al., 1992). RNA 2 codes for a nonstructural (NS2) protein of unknown function on the viral strand and on the viral complementary strand for a large protein (vcNS2) with similarities to the glycoprotein precursor gene of Uukuniemi phlebovirus (family *Bunyaviridae*) (De Miranda et al., 1996, Estabrook et al., 1996). RNA 3 codes for the nucleocapsid protein (N) and a non-structural (NS3) protein (de Miranda et al., 1994). RNA4 encodes two nonstructural proteins (vcNS4 and NS4) (Ramirez et al., 1993) of which vcNS4 has recently been demonstrated as viral cell-to-cell movement protein in Rice stripe virus (Xiong et al., 2009).

Tomato spotted wilt virus (TSWV) is the type species of the *Tospovirus* genus within the arthropod-born *Bunyaviridae*, a family of primarily animal-infecting viruses. Tospoviruses have a broad host range of more than 1000 different plant species worldwide, including many economically important agricultural crops. They are transmitted by thrips (*Thysanoptera*: family *Thripidae*) in a propagative manner, with *Frankliniella occidentalis* Pergande (Western flower thrips) and *Thrips tabaci* (onion thrips) as main vectors (Falk & Tsai, 1998, Wijkamp et al., 1993).

It has a tripartite genome organization, which is similar to RHBV (Figure 1-4) with an ambisense nature. The L RNA is of entire negative polarity and encodes the RdRp (de Haan et al., 1991). The Glycoproteins (Gn and Gc) and movement protein (NSm) are encoded on the ambisense M RNA (Kormelink et al., 1992, Storms et al., 1995). The S RNA contains of the nucleocapsid ORF (N) and the NSs ORF (de Haan et al., 1990).

Tenuiviruses are classified in a floating genus, and although membrane bound virus particles have never been observed for these viruses, they share many structural (genomic and

protein) similarities to members of the *Bunyaviridae* (Espinoza et al., 1993, Ramirez & Haenni, 1994). For TSWV tospovirus and RHBV tenuivirus, this has supported in the identification of a RSS gene at analogous positions in the viral genomes of TSWV and RHBV, i.e. the 52.5 kDa NSs and the 23 kDa NS3 respectively (Bucher et al., 2003). The abundant presence of GFP specific siRNAs during suppression of GFP silencing with RHBV NS3 versus a lack of these during suppression with TSWV NSs (Bucher et al., 2003), though, supported the idea that both RSS proteins exhibit a different mode of action.

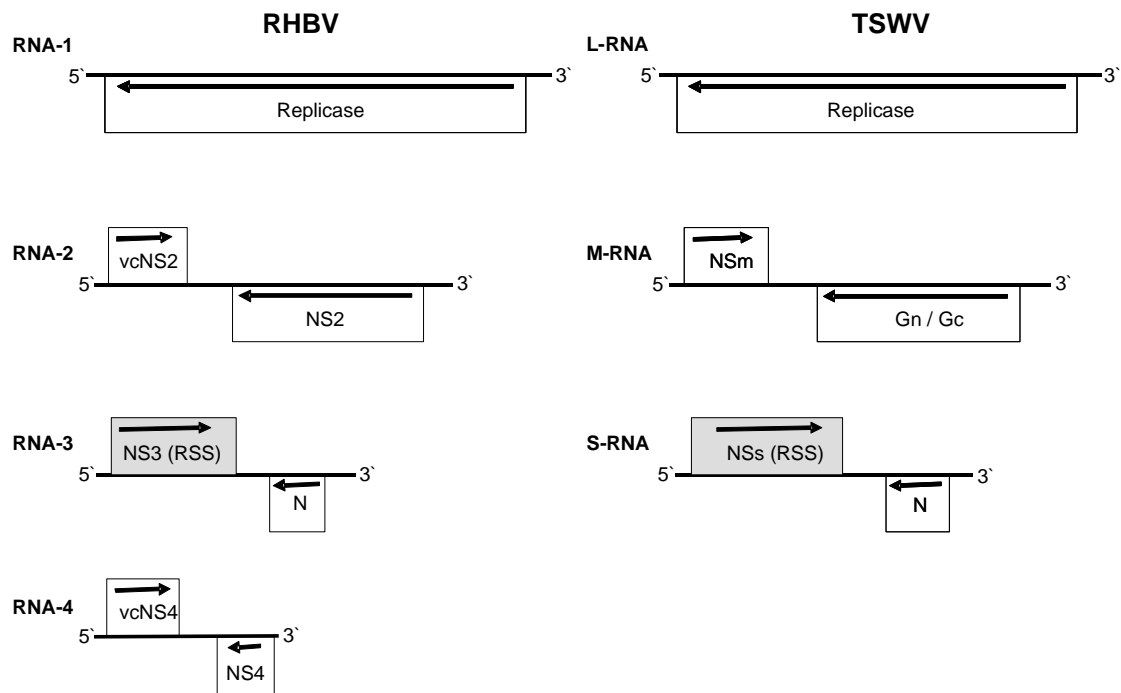


Figure 1-4: Schematic representation of the Rice hoja blanca virus (RHBV) and tomato spotted wilt virus (TSWV) genome. The RHBV RNA genome consists of a negative-stranded RNA 1 and 3 ambisense genome segments (RNA2-4). The TSWV RNA genome is tripartite with a negative-stranded L-RNA and ambisense M- and S-RNA. The RNA silencing suppressor (RSS) proteins, NS3 of RHBV and NSs of TSWV are indicated as grey boxes. NS, non-structural protein; vc, viral complementary; RSS, RNA silencing suppressor; N, nucleocapsid protein; NSm, movement protein; Gn/Gc, Glycoproteins.

Outline of the thesis

At the onset of this thesis, only limited information about the interaction between RSS proteins and different silencing pathways was available. For RHBV NS3, biochemical analysis had revealed a high affinity for siRNA and miRNA/miRNA* molecules, which implied that NS3 could possibly interfere on two distinct RNA silencing pathways. Next to the RSS activity in plants, NS3 was also shown to have similar activities in insect cell reporter-based assays (Hemmes et al., 2007). Hardly any information was available on the mode of action of TSWV NSs at the start of this thesis. The reported results, showed RSS activity of TSWV NSs in plants and insect cells (Bucher et al., 2003, Garcia et al., 2006, Takeda et al., 2002).

The focus of this thesis research was to further analyze if and how NS3 and tospovirus NSs would interfere in different RNA silencing pathways and if this was limited to plant and insect cell systems. **Chapter 2** describes an alanine scanning mutagenesis resulting in the identification of amino acids important for RSS activity of NS3 and their importance on the reported siRNA affinity. Mutants having lost their RSS activity in plants were further investigated for their affinity to siRNAs. Small interfering RNA molecules are key molecules of the RNA silencing pathway and thereby conserved in all eukaryotes. Therefore, **chapter 3** shows the functionality of NS3 as RSS protein in non-host mammalian cells. The importance of siRNA binding in this was confirmed by testing a NS3 mutant from chapter 2, which lacked RSS activity in plants and the affinity for siRNAs. Mammals trigger an interferon pathway in response to viral infections. On the other hand they contain a functional RNA silencing pathway and at least some mammalian viruses encode a protein with RSS activity. In **chapter 4**, it was investigated whether the production of a mammalian-infecting virus, HIV-1, is indeed being inhibited by RNA silencing and if the proposed RSS protein, HIV-1 Tat, is needed to counteract this response. A Tat-negative HIV-1 mutant was trans-complemented with NS3, resulting in virus titers compared to those from wildtype HIV-1. To rule out NS3 interference on the interferon pathway its antagonistic properties in an interferon-induced reporter assay was analyzed. NS3 was previously shown to additionally bind miRNA/miRNA* duplexes *in vitro* and a possible antiviral activity of the miRNA pathway, at least in mammals, has been proposed. Therefore the interference of NS3 on other small dsRNA silencing pathways with structural similarities to siRNAs, in specific the miRNA and endo-siRNA pathways, was investigated in mammalian cells (**Chapter 4**), plants and insects (**Chapter 5**). Transient transfection and reporter-based assays were used for this investigation. **Chapter 6** describes experiments to investigate the mode of action of the TSWV NS3 analogue, NSs and those from several other tospoviruses. Their affinity to different dsRNA molecules, *in vitro* and their silencing suppressor activity on e.g. Dicer cleavage and miRNA-based reporter assays was determined. The results are discussed in light of evolutionary divergence and genetic relationship between the plant infecting Tospovirus and the animal-infecting species belonging also to the *Bunyaviridae*.

Finally, **chapter 7** is a general discussion linking and evaluating the results of the experimental chapters to literature data regarding the current view on RSS proteins on different RNA silencing pathways. The potential effects during a natural viral infection are assessed and evolutionary events resulting in different mode of actions of rather related RSS proteins are highlighted.

Chapter 2

Binding of siRNA molecules is crucial for RNA silencing suppressor activity of Rice hoja blanca virus NS3 in plants

Abstract

The NS3 protein of Rice hoja blanca tenuivirus represents a viral suppressor of RNA silencing that sequesters small interfering (si)RNAs *in vitro*. To determine whether this siRNA binding property is the critical determinant for the suppressor activity of NS3, an alanine substitution analysis was performed and the resulting mutant proteins were tested for both siRNA binding ability and RNA silencing suppressor activity in plants. Alanine substitutions of lysine residues at position 173 - 175 resulted in mutant proteins that lost both their affinity for siRNAs and their RNA silencing suppressor activity *in planta*. This indicates that siRNA binding of NS3 is indeed essential for the suppressor function of NS3 and that residues at position 173-175 are involved in the siRNA binding and suppressor activities.

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Introduction

As a response to antiviral RNA silencing, plant viruses encode antagonistic proteins, often referred to as RSS proteins, which counteract or evade this host defense mechanism (reviewed by Ding & Voinnet, 2007, Voinnet, 2005a). A typical suppressor action adopted by plant viruses is the binding to dsRNA molecules, either in a size specific manner to 21-26 nt siRNA or in a non-size specific manner (Lakatos et al., 2006, Merai et al., 2006). The 21 nt siRNA molecules play important roles in different RNA silencing pathways (Brodersen & Voinnet, 2006, Vaucheret, 2006). One strand of the siRNA duplex is incorporated in RISC to guide the sequence specific recognition of complementary targets, resulting in cleavage (Tomari & Zamore, 2005). By sequestering siRNAs, a considerable number of viral RSS remove these molecules from the RNA silencing pathway, thereby preventing RISC assembly (Lakatos et al., 2006) and probably the systemic silencing signal (Dunoyer et al. 2010).

RHBV is a member of the floating genus *Tenuivirus*, that infects rice and is propagatively transmitted by an insect vector, the plant hopper *Tagosodes orizicolus* (Ramirez et al., 1993, Ramirez et al., 1992). The virus has an ambisense RNA genome which is divided in 4 segments (Figure 1-4). As RHBV replication takes place in both plants and the insect vector (Falk & Tsai, 1998), a relatively unique feature among plant viruses, it likely induces antiviral RNA silencing in both.

The RSS protein (NS3) of RHBV (Bucher et al., 2003) inhibits antiviral RNA silencing in both plant and insect cells and efficiently binds 21 nt siRNA *in vitro* (Hemmes et al., 2007). As siRNAs are key molecules of the antiviral RNA silencing pathway in plants and insects, it is likely that binding siRNAs *in vivo* is the crucial biochemical activity of this protein to suppress RNA silencing in these different organisms. If this would be the case, loss of RSS activity should coincide with loss of binding affinity to siRNA molecules. To test this, an alanine point mutagenesis approach within the NS3 sequence was performed and the resulting mutant proteins were monitored for RSS activity in plants and siRNA binding capacity *in vitro*.

Results

Identification of RNA binding domains in the NS3 protein

A sequence alignment, using the computer program ClustalX, of the available tenuiviral NS3 proteins (Rice stripe virus (RSV), Maize stripe virus (MStV), RHBV, Echinochloa hoja blanca virus (EHBV), Urochloa hoja blanca virus (UHBV), Rice grassy stunt virus (RGSV)) and a prediction of RNA-binding residues of RHBV NS3 was made, using the online prediction tool BindN, to identify regions or sequence motifs potentially important for RNA binding (Figure 2-1).

To check the usability of the RNA binding residue prediction by BindN, known RNA binding RSS proteins, like DCV 1A, were analyzed too. Since the program well predicted the experimentally identified residues essential for RNA binding in DCV 1A (data not shown, Rij et al., 2007), the predictions for NS3 were used for mutational analysis of NS3.

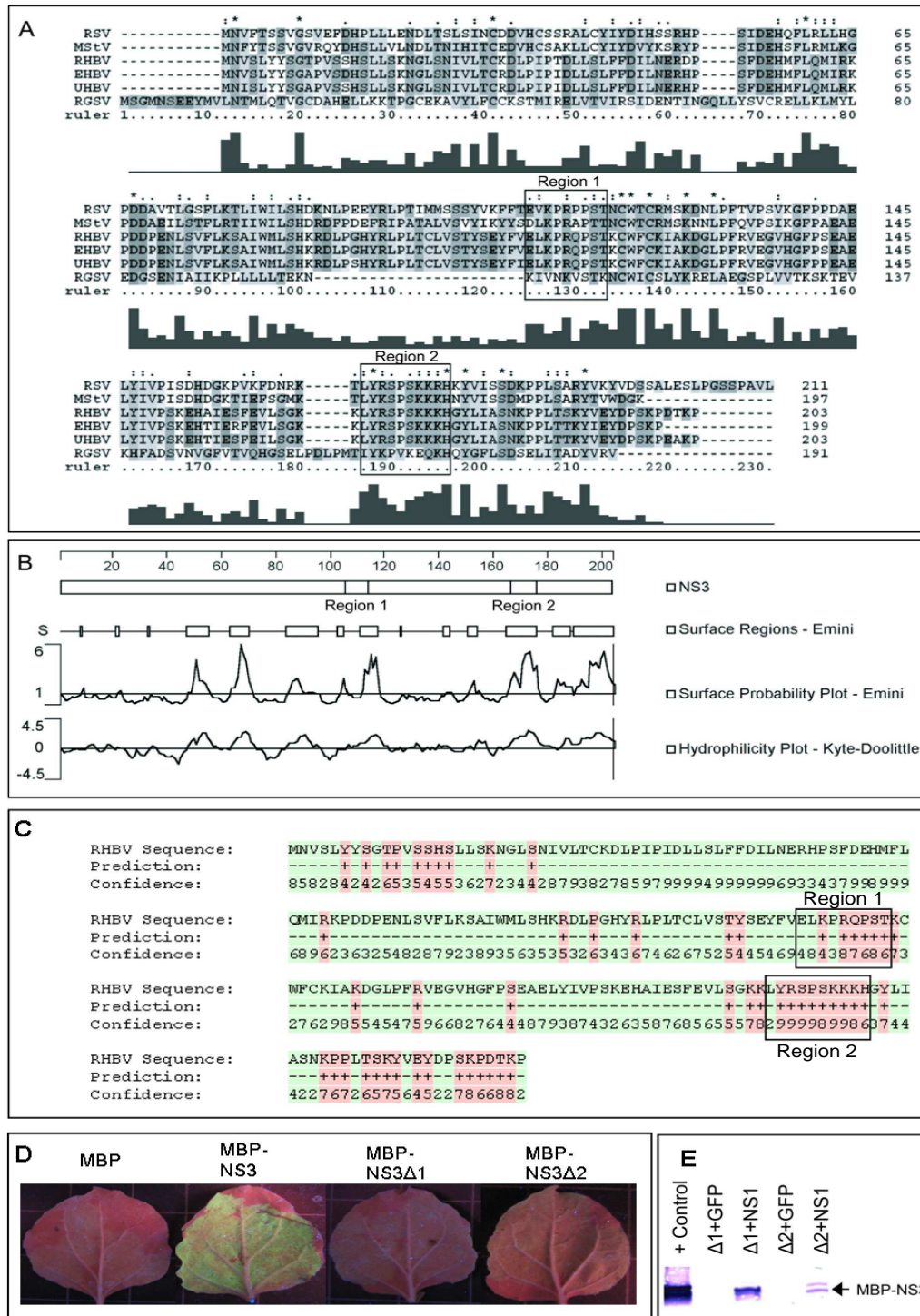


Figure 2-1: Analysis of the tenuiviral NS3 proteins. (A) Sequence alignment of six NS3-orthologs from rice stripe virus (RSV; NS3), maize stripe virus (MStV; p3), rice hoja blanca virus (RHBV; NS3), echinocloa hoja blanca virus (EHBV; pv3), urochloa hoja blanca virus (UHBV; pv3) and rice grassy stunt virus (RGSV; p5), using the ClustalX computer program. Amino acid identity: identical amino acids, conserved substitutions and semi-conserved substitutions are indicated by asterisk (*), double dots (:) and dots (.), respectively. (B) Surface probability plot and hydrophilicity plot of the RHBV NS3 protein, determined with the Protean (DNASTAR) computer program. (C) Prediction of RNA-binding residues in the RHBV NS3 sequence, using the BindN program. Predicted binding residues are labeled in red with "+" and non-binding residues are labeled in green with "-". The confidence of the prediction ranges from low (0) to high (9). (D) RNA silencing suppression assay on *N. benthamiana* leaves. GFP expression was visualized 5 days post-infiltration, in leaves co-infiltrated with *Agrobacterium tumefaciens* harboring binary vectors encoding mGFP and NS3, MBP, MBP-NS3, MBP-NS3Δ1 (Δ1) or MBP-NS3Δ2 (Δ2), respectively. (E) Detection of MBP-NS3Δ1 and MBP-NS3Δ2 expression by western blot analysis, using a MBP specific antibody, in leaf samples co-infiltrated with the NS1 protein of influenza A virus. As positive control bacterial purified MBP-NS3 was used. MBP, maltose binding protein; NS, non-structural; mGFP, modified green fluorescent protein.

A common feature of amino acids interacting with RNA molecules in a broad range of proteins is that they are polar and positively charged (Haasnoot et al., 2007, Hartman et al., 2004, van Rij et al., 2006, Wang et al., 1999). Several conserved areas were found in the alignment of which only a few matched with the requirement to be conserved, surface exposed, containing positively charged residues (K, R, H) (Figure 2-1 A and B) and reaching a high score in RNA binding prediction (Figure 2-1C). One of these regions (residues 106-114; region 1) located centrally in the protein, the other (residues 167-176; region 2) near the C-terminus. Region 2 contained a marked cluster of three positively charged lysine (at positions 173-175).

Single alanine substitutions in region 1 and 2 do not effect RSS activity

To further fine map essential amino acids within region 1 and 2 required for RSS activity single alanine substitutions were made. Based on surface probability, conservation and positive charge, five single amino acid substitutions were made in region 1 (E110A, L111A, K112A, P113A and R114A) and three in region 2 (K173A, K174A and K175A) using standard PCR technology. Single point mutations at positions 111 and 114 (L111A and R114A, respectively) repeatedly failed to yield a stable mutant, hence in total six single alanine mutants were tested for loss of RSS activity and siRNA binding. Five days post infiltration wild type levels of GFP were scored for all mutants in three independent repetitions, indicating that no single residue in region 1 or 2 was critical for RSS activity *in planta* (Figure 2-2A, upper panel). Northern blot analysis showing increased GFP specific mRNA underscored their suppression activity (Figure 2-2A, central panel).

GFP specific siRNAs, extracted and enriched as described previously (Bucher et al., 2003), were present in each sample and taking the loading control into account, no significant differences in siRNA concentration were detected compared to the positive control (wt NS3) (Figure 2-2A, lower panel).

In line with these results, all single alanine substitution mutants retained a high affinity for siRNA molecules, represented by a low dissociation constant (K_d): E110A, 133 ± 9.2 nM; K112A, 2.8 ± 0.3 nM; P113A, 136 ± 8.3 nM; K173A, 7.7 ± 2.0 nM; K174A, 12.4 ± 3.2 nM; K175A, 10.0 ± 2.1 nM (calculated from three independent electrophoretic mobility shift assays (EMSA, data not shown)). As controls MBP-NS3 (K_d 4.7 ± 0.5 nM; see Figure 3A) and MBP were used. MBP showed no binding to siRNAs at all tested concentrations (data not shown).

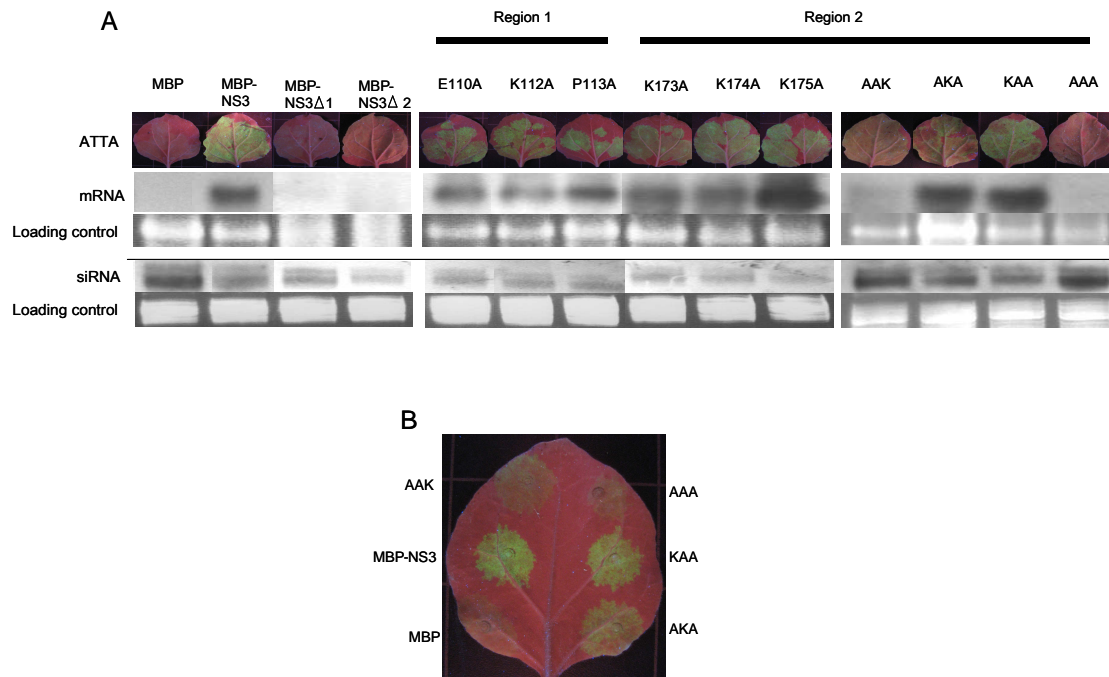


Figure 2-2: Testing alanine substitution mutants of Rice hoja blanca virus NS3 for demonstrating RNA silencing suppressor activity in *N. benthamiana*. (A) *Agrobacterium* harboring vectors encoding mGFP were co-infiltrated in *N. benthamiana* leaves with MBP (negative control), wt NS3 or one of the NS3 mutated constructs, respectively. Upper panel: GFP expression in infiltrated leaves 5 days post-infiltration. The corresponding GFP mRNA and siRNAs levels were detected by northern blot analysis of the total RNA, using a DIG-labeled DNA probe and are presented in the middle and lower panel respectively. As loading control, ethidium bromide-stained RNA was used. KAA, K173/A174/A175, AKA, A173/K174/A175, AAK, A173/A174/K175, AAA, A173/A174/A175. (B) For better comparison the various NS3 constructs and controls were co-infiltrated in different patches within a single leaf and GFP expression visualized by UV 5 days post infiltration. MBP, maltose binding protein; NS, non-structural; mGFP, modified green fluorescent protein; wt, wild type; siRNA, small interfering RNA; mRNA, messenger RNA.

Triple lysines in region 2 are important for siRNA binding and RSS activity of NS3

As single mutations proved insufficient to destroy the RNA binding ability of NS3, as also reported in other cases (Wang et al., 1999) and clustered basic amino acids have previously been shown to be important for the suppressor activity of Influenza A virus NS1, tombusvirus P19 protein and DCV 1A (Bucher et al., 2004, Chu et al., 2000, Haasnoot et al., 2007, van Rij et al., 2006, Vargason et al., 2003, Wang et al., 1999), double and triple alanine substitutions were considered. NS3 encompasses a cluster of positively charged residues in region 2, i.e. 3 conserved lysines on positions 173-175 (Figure 2-1A) with high similarity to DCV 1A protein. The latter was shown to simultaneously lose its RSS activity and affinity to long dsRNA after a double mutation in the cluster of basic residues (K73A/K74A) (van Rij et al., 2006). For this reason, double and triple alanine substitutions were introduced into region 2 of NS3 by PCR, resulting in four possible mutant proteins (A173/A174/K175; AAK, K173/A174/A175A; KAA, A173/K174/A175; AKA, A173/A174/A175; AAA). Testing these mutants for RSS activity in *N. benthamiana* leaves, revealed that double mutant AAK and triple mutant AAA, but not the two other double mutants (KAA and AKA), were defective in RSS, as revealed by lack of GFP expression (Figure 2-2A upper panel and Figure 2-2B) and absence of GFP mRNA (Figure 2-2A central panel).

Regarding siRNA binding ability, mutants KAA and AKA showed a K_d in the order of wild type protein (56.9 ± 8.3 nM and 10.8 ± 1.2 nM, respectively; Figure 2-3B, C and F) while mutants AAK and AAA completely lost their siRNA binding capacity ($K_d \gg 750$ nM, Figure 2-3D, E and F).

Discussion

In all, the presented data unequivocally demonstrate that siRNA binding capacity, which can be disrupted with double and triple mutations in a triple lysine motif in region 2, is essential for NS3 RSS activity in plants. Prediction of the secondary structure, using the online tool PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>), revealed only a small and similar change for all four mutants (data not shown). Hence, it is very likely that the triple K motif at positions 173 - 175 is specifically involved in siRNA binding, rather than that gross alterations in the NS3 folding would have crippled the protein upon the amino acid substitutions. Since for other dsRNA-binding viral RSS proteins clustered positively charged amino acids have been found to be important for their activity (Bucher et al., 2004, Chu et al., 2000, Haasnoot et al., 2007, van Rij et al., 2006, Wang et al., 1999), it is tempting to assume that the triple K motif represents a key part of the siRNA binding domain. Solving the crystal structure of the NS3 protein may confirm this notion.

Single alanine substitutions of the investigated residues, either in region 1 or 2, showed no effect on RSS activity in the infiltrated *N. benthamiana* leaves and the binding affinity for siRNA did not show any drastic decrease by these alterations either. This is consistent with observations for other RSS proteins, known to act through dsRNA binding, including NS1 of Influenza A virus (Bucher et al., 2004, Wang et al., 1999), VP35 of Ebola virus (Haasnoot et al., 2007) and DCV 1A (van Rij et al., 2006), which all required at least a double mutation to remove their RSS activity.

It has to be noticed that, based on the results of the EMSA, the K_d for siRNAs of NS3 proteins with single alanine substitutions or AKA and KAA ranged in the order of 3-130 nM, which is higher than that of wild type NS3 (4.7 ± 0.5 nM). However, even a protein with a K_d of 130 nM is considered to have a significant affinity for the target molecule. These results, together with the fact that these mutants still significantly suppress RNA silencing in plants, may indicate that the K_d of plant pre-RISC (or RISC loading) complexes for siRNAs is higher than 130 nM. Alternatively, in the chosen experimental setup the NS3 mutant proteins may have accumulated to high levels compared to RISC (loading), making proteins with a relatively low affinity sufficiently strong RSSs. In this context, it would be interesting to see how these mutations behave in the context of a virus background in natural infections, but at present this is not possible due to the lack of a reverse genetics system for tenuiviruses. The mutational analysis has also revealed at least one additional region (denoted region 1, Fig.2-1) that may be involved in the functionality of NS3. Deletion of this region also caused loss of suppressor activity *in planta*.

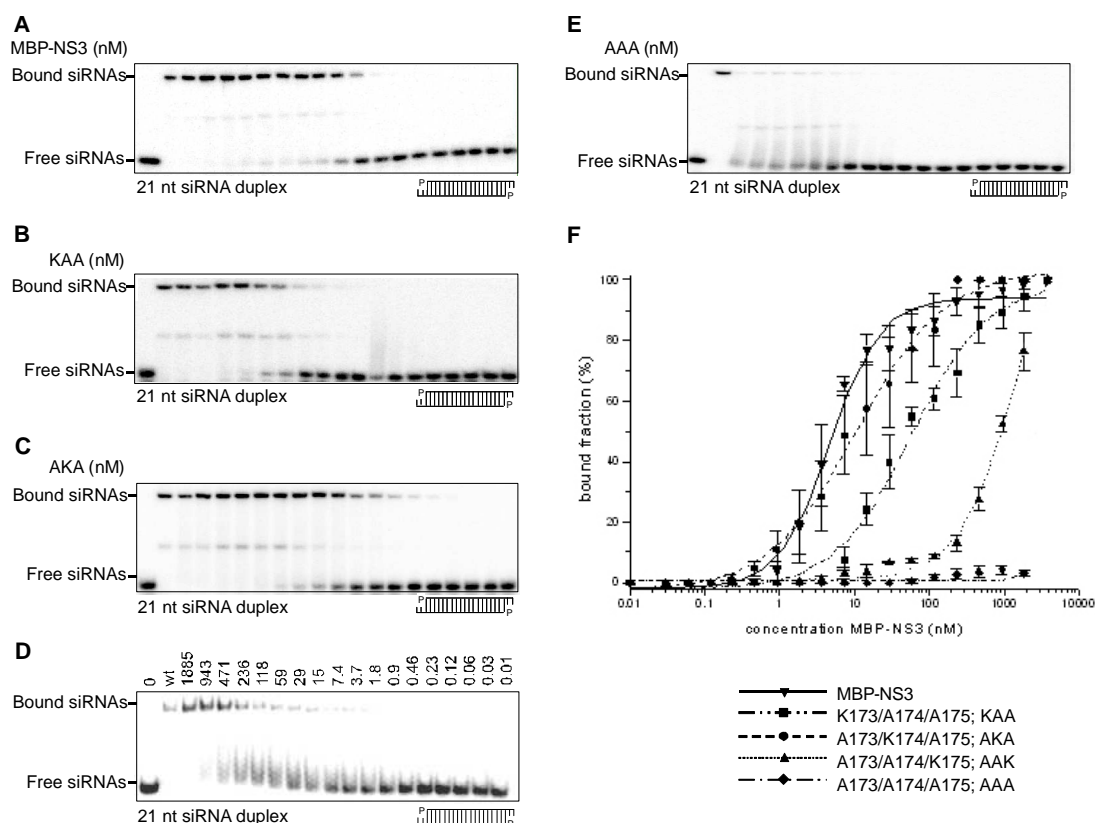


Figure 2-3: Affinity of MBP-NS3 mutants for siRNA duplexes.

A dilution series (0.01-3770 nM) of bacterial expressed and purified N-terminally fused NS3 mutated protein, K173/A174/A175; KAA (B), A173/K174/A175; AKA (C), A173/A174/K175; AAK (D) or A173/A174/A175; AAA (E), was incubated with 100 pM of 32 P-labelled 21 nt siRNA duplexes in binding buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM DTT, 2.5 mM $MgCl_2$, 10% (vol/vol) glycerol) for 20 minutes, then resolved onto a 5% native acrylamide gel. The gel was exposed overnight to a phosphor screen and scanned by a Molecular Dynamics Typhoon Phosphor imager (Amersham Biosciences). Bands were quantified using Genius Image Analyser software (Syngene). As control wild type MBP-NS3 was used (A). The K_d was determined for the MBP-NS3 (mutant) proteins by plotting the bound RNA fraction as function of the MBP-NS3 (mutant) concentration. The K_d of the different MBP-NS3 (mutant) proteins represents the protein concentration where 50% of the siRNA was bound (F). MBP, maltose binding protein; NS, non-structural; K_d , dissociation constant.

Several RSS proteins that act by binding to dsRNA molecules, like tombusvirus P19 (Lakatos et al., 2004), Beet yellows virus P21 (Ye & Patel, 2005), FHV B2 (Lingel et al., 2005) or NoV B2 (Korber et al., 2009), have been shown to dimerise or even oligomerise. The NS3 ortholog p5 of RGSV has been shown to homodimerise through the N-terminal domain (Chomchan et al., 2003). Another NS3 ortholog of Rice stripe tenuivirus has recently been identified as RSS protein in plants (Xiong et al., 2009) and observed to oligomerize in infected plants and insect cells (Takahashi et al., 2003). By gel-filtration analysis the RHBV NS3 has been observed to bind siRNA molecules as dimer (Hemmes et al., 2007). Based on these observations, it is tempting to speculate that region 1 could be required for oligomerization. However, the identification of other conserved amino acids and regions within NS3 predicted with high surface probability does not rule out the involvement of more residues for RSS activity and dimer formation. To give a final answer, the crystal structure of NS3 has to be solved. However, attempts (in collaboration with the University of Frankfurt) to purify large amounts of

NS3 for this purpose, so far has failed due to instability and insolubility of bacterial produced (fusion-) NS3 proteins.

As previously mentioned, siRNAs are not only key molecules of the RNA silencing pathway in plants and insects, but in a variety of other organisms as well. If siRNA binding is the crucial biochemical activity of NS3, it would be expected to act as RSS protein even in non-host organisms with a functional RNA silencing pathway. Whether NS3 would exhibit RSS activity in e.g. animal cells, remains to be investigated.

Materials and methods

Plasmid constructs

The coding sequence of RHBV NS3 was amplified by PCR and cloned into the pQE30 vector (Qiagen) as *Bam*HI-*Kpn*I fragment. To create the bacterial expression vector, NS3 was cut out of the pQE30 by *Bam*HI and *Pst*I and cloned in frame with the MBP coding sequence into the pMal-c2x vector (NEB).

PCR reactions were used to introduce deletions or alanine substitutions (single or multiple) in NS3, either in region 1 (amino acids 106-114) or region 2 (amino acids 167-176). To this end, a region specific primer (region 1 or region 2) was combined with a primer harboring the specific mutation (Table 2-1). The NS3 mutants obtained were verified by sequencing analysis for the presence of alanine substitutions.

Table 2-1: Primers and templates used for construction of mutated versions of the RHBV NS3.

Region 1 (amino acids 106-114)			Template
	Region specific primer	5`cag cca tca aca aaa tgc tg 3`	MBP-NS3 wildtype
1	Δ1	5`act ata tgt tga aac caa aca agt cag agg 3`	MBP-NS3 wildtype
2	E110A	5`ccg agg ctt tag tgc aac aaa ata ctc 3`	MBP-NS3 wildtype
3	L111A	5`ccg agg ctt tgc ttc aac aaa ata 3`	MBP-NS3 wildtype
4	K112A	5`ccg agg cgc tag ttc aac aaa ata 3`	MBP-NS3 wildtype
5	P113A	5`ccg agc ctt tag ttc aac aaa ata 3`	MBP-NS3 wildtype
Region 2 (amino acids 167-176)			Template
	Region specific primer	5`gga tat ttg atg gct tca aat aag 3`	MBP-NS3 wildtype
6	Δ2	5`ctt ctt ccc gct gag cac ttc aaa g 3`	MBP-NS3 wildtype
7	K173A	5`atg ttt ctt cgc gct agg aga tct gta taa 3`	MBP-NS3 wildtype
8	K174A	5`atg ttt cgc ctt gct agg aga tct gta taa 3`	MBP-NS3 wildtype
9	K175A	5`atg tgc ctt ctt gct agg aga tct gta taa 3`	MBP-NS3 wildtype
10	K173/A174/A175 (KAA)	5`atg tgc cgc ctt gct agg aga tct gta taa 3`	MBP-NS3 K175A
11	A173/K174/A175 (AKA)	5`atg tgc ctt cgc gct agg aga tct gta taa 3`	MBP-NS3 K175A
12	A173/A174/K175 (AAK)	5`atg ttt cgc cgc gct agg aga tct gta taa 3`	MBP-NS3 K174A
13	A173/A174/A175 (AAA)	5`atg tgc cgc cgc gct agg aga tct gta taa 3`	MBP-NS3 K174A

Codons for alanine are indicated in bold.

Binary vectors containing the NS3 gene and its mutant derivatives were generated by gateway cloning following the manufacturers' protocol. The attB recombination sides were introduced by PCR to the MBP-NS3 coding sequence, using the bacterial expression vectors as template. pDonor207 (Invitrogen) was used as backbone of the Entry vector and the pK2GW7 (Karimi et al., 2002) as destination vector.

Agrobacterium tumefaciens transient transformation assay (ATTA) and expression analysis

Agrobacterium infiltration was performed as previously described (Bucher et al., 2003). For RNA silencing suppressor assays, *N. benthamiana* leaves were co-infiltrated with *Agrobacterium* (at an $OD_{600}=0.5$) harboring binary vectors encoding mGFP and the suppressor construct of interest. The GFP expression in the leaves was visualized 5 days post infiltration with a hand-held UV lamp and a Canon Power shot A710IS digital camera, using the high fluorescent program.

Expression of NS3 was detected by western blot analysis. A leaf disc of the infiltrated leaves was collected, grinded in 25 μ l PBS and 25 μ l 2x SDS-loading buffer, heated for 5 minutes at 95°C and centrifuged for 3 minutes at 14000 rpm. The proteins were separated by a SDS-Page gel, transferred to Immobilon-P (Millipore) by semi-dry blotting and the MBP-NS3 protein was detected by a MBP-specific primary antibody (BioLabs), followed by an alkaline phosphatase conjugated secondary antibody. The protein was visualized with NBT-BCIP as substrate (Roche) according to manufacturers' protocol.

Northern blot analysis

RNA was isolated from *Agrobacterium* infiltrated leaves as previously described by Bucher (Bucher et al., 2004). To this end, seven μ g of total RNA mixed with formaldehyde loading buffer and heated for 5 minutes at 70°C, was separated on a 1 % agarose gel. The RNA was transferred onto Hybond N (Pharmacia-Biotech), followed by UV-cross linking.

For the siRNA blot, RNA was enriched for small RNAs as described (Hamilton & Baulcombe, 2004) and five μ g of this fraction was resolved on a 20%, 0.5x TBE denaturing acrylamide gel. Following separation, the RNA was electroblotted onto Hybond-N+ (Pharmacia-Biotech) and crosslinked by UV-light. Hybridization of both blots (mRNA and siRNA) was performed overnight at 48°C in modified church buffer with mGFP-specific Dig-labeled DNA probe. The blots were washed briefly three times with 2x SSC and three times for 15 minutes with 2xSSC and 0.2% SDS at 48°C.

The labeled probe was detected by western blot analysis using a Dig-specific antibody conjugated to alkaline phosphatase in blocking buffer (maleic acid buffer + 1% blocking reagent) and CSPD as substrate (Roche) according to the manufacturers' recommendations.

Recombinant protein expression and electrophoretic mobility shift assay (EMSA)

The wild type and mutant MBP-NS3 proteins were expressed in BL21 DE3 cells and purified according to manufacturers' protocol and as previously described by Hemmes et al. (2007). Purification was checked by SDS-page gel followed by commassie brilliant blue staining. Protein concentrations of the eluted fractions were determined by the Bradford assay (Biorad).

EMSA was at least performed in triplicate with 100 pM 32 P-labelled 21 nt siRNA molecules and bacterial expressed MBP-NS3 (mutant) proteins, as previously described (Lakatos et al.

2006; Hemmes et al., 2007). Complexes were separated on an 8% native acrylamide gel and after drying exposed overnight to a phosphor screen. The screen was scanned using a Molecular Dynamics Typhoon Phosphor imager (Amersham Biosciences) and a representative picture was shown. Visual bands were quantified using Genius Image Analyser software (Syngene) and the K_d was determined by plotting the bound RNA fraction as a function of the protein concentration.

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Chapter 3

The NS3 protein of Rice hoja blanca virus suppresses RNA silencing in mammalian cells

Abstract

The NS3 protein of the tenuivirus Rice hoja blanca virus has previously been shown to represent the viral RNA silencing suppressor protein and is active in both plant and insect cells by binding siRNAs *in vitro*. Using firefly luciferase-based silencing assays it is shown that NS3 is also active in mammalian cells. This activity is independent of the used inducer molecule. Using either synthetic siRNAs or a short hairpin RNA construct, NS3 was able to significantly suppress the RNA-mediated silencing of luciferase expression in both monkey (Vero) and human (HEK293) cells. These results support the proposed mode of action of NS3 to act by sequestering siRNAs, the key molecules of the RNA silencing pathway conserved in all eukaryotes. The possible applications of this protein in modulating RNA silencing and investigating the proposed antiviral RNA silencing response in mammalian cell systems are discussed.

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Introduction

RNA silencing is a conserved eukaryotic gene regulation mechanism comprising endonucleolytic cleavage of long dsRNA into siRNA molecules of 21-26 nt by Dicer (Bernstein et al., 2001). After incorporation of the siRNAs in RISC, present in the cytoplasm, they are unwound and the retained (guide) strand is used for sequence-specific recognition and degradation of RNA targets (reviewed by Sontheimer, 2005). Although most eukaryotes encode a functional RNA silencing pathway with conserved parts, there are differences between the kingdoms (Dykxhoorn et al., 2003). Over time, different biological processes involving this mechanism have been identified (reviewed by Herr, 2005; Sontheimer, 2005, Tomari, 2005, Voinnet, 2005).

In plants, nematodes and insects, RNA silencing has been demonstrated to serve as an innate antiviral defense response, but RNA silencing has not been confirmed to operate as such in mammalian cells (Li et al., 2002, Voinnet, 2001, Zambon et al., 2006). To counteract this antiviral response, many plant and insect viruses encode proteins that interfere with the RNA silencing pathway, so called RSS proteins. These proteins, have been identified and characterized in negative and positive stranded RNA viruses as well as DNA viruses (reviewed in the introduction & by Lecellier & Voinnet, 2004). Similar to these RSS proteins of plant and insect viruses, some proteins of human-infecting viruses have also been demonstrated to act like this. The NS1 protein of human Influenza A virus was shown to act as RSS protein in plants and insect cells (Bucher et al., 2004, Li et al., 2004). Next to the cross-kingdom activity by human infecting viruses, some intra-species RNA silencing suppression was demonstrated too. The HIV-1 Tat protein or the core and E2 protein of HCV, for example, show RSS activity in cultured human cells (Bennasser et al., 2005, Ji et al., 2008). A considerable number of viruses are able to infect hosts belonging to two different kingdoms such as insects and mammals or plants and insects. Until now, only limited information is available on the question if their RSS proteins are active in both organisms and how they act. The Nodamura virus infects insects and mammals and encodes an RSS protein (B2) which has been demonstrated to be active in mammalian cells (Sullivan & Ganem, 2005). Moreover, the crystal structure has shown that it binds dsRNA molecules, a key molecule of the RNA silencing pathway and thereby inhibits Dicer cleavage (Korber et al., 2009, Sullivan & Ganem, 2005). RHBV is another virus capable of infecting and replicating in two different kingdoms, i.e. plants (rice) and its insect vector (plant hopper, *Tagosodes orizicolus*) (Falk & Tsai, 1998) and likely induces antiviral RNA silencing in both. The RSS protein of RHBV, NS3, has been shown to act in plants (Bucher et al., 2003) and more recently in cultured insect cells (Hemmes et al., 2007). NS3 exhibits a high affinity for 21 nt siRNAs (Hemmes et al., 2007) and this binding capacity is crucial for its RSS activity in plants, as shown in chapter 2.

In mammalian cells, RNA silencing can be induced by endogenous or exogenous dsRNA molecules. A single Dicer enzyme processes these long dsRNA molecules into 21 nt siRNAs (Bernstein et al., 2001), with similar biochemical properties (2 nt 3'-overhang and

5'-phosphorylated) as those from plants and insects. The results of chapter 2 supported the idea that the RSS activity of NS3 solely relies on siRNA binding. If this is true, NS3 should also be operational in other organisms encoding a functional RNA silencing pathway, like mammalian cells. To test this hypothesis, a RSS assay was established in mammalian cells using different silencing molecules and the RSS capability of NS3 and the NS3 mutant lacking the RSS activity in plants and siRNA binding affinity (AAA; Chapter 2) was analyzed.

Results

Establishment of a reporter gene RNA silencing assay in mammalian cells

Prior to the analysis of NS3 RSS activity in mammalian cells, wt NS3 or NS3 mutant (K173A/K174A/K175A; AAA) (as described in chapter 2) were N-terminally fused to MBP and cloned in a mammalian expression vector. Their expression in mammalian Vero cells was verified by western blot analysis.

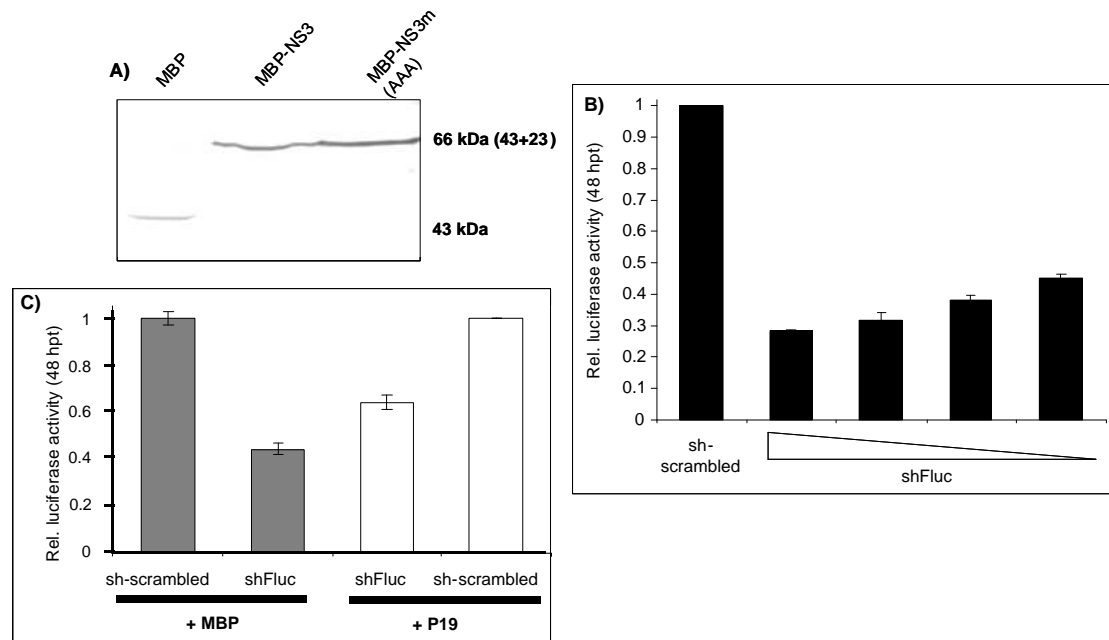


Figure 3-1: A) Western blot analysis of MBP, MBP-NS3 or MBP-NS3 mutant (MBP-NS3m) expressed in Vero cells B) Concentration dependent short hairpin induced silencing. Plasmids encoding for Renilla luciferase (Rluc), Firefly luciferase (Fluc) and different concentrations of either unspecific (sh-scrambled) or specific (shFluc) short hairpin RNA were co-transfected in Vero cells. C) Suppression of short hairpin RNA-induced Fluc silencing by tombusvirus P19. Vero cells were co-transfected with expression plasmids encoding Rluc, Fluc, a non-specific (sh-scrambled) or specific (shFluc) Fluc short hairpin RNA and either MBP or tombusviral P19. MBP, maltose binding protein; NS, non-structural.

As negative control, pEF5/V5-based expression plasmid encoding the MBP alone was included (Figure 3-1A). Next a mammalian cell reporter gene RNA silencing assay was developed based on the Firefly luciferase (Fluc). To this end, Vero and Human Embryonic Kidney (HEK)293 cells were co-transfected with plasmids encoding Fluc and a short hairpin (sh)RNA construct (Paddison et al., 2002) specifically targeting Fluc (shFluc) or a non-specific (scrambled) shRNA. To ensure comparability, a Renilla luciferase (Rluc) expression vector (pRL-CMV; Promega) was used as an internal control. The Dual luciferase assay (DLR;

Promega) determined 48 hours post transfection (hpt) the luciferase expression levels. Cells co-transfected with Fluc- and shFluc-encoding plasmids showed a drastic decrease in Fluc expression levels, which was not observed in cells expressing scrambled shRNA (Figure 3-1B, data not shown). This decrease was dependent on the amount of the RNA silencing inducer (Figure 3-1B), and enabled a maximum silencing of approximately 80% of the original Fluc expression level. Addition of more RNA silencing inducer plasmid did not support a further increase in the silencing of Fluc expression (data not shown).

To validate the assay, Vero cells were co-transfected with Fluc, shFluc or scrambled shRNA and the tombusvirus P19 siRNA-binding RSS-encoding plasmid, demonstrated to be active in plant and mammalian cells (Dunoyer et al., 2004, Lakatos et al., 2004). To observe the most optimal RNA silencing suppression of P19, a sh-construct concentration was chosen not oversaturating the RNA silencing pathway and giving a silencing of approximately 60% at 48 hours post transfection (hpt). Using this set-up the observed luminescence in Fluc silenced cells additionally expressing P19 was significantly higher than in cells expressing MBP, whereas non-silenced cells did not show any differences, irrespective of the presence or absence of P19 (Figure 3-1C). These results demonstrated that the designed experimental set-up could be used to determine RSS activity in mammalian cells.

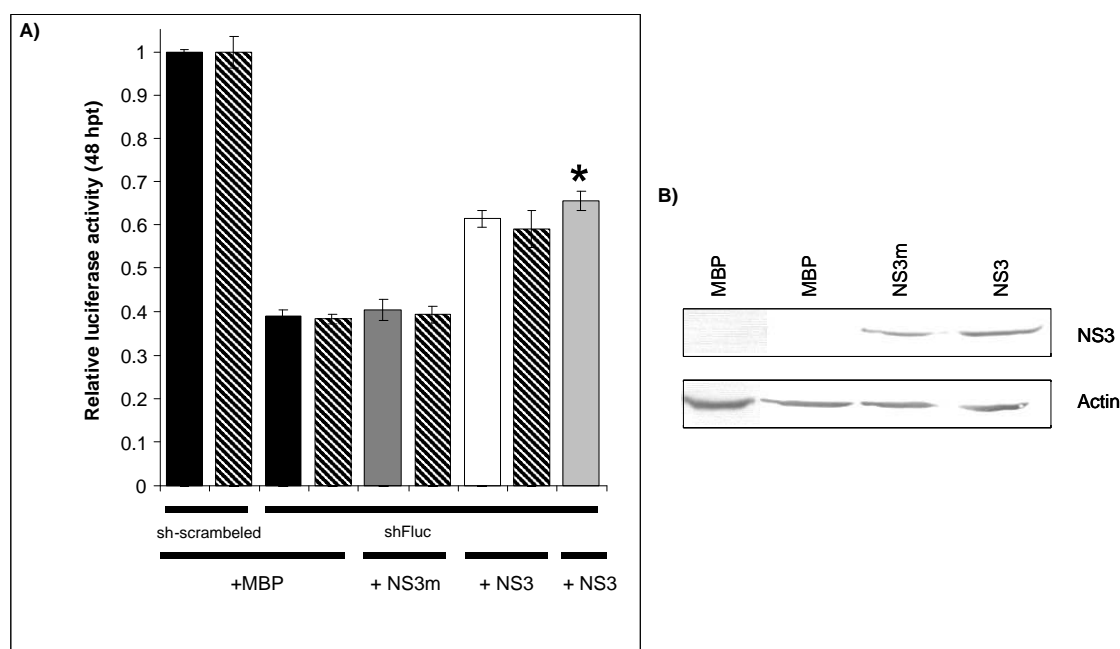


Figure 3-2: Effect of NS3 expression on short hairpin RNA induced silencing.

HEK293 (dashed) or Vero (solid) cells were co-transfected with Firefly luciferase (Fluc), a nonspecific (sh-scrambled) or specific (shFluc) Fluc shRNA, and either MBP, MBP-NS3 mutant (NS3m) or NS3 (A), either N-terminally MBP-tagged or untagged (*). Each treatment was performed in duplicate in at least two independent assays and the Fluc activity was normalized to Renilla luciferase (Rluc) for each of these treatments. Western blot confirmation of NS3 expression within the samples analyzed in panel A (B). Immunological detection of β -actin served as loading control. MBP, maltose binding protein; NS, non-structural.

RHBV NS3 suppresses siRNA and shRNA induced silencing in mammalian cells

Next the RSS activity of NS3 in Vero cells (Figure 3-2A) was tested. Similar as to the results with P19, a significant and reproducible (partial) recovery of luminescence was observed in the presence of wildtype NS3, either tagged or untagged, but not with NS3mutant (expressed at similar levels as wt NS3; Figure 3-2B), indicating that this protein was able to suppress RNA silencing in mammalian cells. Using HEK293 cells instead of Vero cells, similar results were obtained for NS3 (Figure 3-2B) and P19 (data not shown), respectively.

Since NS3 has been proposed to exhibit its RSS activity by sequestering siRNAs (Chapter 2), instead of shRNAs, also synthetic siRNAs were used to test for specific gene silencing in cultured mammalian cells. Whereas shRNAs homologous to Fluc yielded a maximal silencing effect of 80%, siRNAs achieved over 95% of silencing (Figure 3-3 A). This is in agreement with earlier reports that have described siRNAs as more potent RNA silencing inducers (Paddison et al., 2002). Furthermore, a concentration-dependent decrease in the Fluc expression was detected in cells transfected with Fluc plasmid DNA and Fluc-specific siRNAs (siLuc), compared to cells transfected with Fluc and unspecific (scrambled) siRNAs (data not shown).

Having demonstrated sequence specific silencing using synthetic siRNAs (Figure 3-3A), timing of NS3 action was investigated. As it proved impossible to suppress silencing at the highest siRNAs concentrations, optimal conditions for measuring RNA silencing suppression were determined. RNA silencing was not suppressed in cells in which the NS3 plasmid was co-transfected with the Fluc plasmid and siLuc (Figure 3-3B). However, a significant increase in Fluc expression was observed in cells initially transfected with the NS3 plasmid and sequentially transfected after 24 hours with Fluc and siLuc (Figure 3-3C). The combined presence of proteins from a pre-transfected encoding plasmid and siRNA in transfected cells was verified by an YFP-expressing plasmid and transfection of rhodamine labeled siRNAs, revealing exclusively double transfected cells at a transfection efficiency of approximately 60% (Figure 3-4). As already observed for the short hairpin induced silencing experiments, no Fluc increase could be detected in the presence of NS3m (Figure 3-3B). This indicated that the NS3 protein was able to suppress siRNA induced silencing but only when being present before the accumulation (or transfection) of siRNAs, as similarly reported for the B2 RSS protein of FHV in insect cells (Li et al., 2004).

RHBV NS3 does not inhibit Dicer cleavage *in vitro*

Although, Dicer action is most probably not needed if siRNAs are used as inducer molecules it can not be ruled out that NS3 additionally acts by inhibition of Dicer needed during RNA-hairpin transfection. The well established *in vitro Drosophila* Dicer cleavage assay (Bernstein et al., 2001) was used to investigate the effect of NS3 on the Dicer cleavage of a radio labeled 114 nt dsRNA substrate into 21 nt siRNA molecules (Merai et al., 2006). Data obtained showed no inhibition of Dicer cleavage in the presence of bacterial produced MBP-NS3 protein, even at high concentrations (Figure 3-3D). These results suggested that the

RSS activity of NS3 is most likely limited to sequestering of siRNAs in mammalian cells. This idea is being supported by the observation of an increase in the silenced status of luciferase, in the presence of equal amounts of the NS3 encoding plasmid, but increasing amounts of siluc (data not shown).

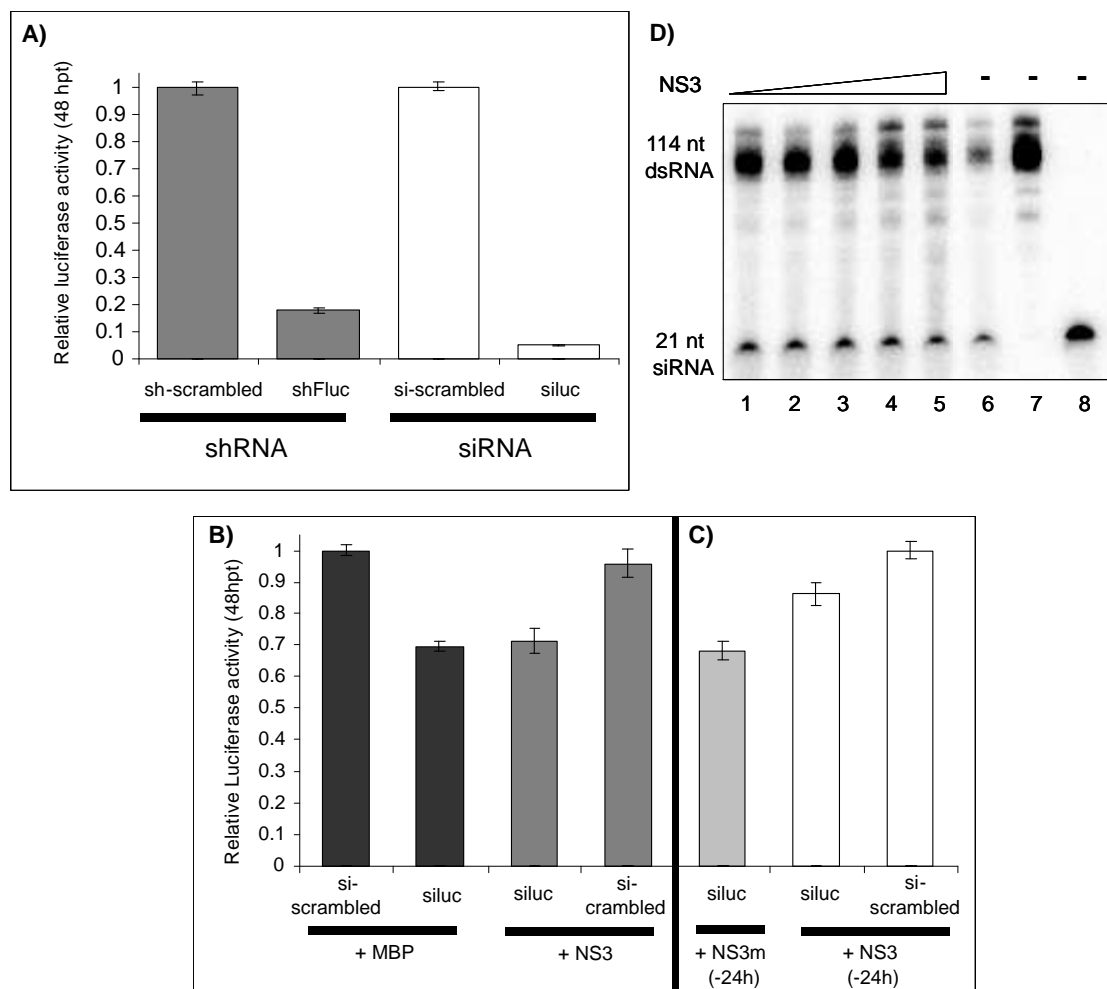


Figure 3-3: A) siRNA or short hairpin (sh) RNA based silencing in Vero cells co-transfected with either specific (luc) or unspecific (scrambled) RNA silencing inducer, together with Firefly luciferase (Fluc) and Renilla luciferase (Rluc) plasmids. B) Effect of NS3 co-transfection on siluc-induced Fluc silencing. Vero cells were co-transfected with vectors encoding Fluc, Rluc and either MBP or MBP-NS3 as well as siRNA specific (siluc) or unspecific (si-scrambled) for Fluc. C) Effect of NS3 pre-transfection (24 hrs) on siluc-induced Fluc silencing. Vero cells were transfected with Fluc and Rluc expression vectors, together with siRNA either specific against Fluc (siluc) or unspecific (si-scrambled). Fluc activity was normalized to Rluc activity independently for each treatment. The relative luciferase activity was obtained from at least two independent assays, whereas each assay was performed in duplicate. D) Effect of NS3 on Dicer cleavage *in vitro*. Radioactively labeled dsRNA (lane 7) was cleaved into siRNAs by Dicer from *Drosophila* embryo extract in the absence (lane 6) or increasing presence of bacterial purified MBP-NS3 protein (lane 1 to 5). siRNAs were included as control (lane 8). MBP, maltose binding protein; NS, non-structural; dsRNA, double stranded RNA.

Discussion

Here it has been shown that NS3 is able to suppress induced RNA silencing in (non-host) mammalian cells, like earlier demonstrated in its natural host plant and insect cells (Bucher et al., 2003, Hemmes et al., 2007). For this suppressor activity, NS3 strongly depends on its high affinity for 21 nt siRNA as already observed for plants (Chapter 2), independent of the

used inducer molecules. The majority of RSS proteins studied so far bind either long or short dsRNA (Lakatos et al., 2006, Merai et al., 2006), both representing conserved molecules of the RNA silencing pathway in all eukaryotic organisms.

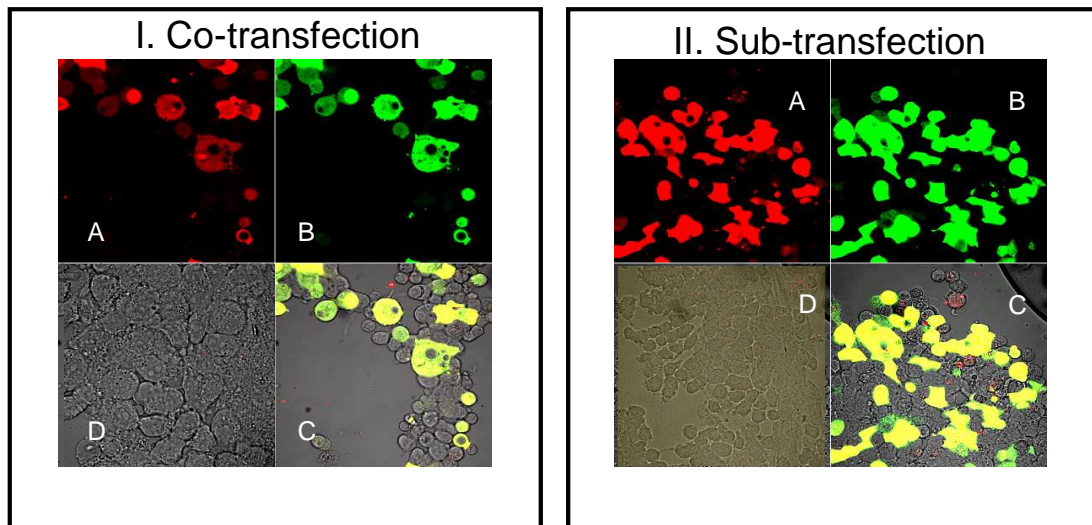


Figure 3-4: Transfection efficiency of DNA plasmids and siRNAs in human embryonic kidney (HEK) 293 cells after co-transfection (left panel) or pre-transfection (right panel). Cells were transfected with yellow fluorescent protein (YFP) plasmid and either co- (left panel) or 24 hours later transfected (right panel) with rhodamine labeled siRNAs. After twenty four hours, fluorescent cells were monitored by a Zeiss laser scanning microscope LSM510. A) rhodamine detection, B) YFP detection, C) merge of A, B and D) bright field image.

Inactivating such essential molecules within the RNA silencing pathway offers a RSS protein the possibility to be active in different host organisms and possibly reduces the chance of these different hosts to evade the RNA silencing suppression. On the other hand, it implies that relatively high amounts of RSSs are possibly needed in the cytoplasm of cells to reach a good level of suppression when compared to a presumed suppressor protein that would inhibit specific proteins of the RNA silencing machinery by direct interaction. Alterations in the host protein that interacts with the latter type of RSS protein, though, may easily result in loss of RNA silencing suppression and hence avirulence. Whereas the virus benefits from encoding a RSS protein, a highly active RSS protein results in a pathogenic virus that not only kills its host and/or vector rapidly, but also would reduce its survival and dissemination.

Regarding the fact that RHBV is facing antiviral RNA silencing in both plant and insect hosts, it is not surprising that NS3 interferes with a conserved element of the RNA silencing pathway present in both hosts and not with a specific protein of one host. This hypothesis is in line with the observation that the NS3 RSS activity is also exhibited in mammalian cells. As the effects of synthetic siRNAs and a shRNA construct were similar, it is tempting to assume that NS3 acts downstream of both RNA silencing inducer molecules or on the inducer molecule itself. This is in agreement with data from previous studies *in vitro* and in plants, which demonstrated that the biochemical activity of NS3 most likely and entirely relies on the binding to siRNA molecules (Hemmes et al., 2007). It is possible that NS3, due to its higher affinity for siRNA, may extract siRNAs from the intermediate RISC complexes (R1 and RLC), where the siRNAs are still double stranded. However, ss siRNAs present in mature, pre-

assembled RISC complexes can not be dissociated by NS3; consequently NS3 can not compete for ss siRNA in these complexes (Hemmes et al., 2007) and only shows RSS activity if expressed 24 hours prior siRNA induced silencing (Figure 3-3A & B).

Using plant, insect or mammalian cell-based assays, a number of innate immunity suppressors, like interferon antagonists encoded by mammalian viruses, have been demonstrated to have RSS activity. Some of the best studied examples so far are NS1 of Influenza A virus (Bucher et al., 2004, Li et al., 2004), VP35 of Ebola virus, E3L of Vaccinia virus, tat of HIV-1, NSs of La Crosse virus, Tas of Primate foamy virus-1, core and E2 of HCV and NoV B2 (Haasnoot et al., 2007, Schutz & Sarnow, 2006, Soldan et al., 2005). Furthermore, it has been shown that the interferon antagonists VP35, NS1 and E3L are RSS proteins in human cells that are capable of restoring the production of a HIV-1 strain defective in the Tat gene (Haasnoot et al., 2007).

These results indicate that RNA silencing, like the interferon pathway, may be an important innate antiviral defense response in mammals, and that mammalian viruses, similar to plant and insect viruses, need to counteract this response in order to replicate. Although not studied into great detail, the RSS proteins from mammalian viruses seem to bind longer dsRNAs with a higher affinity than siRNAs (Hemmes, 2007). Besides serving as RNA silencing inducers, long cytoplasmic dsRNAs induce the replication-dependent antiviral interferon pathway in mammalian cells (Kato et al., 2005, Marques et al., 2006). It is therefore difficult to separate these two pathways as well as to unravel the effect of long dsRNA binding proteins with respect to both pathways. However, using a protein like NS3, exclusively binding siRNAs, provides a promising strategy to distinct between these two and study the possible presence of an antiviral RNA silencing pathway in mammalian cells. The use of NS3 could also give information about the relative importance of each antiviral pathway, by determining its complementing effect on replication level of viruses defective in their innate antiviral suppressor protein. Next to the possibility to reveal novel aspects of the virus-host interaction, the *in trans* complementation of viruses defective in their own innate antiviral suppressor genes opens the chance of virus particle production in plants, mammalian and insect cells, for example, for attenuated vaccine strains. Until now, a problem faced during the production cycle of attenuated viruses, most probably having defects in their innate antiviral suppressor genes, are the low virus titers reached in culture. In insect cells it was shown that virus titers can be increased by complementation, using either the virus' own suppressor protein or even cross-kingdom suppressor proteins (Li et al., 2004). Recent results have shown that this is also possible by using cell lines stably expressing an RSS protein, such as Tat of HIV-1 (de Vries et al., 2008). A main drawback of using mammalian encoded RSS proteins, like VP35 and NS1, may in this case be the risk of wildtype rescuing due to recombination. The use of NS3 based producer cell lines would avoid such drawbacks and improve the biosafety of such an approach. Further research is needed to confirm the potential application of NS3 in mammalian virus research and production strategies.

Materials and methods

Plasmid construction

The mammalian expression plasmids, MBP, MBP-NS3 and MBP-NS3 mutant were generated using the described Entry clones (Chapter 2), the pEF5/v5 (Invitrogen) destination vector and LR clonase according to the manufacturers' protocol of the gateway technology.

The P19 of Carnation italian ring spot virus (CIRV) and NS3 (RHBV) open reading frames were cloned into the mammalian expression vector pEF5-v5-dest containing the human EF1 α promoter using gateway technology. The expression plasmid of shLuc has been described by Paddison et al. (2002) and the scrambled shRNA by Konstantinova et al. (2006).

The bacterial expression vector of MBP-NS3 has been described in chapter 2.

Cell culture and transfection

Human embryonic kidney (HEK293) cells or African green monkey kidney Vero cells were grown as a monolayer in DMEM (Gibco, BRL) supplemented with 10% fetal calf serum (FCS) (Gibco), streptomycin (100ug/ml) and penicillin (100U/ml) at 37°C and 5% CO₂. To reach a confluence of 60-70% at the time of transfection, cells were trypsinated 24 hours pre-transfection and seeded in a 24-well plate at a concentration of 2.2×10^5 or 1.1×10^5 cells per well, respectively. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions.

For the RNA silencing suppressor assays using short hairpin constructs as inducer molecules, cells were transfected with luciferase expression plasmids, i.e. 100 ng Firefly luciferase (GL3; Promega) and 2 ng Renilla luciferase (pRL-VMV; Promega), and 4 ng of short hairpin encoding plasmids, either non-specific or Firefly luciferase specific (pShh1-Ff1; Paddison et al., 2002). Next to this, cells were also co-transfected with 450 ng of the RNA silencing suppressor expressing plasmid (MBP, MBP-NS3, MBP-NS3mutant, P19 or NS3).

For the siRNA based suppressor assays, cells were transfected with 450 ng suppressor expression plasmids (MBP, MBP-NS3 or MBP-NS3mutant) and dependent on the experimental set-up directly or 24 hours later with 100 ng Firefly luciferase expression plasmid, 2 ng Renilla luciferase expression plasmid and 0.15 ng siRNA molecules, either Firefly luciferase specific (Qiagen) or unspecific (Eurogentec).

To determine transfection efficiencies, HEK293 cells seeded on coverslips were transfected with 450 ng yellow fluorescent protein (YFP)-expression plasmid and either co- or sub-transfected 24 hours later with 150 ng rhodamine labeled siRNA molecules. Fluorescence was determined 24 hours later with a Zeiss laser scanning microscope (LSM510), using the multitrack setting and the corresponding lasers and filters.

Luciferase assays and expression analysis

Cells were lysed 2 days post (2nd) transfection and luciferase expression was determined using the Dual luciferase reporter assay (Promega), according to manufacturers' protocol.

Expression of MBP-tagged proteins was analyzed by western blot analysis. Lysate prepared for the Dual luciferase assay was resolved on a SDS-page gel. Proteins were semi-dry transferred to Immobilon-P (Millipore) and MBP-tagged proteins subsequently detected using a MBP-specific rat primary and goat alkaline phosphatase conjugated secondary antibody. To verify for protein loading, an internal control protein, β -actin, was detected by a specific monoclonal primary antibody, followed by a secondary antibody conjugated to alkaline phosphatase. The protein-IgG complexes were visualized with NBT-BCIP as substrate (Roche) according to the manufacturers' protocol.

dsRNA preparation

Double stranded RNA was generated by T7 RNA polymerase (Promega) based *in vitro* transcription with α -³²P-CTP (Perkin Elmer) using gel purified PCR products (GE Healthcare) as template. The promoter sequence for T7 RNA polymerase was introduced by PCR at each end of the eGFP specific PCR product using primers T7-dsRNA 114nt F (5' gta ata cga ctc act ata ggg ggc gtc cag tgc ttc agc cgc 3') and T7-dsRNA 114nt RV (5' gta ata cga ctc act ata ggg gcc gtc gtc ctt gaa gaa gat gg 3'). After PCR amplification and T7 transcription, RNA transcript molecules were incubated at 70°C for 10 minutes and left at room temperature to anneal (slowly cooled down to room temperature). Remaining single stranded RNA and DNA template were removed by treatment with DNase I and RNase A. The resulting dsRNA was gel purified from an 8% native 0.5x TBE acrylamide gel. Annealing and labeling of 21 nt siRNA molecules was performed as described by Haley (Haley et al., 2003).

Recombinant protein expression and Dicer cleavage assay

The RHBV NS3 protein was expressed in BL21 DE3 cells according to the manufacturers' protocol and purified as previously described (Chapter 2).

The *Drosophila* embryo extract used for Dicer cleavage assays was prepared as previously described (Haley et al., 2003). A typical Dicer cleavage reaction was performed in 10 μ l and contained 2 μ l *Drosophila* embryo extract, 125 pmol 114nt dsRNA and various concentrations of bacterial expressed and purified MBP-NS3, and left to incubate for 60 minutes at 25°C (Haley et al., 2003), in buffer lacking KCL. Samples were deproteinized, purified by phenol/chlorophorm, precipitated by ethanol and the resulting RNA resolved on a 12% denaturing polyacrylamide gel in 0.5X TBE. After electrophoresis, the gel was dried, exposed overnight to a phosphor screen and subsequently scanned by Molecular Dynamics Typhoon Phosphorimager (Amersham Biosciences).

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Chapter 4

The NS3 protein of Rice hoja blanca virus complements the RNA silencing suppressor function of HIV-1 Tat

Abstract

The question whether RNA interference or RNA silencing serves as antiviral mechanism in mammalian cells remains controversial. The antiviral interferon response cannot easily be distinguished from a possible antiviral RNA silencing pathway due to the involvement of double stranded RNA as common inducer molecule. The NS3 protein of Rice hoja blanca virus is an RNA silencing suppressor (RSS) that exclusively binds small double stranded RNA molecules. Here, it is demonstrated that this plant viral RSS lacks interferon antagonistic activity, yet is able to substitute the RSS function of the Tat protein of human immunodeficiency virus type 1. An NS3 mutant that is deficient in RNA binding and its associated RSS activity is inactive in this complementation assay. This cross-kingdom RNA silencing suppression in mammalian cells by a plant viral RSS indicates the significance of the antiviral RNA silencing response in mammalian cells and the usefulness of well defined RSS proteins.

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** first shared author*

Introduction

RNA silencing serves as an antiviral response in plants, insects and invertebrates (Ding & Voinnet, 2007, Galiana-Arnoux et al., 2006, van Rij et al., 2006, Voinnet, 2001, Wang et al., 2006a). RNA silencing is induced by dsRNA viral replication intermediates and extended secondary structures in viral RNA (Voinnet, 2005a). These dsRNA molecules are recognized by Dicer proteins and processed into siRNAs, which guide RISC to inactivate a target RNA in a sequence-specific manner (Sontheimer, 2005). To counteract this antiviral response, plant and insect viruses encode RSS proteins (Voinnet, 2005a). Most plant viral RSS proteins have dsRNA binding domains for short dsRNAs (Lakatos et al., 2006, Merai et al., 2006, Voinnet, 2005a) or longer dsRNAs (Deleris et al., 2006, Merai et al., 2005). Other plant viral RSS proteins interfere with protein components of the RNA silencing machinery (Deleris et al., 2006, Levy et al., 2008).

Mammalian cells possess a functional RNA silencing pathway that can be instructed to become antiviral upon transfection with siRNAs or constructs that express shRNAs against viral sequences (reviewed by Haasnoot et al., 2007, Marques & Carthew, 2007). However, the potential role of RNA silencing as a natural antiviral defense mechanism, in mammalian cells, remains controversial. The hallmark of antiviral RNA silencing, that is virus-derived siRNAs, could not be identified in infected cells (Pfeffer et al., 2004). However, such molecules were described more recently for several endogenous and exogenous viruses, including HIV-1 (Bennasser et al., 2005, Soifer et al., 2005, Yang & Kazazian, 2006, Parameswaran et al., 2010), yet the significance of these findings is still being debated (Lin and Cullen, 2007).

Next to the siRNA pathway, another similar pathway plays an important role in most, if not all, eukaryotes and which regulates gene expression at the post transcriptional level. Host encoded small RNA molecules, called miRNAs are crucial for this pathway (Bartel et al., 2004; Carrington & Ambros, 2003; Herr et al., 2005). MicroRNAs arise from polymerase II transcribed miRNA genes that fold into stem loop structures called pri-miRNAs. The nuclear protein Drosha cleaves these into pre-miRNAs of approximately 70 nt, in a complex with a dsRNA binding partner called DGCR8, in humans (Lee et al., 2003; Han et al., 2004). Following export to the cytoplasm by exportin 5 (Lund et al., 2004), pre-miRNAs are further processed by Dicer into 21-24 nucleotide mature miRNA/miRNA* duplexes. One strand of these duplex molecules, like with siRNAs, is incorporated into miRISC and subsequently assists in the sensing of (partly) complementary target sequences that become cleaved or translationally arrested (Bartel et al., 2004). There is accumulating evidence that mammalian cells use miRNAs to control viruses (Berkhout & Jeang, 2007). HIV-1 is inhibited by miR-17 and -20a due to down-regulation of histone acetylase PCAF, a co-factor of the transactivator of transcription (Tat) protein (Triboulet et al., 2007). MicroRNAs may also regulate components of the antiviral interferon (IFN) pathway and thus provide a possible link between the RNA silencing and IFN pathways (reviewed by Sonkoly et al., 2008). These combined

findings support the idea that RNA silencing, either siRNA or miRNA based, is part of the innate immune system in mammals.

Consistent with this idea, an increasing number of mammalian viruses have been shown to encode an RSS protein, e.g. the HCV core and envelope protein 2 (Ji et al., 2008, Wang et al., 2006b), Vaccinia virus E3L (Li et al., 2004), Ebola virus VP35 (de Vries & Berkhout, 2008), PFV Tas (Lecellier et al., 2005), Influenza A virus NS1 (Bucher et al., 2004, Haasnoot et al., 2007, Li et al., 2004) and HIV-1 Tat (Bennasser et al., 2005). These RSS proteins suppress RNA silencing-mediated down-regulation of a reporter gene construct. NS1 and VP35 can also trans-complement the production of a Tat-negative HIV-1 variant (Haasnoot et al., 2007). HIV-1 Tat and HCV core were proposed to block Dicer activity (Bennasser et al., 2005, Chen et al., 2008), while NS1, E3L and VP35 most likely act by sequestering dsRNA (Bucher et al., 2004, Haasnoot et al., 2007, Li et al., 2004). Furthermore, stable expression of mammalian RSS proteins have been shown to increase viral replication (de Vries et al., 2008).

Intriguingly, most identified RSS proteins of mammalian viruses also possess antagonistic properties against the extracellular (Toll-like receptor-mediated) or intracellular (PKR, IFN, RIG-I, MDA-5 mediated) defense pathway, and these activities usually map to the RNA-binding domain that is also implicated in the RNA silencing function (Bucher et al., 2004, Wang et al., 1999, Wang et al., 2000). To distinguish between IFN and RNA silencing pathways and to determine if HIV-1 production is restricted by RNA silencing in mammalian cells, here the NS3 protein of RHBV was used that binds exclusively to small dsRNAs (Hemmes et al., 2007), needing this binding for its RSS activity (Chapter 2) and exhibits RSS activity in mammalian cells (chapter 3). Consistent with this property, it is confirmed that this plant virus protein lacks IFN antagonistic activity and yet is able to rescue a Tat-minus HIV-1 reporter virus.

Results

The plant virus NS3 protein complements Tat

Recently, the Ebola virus VP35 protein has been shown to complement a Tat-minus HIV-1 variant in which the Tet-system for doxycycline (dox)-inducible gene expression replaces the transcription function of Tat (Haasnoot et al., 2007). Now this system is used (Figure 4-1 A) to test whether the Tat RSS function can be complemented by the RHBV NS3 protein, which exclusively binds short dsRNA molecules (Hemmes et al., 2007) and is therefore not expected to modulate the IFN pathway. Prior to this, the expression of the constructed NS3 vectors (Chapter 2) in HEK293T cells was verified. To this end, both NS3 and the NS3 mutant (NS3m, AAA), lacking RNA binding and RSS activity in plants and mammalian cells (Chapters 2 & 3), were expressed as a fusion protein of 66 kDa with MBP. The MBP domain (43 kDa) was included as control (Figure 4-1B).

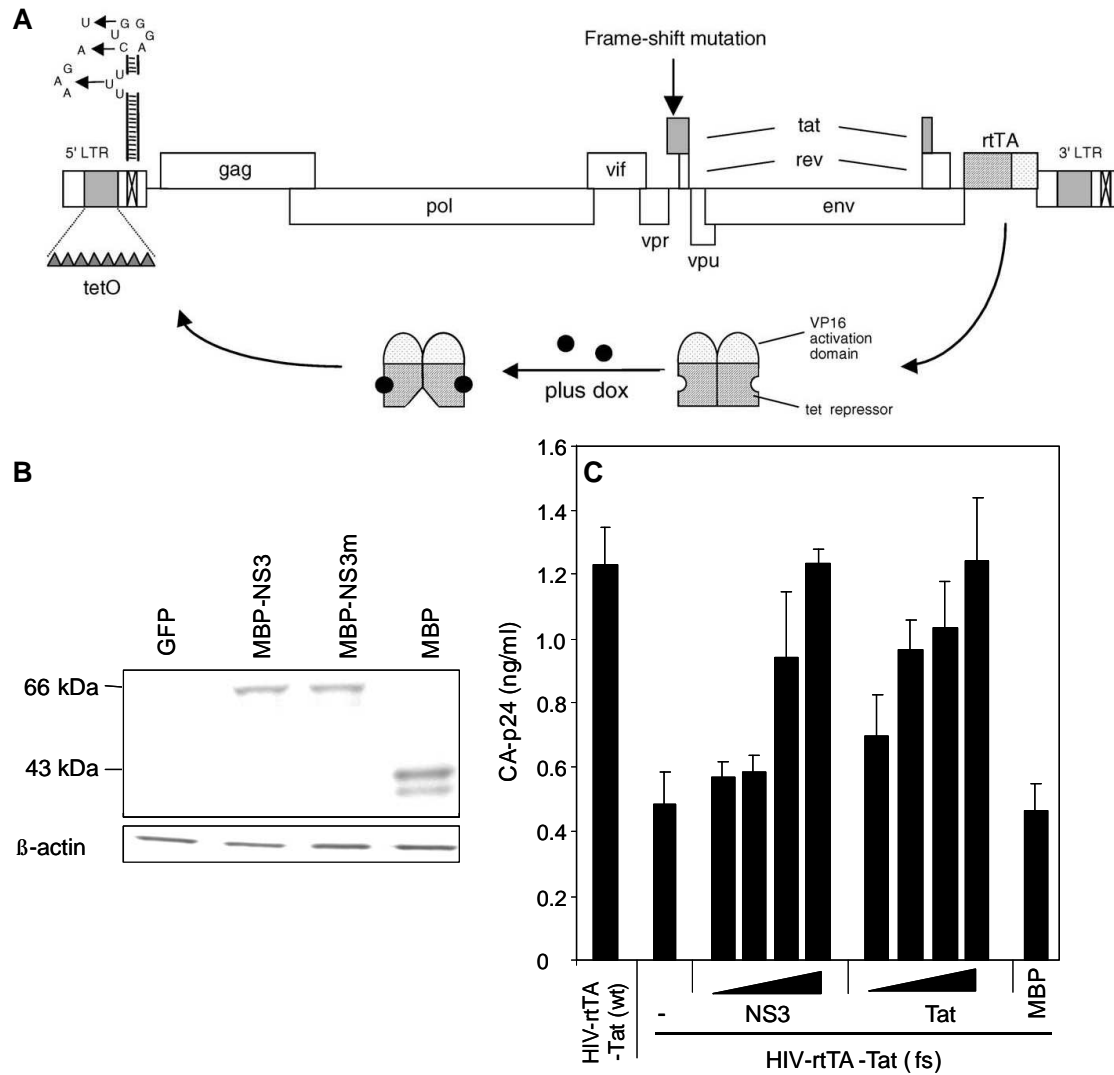


Figure 4-1: Plant viral RNA silencing suppressor protein NS3 complements HIV-1 Tat

Schematic representation of the HIV-rtTA genome (taken of Haasnoot et al., 2007). The Tat-TAR dependent transcription was inactivated by mutations in the TAR region (as indicated) and a frameshift mutation at codon 20 of the tat gene. Transcription was established by the insertion of the rtTA gene instead of the nef gene and 8 tetO sites in the LTR promoter. Addition of doxycycline (dox) results in the binding of rtTA to the tetO sites, starting transcription and viral replication (A). Lysates of HEK293T cells, transfected with expression plasmids for GFP, MBP-NS3, MBP-NS3m or MBP (900 ng), were analyzed for protein expression by western blot analysis, using a rabbit polyclonal antiserum against MBP. Immunological detection of β -actin served as loading control (B). HEK293T cells were co-transfected with HIV-rtTA-Tat^{wt} and HIV-rtTA-Tat^{fs} (100 ng) in combination with increasing amounts (10, 100, 600 and 900 ng) of NS3 or Tat expression plasmids. The vector expressing MBP (900 ng) was used as negative control. HIV-1 production was determined two days post transfection by detecting CA-p24 in the supernatant using ELISA. The mean of at least three independent experiments is shown with standard error (C). ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus type 1; MBP, maltose binding protein; NS3, non structural protein 3; NS3m, NS3 mutant protein; RSS, RNA silencing suppressor; Tat, transactivator of transcription

After co-transfection of increasing amounts of NS3 expression plasmid with the Tat-negative HIV-1 (HIV-rtTA-Tat^{fs}) construct in HEK293T cells, the effect of NS3 on HIV-1 production was monitored. Strikingly, the RHBV NS3 protein was able to rescue virus production in *trans* to approximately the same extent as HIV-1 Tat (Figure 4-1C). The reason that more NS3 than Tat vector is needed could be due to differences in the RSS mechanism (siRNA binding *versus* Dicer blocking) or the proteins' intracellular localization/stability/concentration, but this was not investigated further. Given the established role of NS3 in counteracting antiviral RNA silencing (Bucher et al., 2003, Hemmes et al., 2007) and the fact that binding of only small dsRNA is essential for its biochemical activity (Chapter 2), this result indicated that HIV-1 production is restricted by the RNA silencing mechanism.

NS3 exhibits no IFN antagonistic activity

To formally rule out that the Tat-complementing property of NS3 was based on IFN pathway modulation, this effect was probed in mammalian cells (HEK293T) using a Fluc reporter construct under control of an IFN- β inducible promoter and Rluc as internal control (Figure 2A). As a positive control, Fluc expression as a measure of IFN production was induced by poly I:C, and this stimulatory effect was significantly reduced in the presence of the IFN-antagonistic VP35 protein of Ebola virus (Cardenas et al., 2006). As expected, the RHBV NS3 protein exhibited no IFN antagonistic activity, yielding the same Fluc expression as the negative control (empty vector). We also tested the HIV-rtTA-Tat^{wt} and HIV-rtTA-Tat^{fs} constructs for their ability to induce IFN in this assay. No such activity was measured (results not shown). Next, the possible involvement of the PKR component of the IFN pathway was tested by performing NS3-complementation in the presence of 2-aminopurine (2-AP), a specific PKR inhibitor (Lu & Cullen, 2004). NS3 maintained Tat-complementation activity with 2-AP (Figure 4-2B), confirming that PKR was not involved. Thus, Tat inactivation and NS3 complementation do not have an impact on the IFN pathway.

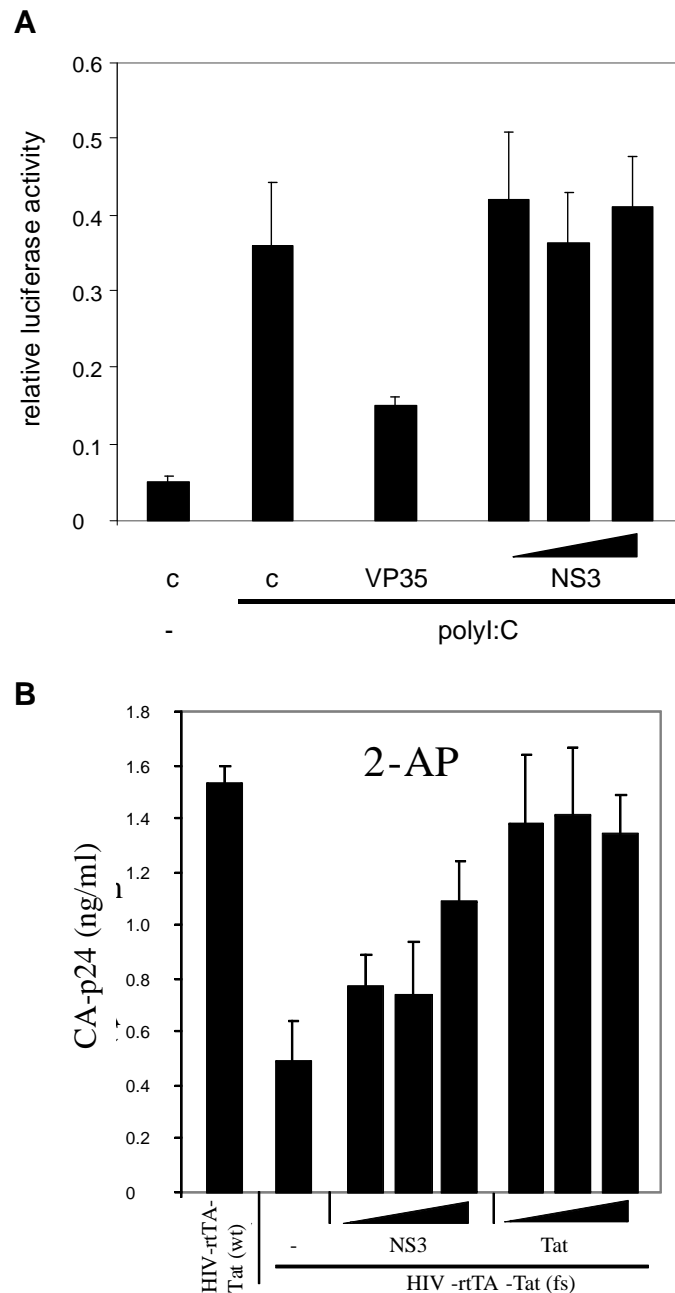


Figure 4-2: NS3 has no interferon and PKR antagonistic properties

Effect of NS3 on interferon inducible construct. HEK293T cells were co-transfected with expression vectors encoding firefly luciferase under control of an IFN- β inducible promoter, renilla luciferase and VP35, NS3 (10, 100, 400 ng) or pBluescript (c), either in the presence (+) or absence (-) of poly I:C. Luciferase expression was measured 3 days post transfection. Shown is relative luciferase expression corrected for the internal renilla control (firefly/renilla). The mean of at least three independent experiments is shown with standard error (A).

Effect of 2-AP on NS3 trans-complementation. HEK293T cells were co-transfected with HIV-rtTA-Tat(wt) and HIV-rtTA-Tat(fs) in combination with increasing amounts (300, 600 and 900 ng) of NS3 or Tat expression plasmids. The PKR inhibitor 2-AP (final concentration of 2,5 mM) was added 4 hours post transfection. HIV-1 production was determined two days post transfection by detecting CA-p24 in the supernatant using ELISA. The mean of three independent experiments is shown with standard error (B). HEK, human embryonic kidney; IFN, interferon; NS3, non structural protein 3; PKR, protein kinase R; ELISA, enzyme-linked immunosorbent assay; HIV-1, human immunodeficiency virus type 1; Tat, transactivator of transcription; 2-AP, 2-aminopurine

NS3 requires dsRNA binding capacity for HIV-1 complementation and interferes with miRNA pathway

Having demonstrated that NS3 was able to complement for Tat, it was tested whether NS3 required its RNA-binding domain for HIV-1 trans-complementation. Therefore, the NS3 mutant (NS3m, AAA) was used, known to be defective in siRNA binding (Figure 4-3A; Chapter 2), suppression of antiviral RNA silencing in plants (Chapter 2) and siRNA-mediated silencing in mammalian cells (Chapter 3). As the anti-HIV effect of the RNA silencing pathway might utilize miRNAs (Triboulet et al., 2007), the NS3m was tested for its ability to bind miRNA/miRNA* duplexes using an electrophoretic mobility shift assay (EMSA) (Figure 4-3B). In comparison to wild type NS3, known to bind miRNA/miRNA* and siRNA duplexes at high affinity (Figure 4-3C & D) (Hemmes et al., 2007), this NS3 mutant was unable to interact with miRNA/miRNA* molecules, even at the highest protein concentration (Figure 4-3B).

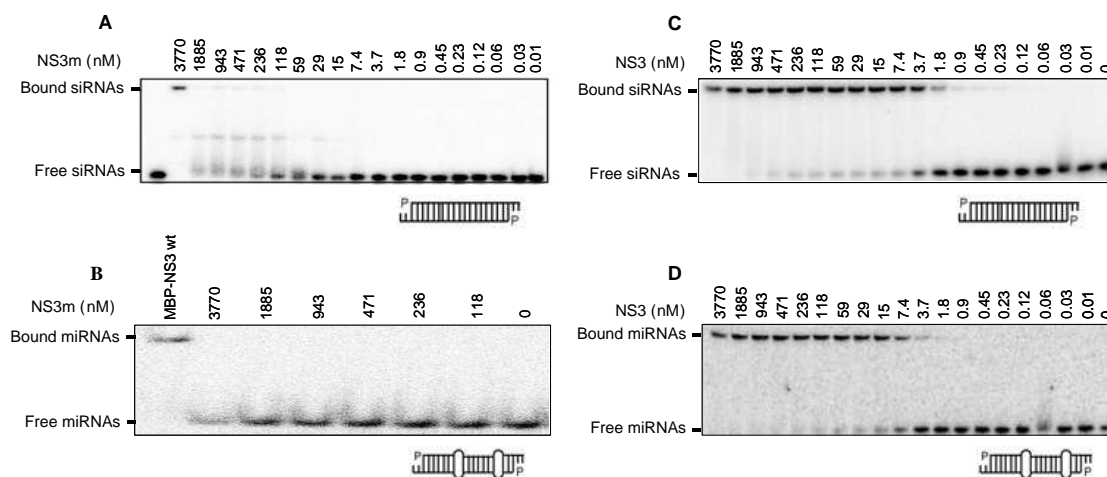


Figure 4-3: NS3 mutant fails to bind siRNA and miRNA/miRNA* duplexes

Different concentrations (0-3770 nM) of bacterially purified NS3 protein, wild-type or mutant (NS3m) protein, were incubated for 20 minutes at room temperature with 100 pM of 32 P-labelled RNA molecules, either siRNA (panels A, C) or *Arabidopsis thaliana* miR171a (B, D). The RNA-protein complexes formed were separated on a native 5% polyacrylamide gel. A representative picture is shown from three independent experiments. MBP, maltose binding protein; miRNA, microRNA; NS3, non structural protein 3; siRNA, small interfering RNA; wt, wild type

The NS3m was subsequently tested in the HIV-1 *trans*-complementation assay (Figure 4-4), after having confirmed that NS3m was expressed at levels comparable to wild type NS3 (Figure 4-1A). Unlike wild-type NS3, NS3m was not able to restore virus production of HIV-rtTA-Tat^{fs} at any of the concentrations tested,

Whether NS3 was able to block the miRNA pathway in human cells was determined by using a Fluc reporter containing multiple miR-30 target sites, for either the sense (pCMV-luc-miR30-P) or antisense (pCMV-luc-miR30-AP) strand. This reporter is normally tested in co-transfection with an excess of miR-30-expression vector (Zeng et al., 2003), which might mask a subsequent RNA silencing suppression effect. HEK293T cells that express endogenous miR-30, where both strands can act as guide strands, (Zeng et al., 2003) were co-transfected with NS3 and either pCMV-luc-miR30-P (Figure 4-5 A) or pCMV-luc-miR30-AP (Figure 4-5B). Several controls were included. First, a control Fluc reporter with randomized miRNA target sites (pCMV-luc-random) (Figure 4-5C) was used. Second, NS3m

and MBP were co-transfected as controls for NS3 RSS activity. Luciferase expression was measured two days post transfection. A modest stimulatory effect of NS3 was observed on pCMV-luc-miR30-P (Figure 4-5A) and AP reporter (Figure 4-5B), but not on the pCMV-luc-random control (Figure 4-5C), suggesting that NS3 was able to inhibit endogenous miRNA action in mammalian cells. HIV-1 Tat has earlier been proposed to interfere with Dicer cleavage (Benasser et al., 2005) and thus could also inhibit the miRNA pathway. To answer this question, the effect of HIV-1 tat expression in HEK293T cells on the luciferase based miRNA30 sensor constructs was analyzed, in analogy to NS3 (Figure 4-5). Like for NS3, higher expression levels for luciferase were observed from pCMV-luc-miR30-P in the presence of HIV1 tat (Figure 4-5A), but not with pCMV-luc-random (Figure 4-5C) as control. Furthermore, the stimulatory effect of HIV-1 Tat was significantly higher compared to that for NS3, most likely due to similar reasons (localization/stability/concentration or differences in RSS activity) that explains the higher amounts of NS3 vector needed in the complementation assays (Figure 4-1).

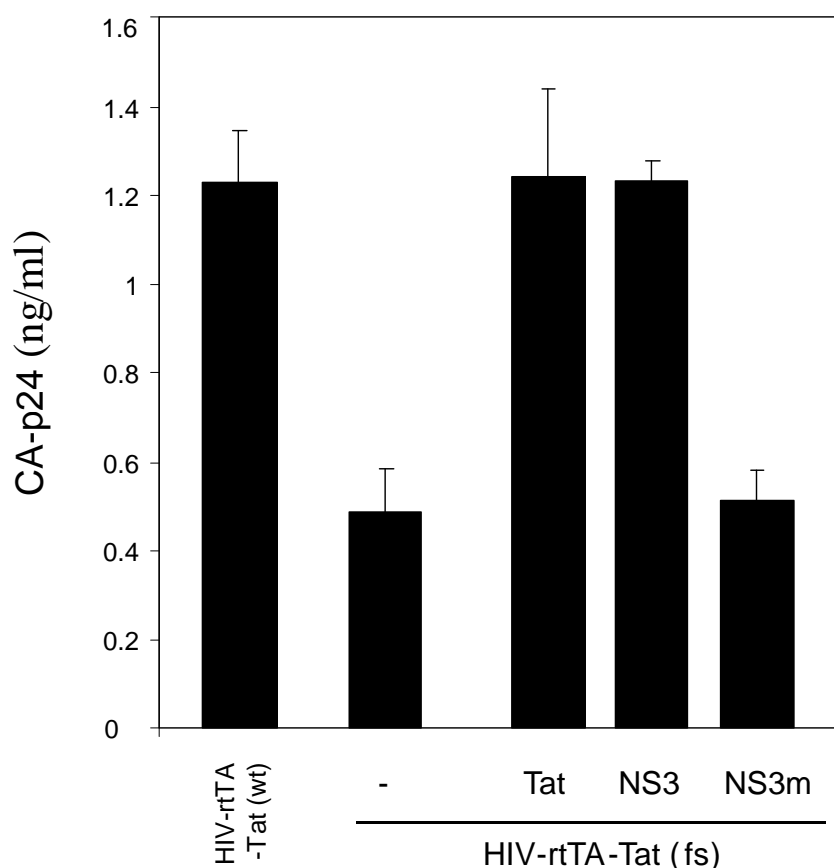


Figure 4-4: dsRNA binding of NS3 is required for HIV-1 Tat complementation
HEK293T cells were transfected with HIV-rtTA-Tat^{wt} or HIV-rtTA-Tat^{fs} in combination with Tat, NS3, NS3 mutant (NS3m) or pBluescript (-) (900 ng). CA-p24 in the culture supernatant was measured at 2 days post transfection. The mean of at least three independent experiments is shown with standard error. dsRNA, double stranded RNA; HEK, human embryonic kidney; HIV-1, human immunodeficiency virus type 1; NS3, non structural protein 3; Tat, transactivator of transcription

Discussion

In this study, it has been demonstrated that a plant viral RSS protein that lacks interferon antagonistic properties can functionally replace the HIV-1 Tat RSS function and that this complementation is based on the sequestration of small dsRNA. These results further corroborate the RSS function of HIV-1 Tat (Bennasser et al., 2005, Haasnoot et al., 2007), which has been questioned by others (Lin & Cullen, 2007), and strongly support the idea that HIV-1 is being targeted by antiviral RNA silencing. Although cross-kingdom suppression of RNA silencing has been reported for a number of viral RSS proteins (Dunoyer et al., 2004, Chapter 3), this is the first report of cross-kingdom RSS activity in a mammalian viral complementation assay. The results are also in line with the observation that knockdown of the RNA silencing pathway by means of Drosha or Dicer silencing enhances HIV-1 replication (Triboulet et al., 2007). The ongoing debate about the physiological relevance of RNA silencing as antiviral mechanism is spurred in part because of the presence of the antiviral IFN pathway (reviewed by Gantier & Williams, 2007). The results presented here demonstrate that a plant virus encoded RSS protein with a well defined biochemical activity can be used as powerful tool to dissect the contribution of the antiviral RNA silencing pathway in mammalian systems in the presence of the IFN pathway. Of note, other eukaryotes do also encode an alternative innate immune response next to the RNA silencing pathway (Arbouzova & Zeidler, 2006, Dangl & Jones, 2001). Taken together, it has been demonstrated that HIV-1 production is limited by endogenous small dsRNAs that viral RSS function can counteract this restriction and that HIV-1 Tat can interfere with the miRNA pathway. RHBV NS3 has been shown to interfere with the siRNA pathway in plants (Bucher et al., 2004; Chapter 2) and both, siRNA (Chapter 3) and miRNA pathway (Figure 4-5), in non-host mammalian cells, due to its RNA binding activity. For these reasons, it is likely that NS3 also interferes with the siRNA and miRNA pathway in its vector and host organism.

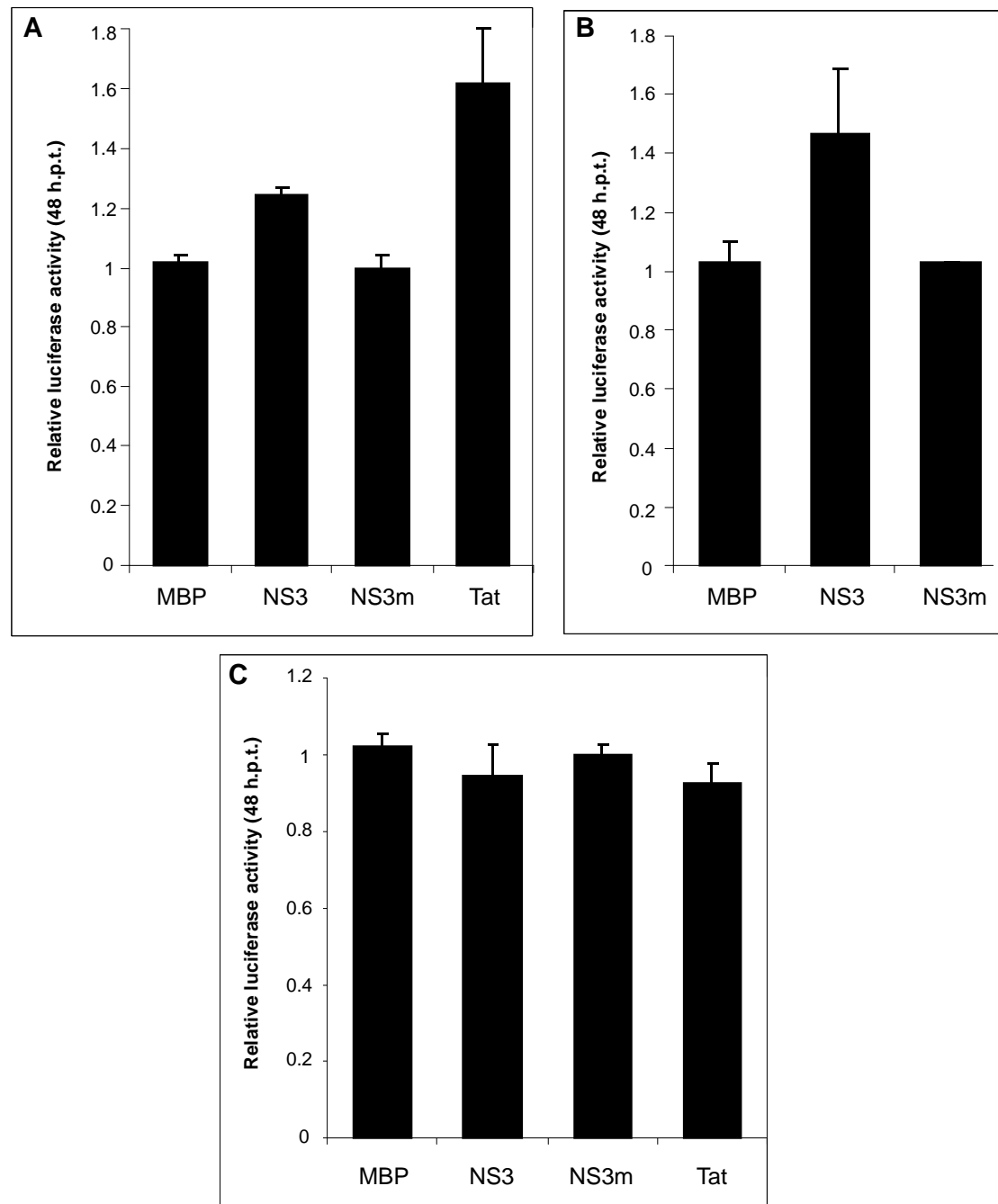


Figure 4-5: NS3 and Tat inhibit endogenous miRNA action

HEK293T cells were co-transfected with expression vectors encoding pCMV-luc-miR30-P (A), pCMV-luc-miR30-AP (B) or pCMV-luc-random (C), in combination with MBP, MBP-NS3, MBP-NS3m or HIV-1 Tat (600 ng). Luciferase expression was measured 2 days post transfection and relative luciferase expression (firefly/renilla) was determined. The luciferase level measured with NS3m was set at 1.0. HEK, human embryonic kidney; MBP, maltose binding protein; miRNA, microRNA; NS3, non structural protein 3; NS3m, mutant protein; Tat, transactivator of transcription

Materials and methods

Plasmid constructs

Mammalian expression plasmids for MBP, MBP-NS3, MBP-NS3m, VP35, Tat, HIV-rtTA-Tat^{wt} and HIV-rtTA-Tat^{fs} were described previously (Haasnoot et al., 2007, chapter 3). Micro RNA based firefly luciferase sensor constructs have been described previously (Zeng et al., 2003). The bacterial expression vectors of MBP-NS3 and MBP-NS3mutant have been described in chapter 2.

Cell culture and transfection

Human embryonic kidney (HEK293T) cells were grown as a monolayer in DMEM (Gibco, BRL) supplemented with 10% fetal calf serum (FCS) (Hyclone), streptomycin (100ug/ml) and penicillin (100U/ml) at 37°C and 5% CO₂. To reach a confluence of 60-70% at the time of transfection, cells were trypsinated 24 hours pre-transfection and seeded in a 24-wells plate at a concentration of 1.5×10^5 cells per well. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions.

For the IFN assay, cells were co-transfected with 500 ng of a Fluc –expression plasmid under control of an IFN beta-inducible promoter, IFNB-luc, 2 ng of an Rluc expression plasmid, 100 ng poly I:C and 400 ng of pBluescript (Stratagene) or plasmids encoding either MBP-NS3, MBP-NS3mutant or VP35. Cells were lysed 3 days post transfection and luciferase expression was determined using the Dual luciferase reporter assay (Promega), according to manufacturers' protocol.

For the miRNA sensor construct assay, cells were co-transfected with 25 ng Fluc expression plasmid, harboring either target sites for sense or antisense of human miRNA-30 (pCMV-luc-miR30-P or pCMV-luc-miR30-AP) or random miRNA target sites (pCMV-luc-random) (Zeng et al., 2003), 0.5 ng of a Rluc expression plasmid and constructs encoding MBP-NS3, MBP-NS3m or MBP. Forty-eight hours post transfection, cells were lysed and assayed for luciferase expression by the Dual luciferase assay (Promega).

For the PKR inhibitor, 2-AP, assay cells were co-transfected with either HIV-rtTA-Tat^{wt} or HIV-rtTA-Tat^{fs} in combination with increasing amounts (300, 600 and 900 ng) of NS3 or Tat expression plasmids. Four hours post transfection, 2-AP (final concentration of 2,5 mM) was added to the transfected cells. Two days post transfection, HIV-1 production was determined by ELISA detection of CA-p24 present in the supernatant.

The HIV-1 Tat complementation assay was performed as previously described (Haasnoot et al., 2007).

Recombinant protein expression and electrophoretic mobility shift assay (EMSA)

Wild type and mutant MBP-NS3 protein were expressed in BL21 DE3 cells and purified as described by Hemmes (Hemmes et al., 2007). EMSA, either with radioactively labeled siRNA or miRNA molecules, was performed in triplicate as previously described (Lakatos et al., 2006). After overnight exposure to a phosphor screen results were visualized after scanning

by a Molecular Dynamics Typhoon Phosphor imager (Amersham Biosciences) and a representative picture was shown.

Protein expression in transfected HEK293T cells was detected by western blot analysis using a rabbit polyclonal antiserum specific for MBP (BioLabs). As loading control β -actin was detected with a mouse monoclonal antibody, after stripping of the blot. For visualization, goat alkaline phosphatase-conjugated secondary antibodies (Dako/Sigma) and NBT-BCIP substrate (Roche) were used according to the manufacturer's recommendations.

Acknowledgments

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Chapter 5

Rice hoja blanca virus NS3 interferes with the miRNA-mediated pathway in different organisms

Abstract

The NS3 protein of *Rice Hoja blanca* tenuivirus acts as RNA silencing suppressor protein on the siRNA pathway in plant, insect and mammalian cells. The protein exhibits a high affinity for small double stranded RNA molecules (siRNA and miRNA/miRNA*) *in vitro* and requires this binding property for its suppressor activity. Whereas NS3 interferes with the siRNA pathway in host and non-host cells, here the interference of NS3 on the miRNA pathway in plants and insects was investigated. It is shown that in plants, NS3 was able to suppress silencing of an eGFP sensor construct, containing target sites for miRNA-1. The appearance of a leaf curling phenotype of *Arabidopsis* transgenically expressing NS3 supported the idea of NS3 interference in endogenous miRNA-mediated host gene regulation in plants. In insect cells, NS3 was able to do so likewise, this time using a Firefly luciferase based-sensor construct. Mutant NS3, lacking affinity for siRNAs and miRNA/miRNA*s, was not able to suppress silencing of these sensor constructs in plant and insect cells. In addition to an earlier observed suppression of a miRNA-sensor in non-host mammalian cells, the results indicate that NS3 interferes in the miRNA pathway of all eukaryotes, by sequestering miRNA/miRNA* molecules. The possible importance of this activity during a natural infection in plants and insects is discussed.

Introduction

RNA silencing involves a sequence specific RNA degradation through the use of homologous small, non-coding RNA molecules. So far, three major types of small dsRNA have been identified, i.e. siRNA, miRNA and piRNA, and their diverging roles in different processes of eukaryotes characterized. siRNAs (21 nt) act in the antiviral defense in plants and insects (Hamilton & Baulcombe, 1999, Li et al., 2002), and derive from cleavage of long dsRNA by DCL proteins or Dicer in plants respectively insects (Bernstein et al., 2001, Deleris et al., 2006, Hamilton & Baulcombe, 1999). The long dsRNA substrate originates from viral replicative intermediates or secondary folding structures in the (viral) RNA (Moissiard & Voinnet, 2006, Molnar et al., 2005). After synthesis, one strand of the siRNA molecules is incorporated into RISC and used to guide the sensing of complementary (viral) RNA molecules, which in turn become degraded (Hammond et al., 2000, Nykanen et al., 2001). Recently, a subclass of siRNA molecules have been discovered in *Drosophila* and mammalian cells, endo-siRNAs (Chung et al., 2008, Czech et al., 2008, Ghildiyal et al., 2008, Kawamura et al., 2008, Okamura et al., 2008a, Okamura et al., 2008b, Tam et al., 2008, Watanabe et al., 2008b). They are similarly sized as viral siRNAs and derive from inverted repeat (IR), multiple repeat or highly structured transgenes by the action of Dicer-2 or Dicer in insects and mammalian cells respectively. Endo-siRNAs contribute to the silencing of transposable elements (TE) and some endogenes in both gonads and somatic tissue (Chung et al., 2008, Czech et al., 2008, Ghildiyal et al., 2008, Kawamura et al., 2008, Okamura et al., 2008a, Okamura et al., 2008b, Tam et al., 2008, Watanabe et al., 2008b).

Host encoded gene transcripts, folding into imperfect stem-loop structures and cleaved through the activity of DCL-1 or Drosha/ Dicer-1 in plants respectively insects result into miRNA molecules (~22 nts). After incorporation of one strand into RISC, the miRISC complex guides translational repression or cleavage of target mRNA to regulate host gene expression (Bartel, 2004, Voinnet, 2009, Wang et al., 2008b).

Most plant and insect viruses counteract the antiviral RNA silencing response by encoding RSS proteins, able to interfere with this pathway (Alvarado & Scholthof, 2009, Li & Ding, 2006). A common strategy of RSS proteins is the binding and thereby sequestering of either small or long dsRNA (Lakatos et al., 2006, Merai et al., 2006). Alternatively, some RSS proteins interact with proteins of the RNA silencing pathway, like AGO or DCL/ Dicer, or even interact at several steps of the pathway, i.e. sequester dsRNA molecules and interact with e.g. AGO (Bortolamiol et al., 2008, Pfeffer et al., 2002, Zhang et al., 2006). Next to their action on the siRNA pathway, some plant and insect virus encoded RSS can interfere with the miRNA pathway as well. Examples are potyviral HC-Pro, tombusviral P19 and poleroviral P0 and the observed developmental defects in transgenic *Arabidopsis* expressing these RSS proteins (Chapman et al., 2004, Dunoyer et al., 2004). On the other hand, none of these RSS proteins interfere on the miRNA pathway in *Drosophila* (Berry et al., 2009). Recently, several plant and insect virus encoded RSS proteins, like FHV B2, DCV 1A and tombusvirus P19,

have been reported to hinder the endo-siRNA pathway in *Drosophila* and thereby impair transposon silencing by endo-siRNAs (Berry et al., 2009).

RHBV is an ambisense negative stranded RNA virus, infecting plants and transmitted by plant hoppers (Ramirez et al., 1993, Ramirez et al., 1992). The virus replicates in its plant host and insect vector and most probably encounters antiviral RNA silencing in both organisms. The NS3 protein acts as RSS protein in plants, insects and even mammalian non-host cells (Bucher et al., 2003, Hemmes et al., 2007, Chapter 3). The protein exhibits high affinity for siRNA and miRNA/miRNA* molecules *in vitro* (Hemmes et al., 2007) and this binding is crucial for RSS activity of NS3 in plant and mammalian cells (Chapters 2 & 3). To test for interference of NS3 on the miRNA pathway in host and vector cells, the effect of NS3 on miRNA-induced silencing was analyzed in plants and insect cells. .

Results

RHBV NS3 interferes with the miRNA pathway in plants

To analyze the effect of NS3 on miRNA-mediated silencing in plants a reporter based miRNA sensor assay was developed. To this end, the coding sequence of eGFP was fused with the 3'UTR of the *par6* gene harboring target sites for miRNA-1, resulting in sensor construct eGFP-3'UTR (Figure 5-1A). Co-expression of the eGFP-3'UTR sensor and pri-miRNA1 constructs in leaves of RDR6 silenced *N. benthamiana* plants by *Agrobacterium* infiltration, resulted in silencing of this sensor construct. Northern blot detection using a DIG labeled PCR product of pri-miRNA1 verified the expression, processing and functionality of the pri-miRNA (Figure 5-1C middle). During *Agrobacterium* infiltration, the host encoded RdRp (*Arabidopsis thaliana* RDR6 homolog) converts (aberrant) sense RNA into dsRNA, which triggers silencing (Dalmay et al., 2000, Parizotto et al., 2004). Plants silenced in RDR6 lack this pathway of silencing induction. As a consequence, similar levels of fluorescence were observed after infiltration of these plants with the eGFP-3'UTR sensor construct in the presence and absence of the NS3 RSS protein (Figure 5-1B upper panel). As expected, Northern blot detection of eGFP RNA showed only low levels of eGFP specific siRNAs (Figure 5-1C). Upon co-infiltration with the pri-miRNA1 construct, but not with MBP as negative control, fluorescence from the eGFP-miRNA-1 sensor disappeared due to silencing, and demonstrated the functionality of this miRNA-induced silencing assay (Figure 5-1B). The additional co-expression of MBP-NS3 restored the fluorescence completely and suggested that the NS3 RSS activity also acted on the miRNA induced silencing pathway in plants. Furthermore, MBP-NS3m was not able to suppress the silencing of the eGFP-sensor construct and demonstrated that the binding of NS3 to small dsRNA was required for this RSS activity (Figure 5-1 B).

Having shown that NS3, but not the mutant, interfered with the miRNA induced silencing pathway in plants, we next tested if NS3 also interfered with the endogenous miRNA pathway in plants. To this end, *Arabidopsis* plants were transformed to constitutively express NS3. After selection, transgenic *Arabidopsis* plants expressing NS3 showed leaf curling and long

petals absent from wild type plants (Figure 5-1D, Hemmes, 2007), indicating a developmental defect likely caused by a possible interference of NS3 with the endogenous miRNA pathway.

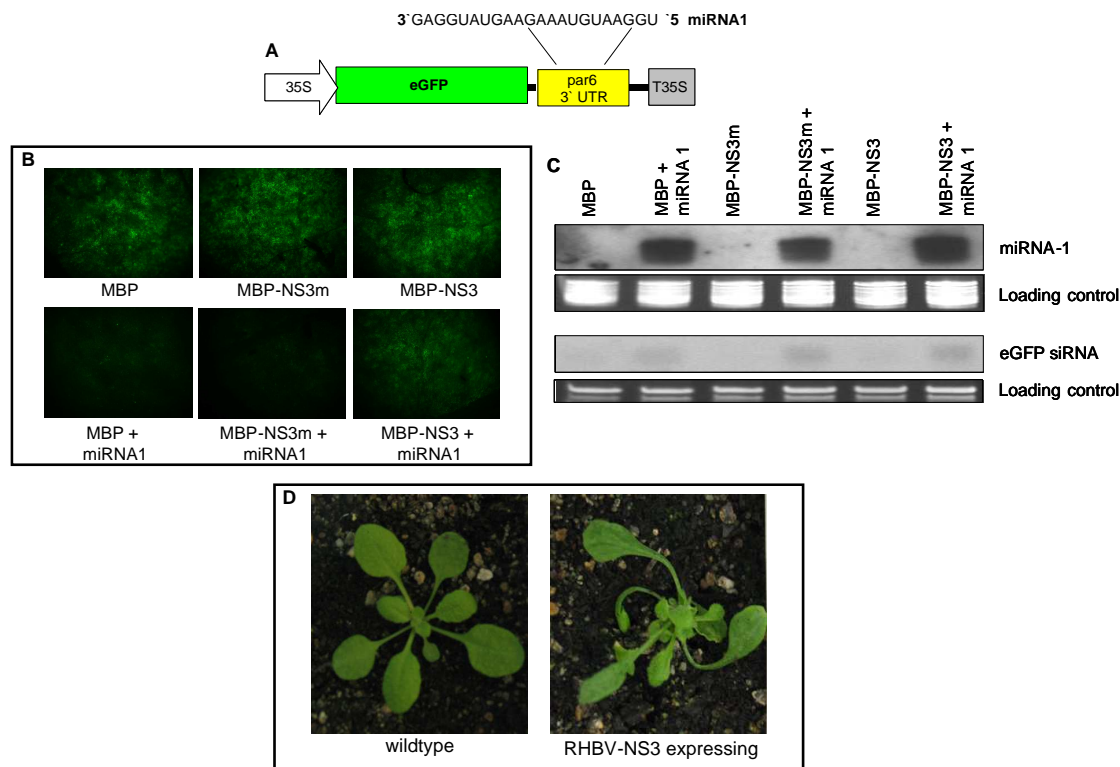


Figure 5-1: RHBV NS3 interference on the miRNA mediated pathway in plants

Schematic representation of the miRNA mediated sensor construct (A).

Agrobacterium harboring vectors for eGFP-3'UTR and MBP, MBP-NS3 or MBP-NS3m were co-infiltrated in RDR6 silenced *N.benthamiana* leaves and eGFP fluorescence visualized 5 days post infiltration. Silencing was induced by co-infiltration of pri-miRNA-1, eGFP-3'UTR and MBP, MBP-NS3 or MBP-NS3m (B). The processed miRNA-1 (C, upper) and eGFP specific siRNAs (C, bottom) were visualized by northern blot analysis using a corresponding DIG-labeled PCR product as probe. Ethidium bromide stained ribosomal RNA or t-RNA was used as loading control.

Developmental defects caused by RHBV NS3 expression in transgenic *Arabidopsis* plants in comparison to wild type plants (D). *Arabidopsis thaliana* Col-0 plants were transformed by the floral dip method (Clough & Bent, 1998) using *Agrobacterium* harboring a translatable NS3 construct. Seed from primary transformants was grown at standard greenhouse conditions under selection for kanamycin resistance. eGFP, enhanced green fluorescent protein; MBP, maltose binding protein; NS3, non-structural protein 3; NS3(m), (mutant of) non-structural protein 3; RDR6, RNA dependent RNA polymerase 6; pri-miRNA1, primary miRNA1; RHBV, rice hoja blanca virus.

MBP-NS3 functions as RNA silencing suppressor protein in insect cells

Having shown that RHBV NS3 interacts with the miRNA pathway in plants it is likely that, due to the conserved nature of the miRNA pathway in eukaryotes, similar activities would be observed in insects. For easier detection and comparison with the plant based experiments, NS3 or NS3 mutant were first N-terminally fused to MBP, cloned in insect pIB-based expression vectors and their expression was verified in *Drosophila melanogaster* Schneider-2 (S2) cells (Figure 5-2A). Earlier, NS3 showed RSS activity on dsRNA induced silencing in insect cells. However, this was based on a visual, non-quantitative assay using GFP as reporter protein (Hemmes et al., 2007). To further substantiate these findings and to assure that the tagged MBP-NS3 had maintained its RSS activity in insect cells, a shRNA-mediated silencing assay of a Fluc reporter gene was developed for insect cells to allow easy

quantification. To this end, S2 cells were co-transfected with inducible plasmids encoding Fluc and Rluc, the latter as internal control, and shRNA constructs specifically targeting Fluc (shFluc) or a non-specific (scrambled) shRNA. Cells co-transfected with MBP, Fluc and shFluc showed a drastic decrease in Fluc expression dependent on the shFluc amount, not observed with sh-scrambled (Figure 2B). In the additional presence of MBP-NS3 or Carnation Italian ringspot virus (CIRV) P19, as a positive control (Berry et al., 2009) Fluc expression was reproducibly restored by 17% or 33% respectively (Figure 5-2C). Similar to what was earlier observed in plants and mammalian cells (Chapters 2 & 3); NS3m was not able to suppress the silenced status of Fluc (Figure 5-2C). Altogether, these data clearly demonstrated that the tagged MBP-NS3 protein was functional, but NS3m not, as RSS protein on the induced siRNA silencing pathway in insect cells.

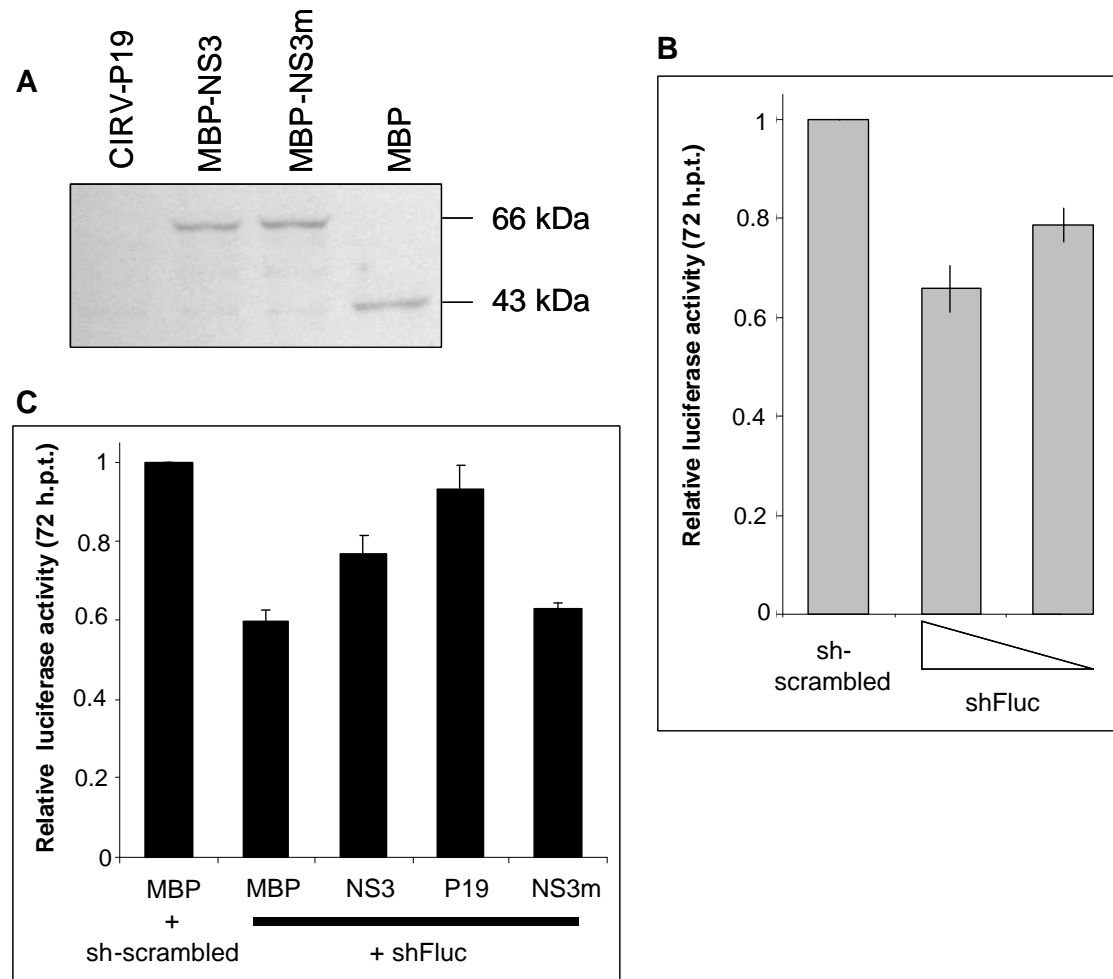


Figure 5-2: MBP-NS3 RNA silencing suppressor activity on the siRNA pathway in insect cells.

Expression of MBP, MBP-NS3 and MBP-NS3m in transfected S2 cells 48 hours post transfection (hpt) was verified by western blot analysis using a MBP-specific antibody (A). S2 cells were co-transfected with pMT-Fluc, -Rluc, two different concentrations of a non-specific (sh-scrambled) or specific (shFluc) Fluc short hairpin RNA (B) and either pIB-MBP, -MBP-NS3, -MBP-NS3m or -Carnation Italian ringspot virus (CIRV) P19. Luciferase expression was induced by 5 μ M CuSO₄ at 48 hpt, and the relative luciferase expression (Firefly/Renilla) was determined 72 hpt. The mean of at least two independent experiments is shown with standard error. The luciferase expression level measured with MBP was set at 1.0 (panel C). Fluc, Firefly luciferase; Rluc, Renilla luciferase; MBP, maltose binding protein; NS3, non-structural protein 3; NS3(m), (mutant of) non-structural protein 3; S2, *Drosophila melanogaster* Schneider-2; RHBV, rice hoja blanca virus.

The RNA silencing suppressor protein, RHBV NS3 acts on the induced miRNA pathway in insects

Having demonstrated MBP-NS3 RSS activity in insect cells, the effect on the miRNA mediated silencing pathway in insect cells was analyzed. Due to the conserved nature of the miRNA pathway in all eukaryotes, and having observed the interference of NS3 with the miRNA pathway in plants, it was likely that NS3 would similarly do so in insect cells. To test this a similar assay as used in plants was designed, however this time using Fluc as reporter gene flanked with a 3'UTR of par6 (together denoted Fluc-3'UTR) containing the target sites for miRNA1. As internal control pMT-Rluc was used and to ensure sequence specific silencing of the sensor construct by miRNA1, a copy of pri-miRNA12 was cloned in the insect pMT expression vector to be used as negative control. Expression of these constructs was induced 48 hpt and luciferase activity was determined 72 hpt. As expected, a concentration dependent decrease in Fluc expression was observed in cells co-transfected with MBP, Fluc-3'UTR and miRNA1, but not when miRNA12 was used instead (Figure 5-3A). Having demonstrated a dosage dependent, sequence specific silencing by miRNA1 the effect of additional NS3 expression was investigated. A significant and reproducible increase in Fluc expression was observed in the presence of NS3, similar to CIRV P19 (Figure 5-3B). As expected, the presence of NS3m did not increase Fluc expression levels (Figure 5-3B). To analyze whether the observed RSS activity of NS3 on the miRNA pathway was concentration dependent, the experiments were repeated but this time different concentrations (50ng and 200 ng) of either NS3 or MBP, as negative control, were applied. Co-transfection with Fluc-3'UTR, miRNA1 and NS3 (Figure 5-3C), but not when MBP was used instead, resulted in a concentration dependent increase of Fluc expression.

Interference with the endogenous siRNA pathway in insects

Endo-siRNA molecules are biochemically similar to siRNAs and it is likely that NS3 also exhibits a high affinity for these molecules, as already observed for several other RSS proteins (Berry et al., 2009).

To analyze whether NS3 also interfered on the endo-siRNA pathway in insect cells a Fluc-based reporter assay was designed based on the endogenous siRNA 2.1 (esi2.1) (Figure 5-4A), known to be highly expressed in Drosophila S2 cells (Czech et al., 2008). Co-transfection of S2 cells with two different concentrations of Fluc-sensor construct, containing two targets sides of esi2.1, and a Rluc construct as internal control, resulted in a concentration dependent reduction of Fluc expression (80% and 70%) compared to a non-functional sensor construct, i.e. Fluc fused to an inverted esi2.1 target site (Figure 5-4B). Having established a functional endo-siRNA-mediated RNA silencing assay, the effect of NS3 on this assay was analyzed. Surprisingly, cells co-transfected with the Fluc-esi2.1 sensor construct and NS3, showed no increase in Fluc. Similar results were observed for CIRV P19 (Figure 5-4C), previously reported to suppress endo-siRNA silencing in flies transgenically expressing these RSS proteins (Berry et al., 2009).

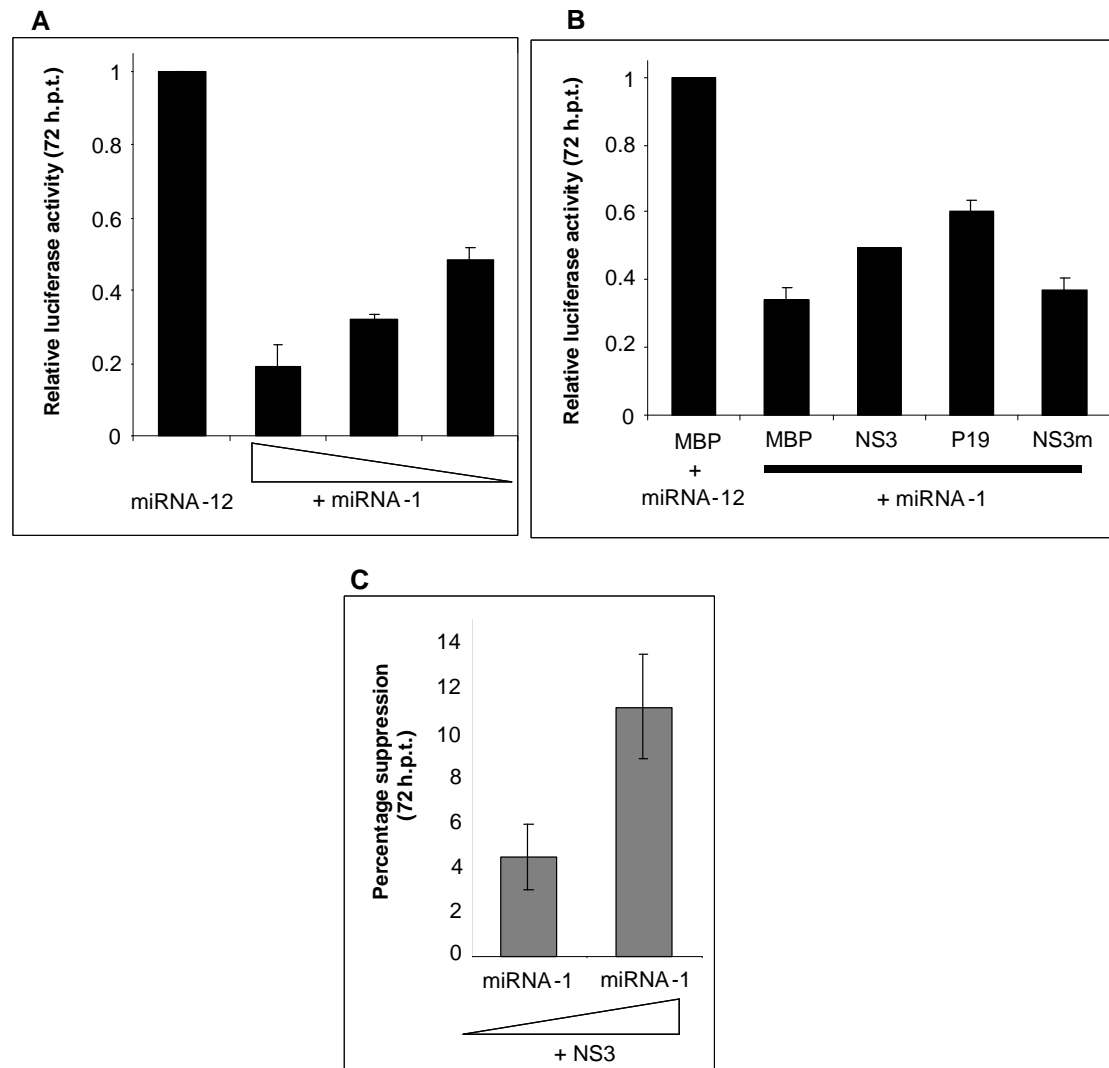


Figure 5-3: RHBV NS3 suppression of miRNA mediated silencing in insect cells.

To measure the effect of NS3 on the miRNA mediated silencing pathway, S2 cells were co-transfected with a pMT-Renilla luciferase (Rluc), pMT-Firefly luciferase (Fluc)-3' UTR (sensor construct), and different concentrations of a specific (miRNA1) or unspecific (miRNA12) primary miRNA (A) in concert with either pIB-MBP, -MBP-NS3, -MBP-NS3m or - Carnation Italian ringspot virus (CIRV) P19. After induction at 48 hpt, relative luciferase expression (Firefly/Renilla) was determined 72 hpt and the mean of at least four independent experiments is shown with standard error (B).

The concentration dependent suppressor activity of NS3 was determined by transfecting S2 cells (as in B) with MBP or MBP-NS3 encoding plasmids at the amount of 50ng and 200 ng. Values were normalized for each concentration of MBP and the relative suppression was plotted (C). MBP, maltose binding protein; NS3, non-structural protein 3; NS3(m), (mutant of) non-structural protein 3; S2, *Drosophila melanogaster* Schneider-2; RHBV, rice hoja blanca virus.

Discussion

The tenuivirus NS3 RSS protein binds siRNA and miRNA/miRNA* molecules *in vitro* and this capacity is crucial for its RSS activity in plants and mammalian cells. Earlier, NS3 RSS activity on the siRNA-induced silencing has been demonstrated in a broad range of host and non-host organisms (Hemmes et al., 2007, Chapter 2-4). Here, it is shown that NS3 is also able to suppress miRNA-induced gene silencing in both plant and insect cells. In addition to the

recent proof for such activity in non-host, mammalian cells (Chapter 4), RHBV NS3 demonstrated to suppress si- and miRNA induced RNA silencing in host and non-host cell systems. Suppression of these pathways is likely accomplished by sequestration of si- and miRNA/miRNA* molecules. In light of the fact that RHBV multiplies in both its plant host and insect vector (Ramirez et al., 1993, Ramirez et al., 1992) it is not surprising that the NS3 RSS protein is able to counteract the antiviral siRNA-induced silencing pathway in both cell types. The observation that NS3 is similarly able to do so in non-host mammalian cells (Chapter 2) only supports the highly conserved nature of this pathway in all eukaryotic organisms. Hence, the capacity of NS3 to additionally interfere in the miRNA-induced silencing pathway is most likely an aberrancy and solely due to the strong structural similarities between siRNA and miRNA/miRNA*. This also supports the idea that NS3 does not interfere with specific miRNA/miRNA* duplexes but more randomly (Chapter 4, Figure 5-1 and 5-3), which is being supported by the appearance of morphological defects in *Arabidopsis thaliana*, transgenically expressing NS3 (Figure 5-1D, Hemmes, 2007).

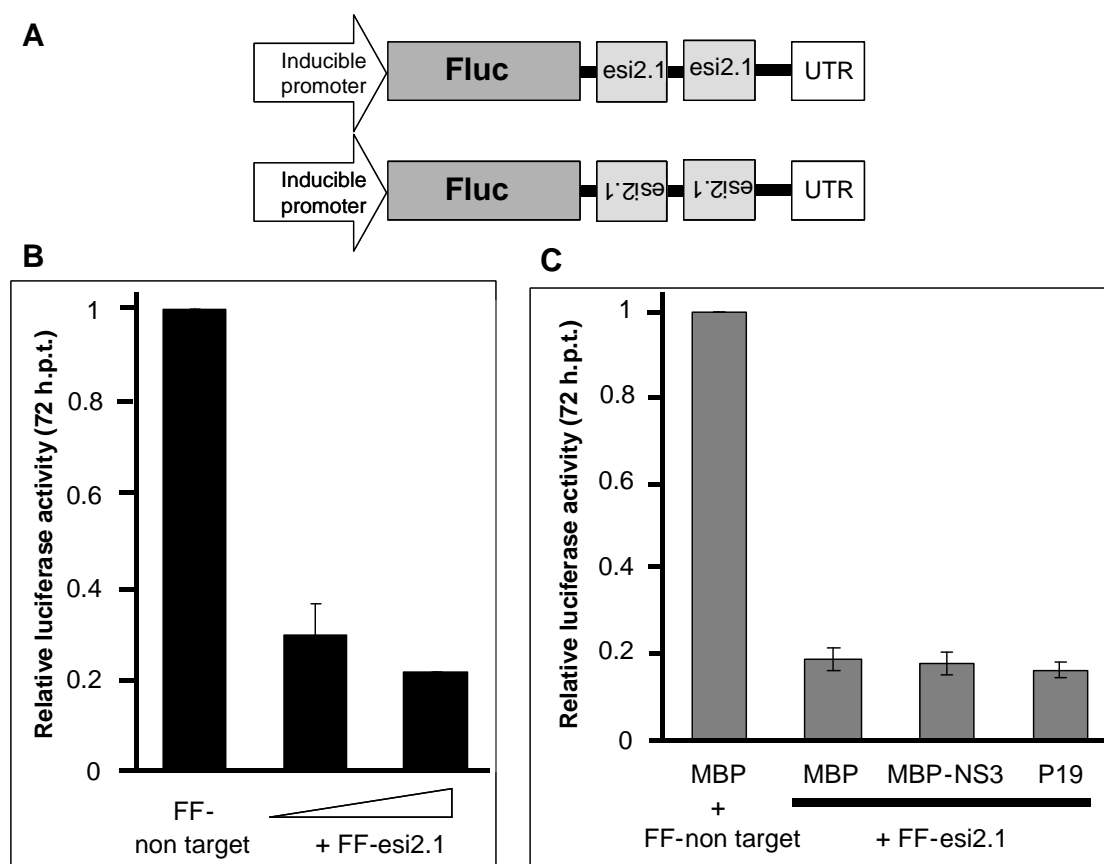


Figure 5-4: Effect of RHBV NS3 on endogenous siRNA mediated pathway in insect cells
S2 cells were co-transfected with two different concentrations (B) of the inducible constructs encoding Fluc-esi2.1 or Fluc-non target (Fluc-inverted esi2.1) (A), Renilla luciferase (Rluc) and constructs constitutively expressing different RSS proteins (MBP, MBP-NS3 or CIRV-P19). Expression of luciferases was induced 2 days post transfection (dpt) and the relative luciferase (Firefly/Renilla) was measured 3 d.p.t. The mean of at least two independent assays is shown with standard error, normalized to cells transfected with the non-target Fluc construct (C). Fluc, Firefly luciferase; MBP, maltose binding protein; NS3, non-structural protein 3; hpt, hours post transfection; dpt, days post transfection; esi2.1, endogenous siRNA 2.1; NS3(m), (mutant of) non-structural protein 3; S2, *Drosophila melanogaster* Schneider-2; RHBV, rice hoja blanca virus; CIRV, Carnation Italian ringspot virus.

Whereas in plants and insects an antiviral function of the miRNA pathway has not yet been established, the interference of mammalian infecting viruses with the miRNA pathway has been widely accepted (Berkhout & Jeang, 2007). MicroRNA-17 and -20a inhibit HIV-1 by down-regulation of histone acetylase PCAF, a co-factor of the Tat protein. The Tat protein interferes with the miRNA pathway during an infection and thereby overcomes this down-regulation of PCAF, resulting in a successful viral replication (Triboulet et al., 2007). Although little information is available about rice encoded miRNA molecules and their targets, computational analysis with the miRanda program revealed a possible interaction between OS-miR528 and the messenger RNA of the nucleocapsid protein of RHBV (Hemmes, 2007). In this context, it would be interesting to see if knockdown of the miRNA pathway in rice would result in higher virus titers. If so, it would strongly suggest that NS3 suppression of the miRNA pathway fulfils a need to support virus replication in rice.

Delphacid planthoppers transmit tenuiviruses in a propagative manner. In several cases replication of the virus in the vector has been studied, transovarial passage observed, and a deleterious effect of the virus on the vector (fecundity, nymph viability and longevity) been demonstrated (Falk & Tsai, 1998, Galvez, 1968, Jennings, 1971, Nault, 1988, Okuyama, 1968, Tsai, 1982). Although speculative, it is possible that the observed deleterious effects in the insect vector could be due to NS3 interference on the miRNA pathway.

RHBV NS3 is the first RSS protein reported to interact with the miRNA pathway in insect cells. Recently several viral RSS proteins have been tested for their interaction with the miRNA pathway in *Drosophila*, but the outcome was negative (Berry et al., 2009). However, here we have observed concentration dependent RSS activity for RHBV NS3 protein on miRNA-induced silencing, and such dosage effect could also explain the contradictory results observed with tombusvirus P19 protein (Figure 5-3 B, Berry et al., 2009). Besides, the observed differences in results for e.g. P19 could also be due to a divergence in amino acid sequence of the used strains, experimental set up, i. e. transgenic flies versus cell culture, and detection methods. This is further supported by the fact that several times negative results were observed regarding the suppressor activity of NS3 on the miRNA mediated silencing pathway in insects (Figure 5-5 A), likely due to a lower expression of NS3 in these experiments (Figure 5-5 B).

Likewise, the failure of NS3 to suppress endo-siRNA induced silencing in insect cells may have been due to relatively low levels of NS3 expression during the assay, combined with relatively high levels of endo-siRNA esi2.1. After all, endo-siRNA molecules and antiviral siRNAs are structural highly similar and like with the interference on the miRNA, a similar effect would hence be expected on the endo-siRNA pathway. This idea is further supported by the fact that CIRV P19 could also not suppress the endo-siRNA induced silencing in cultured S2 cells (Figure 5-4 C), while it has been reported a potent RSS protein on the endo-siRNA pathway in transgenic flies (Berry et al., 2009). If this also applies to NS3 remains to be investigated.

Whether the interaction with NS3 and the miRNA- and possibly endo-siRNA pathway occurs during a natural viral infection and the biological relevance of this interaction remains to be investigated.

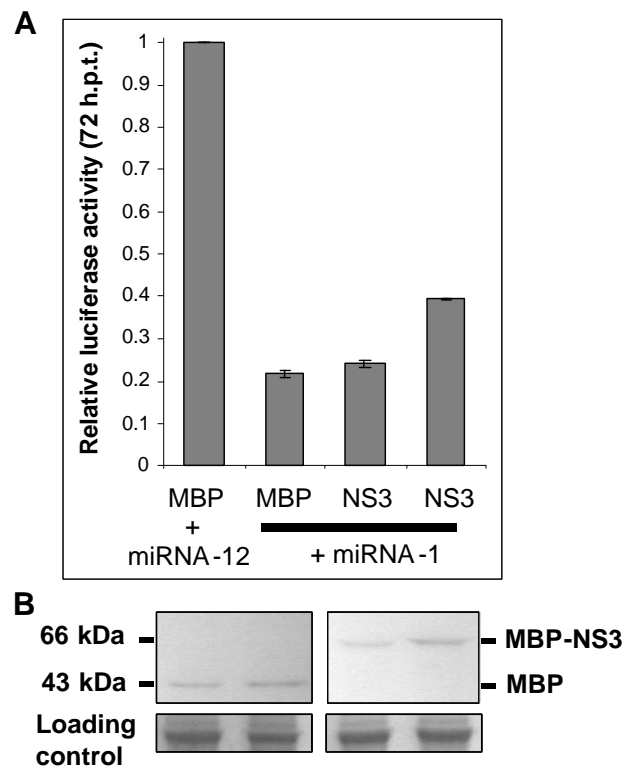


Figure 5-5: Suppression of miRNA mediated silencing in insect cells depends on RHBV NS3 concentration. The effect of NS3 on the miRNA mediated silencing was determined by co-transfection of S2 cells with pMT-Renilla, pMT-Firefly-3' UTR, a specific (miRNA1) or unspecific (miRNA12) primary miRNA and either pIB-MBP or -MBP-NS3. After induction, the relative luciferase expression (Firefly/Renilla) was determined 72 hours post transfection (hpt) and the mean of one experiment in duplo is shown with standard error (A). To correlate the presence or absence of suppressor activity of NS3, determined in A, a western blot analysis with a MBP specific polyclonal antibody was performed using the cell extract of panel A. As loading control, commasie brilliant blue staining and Bradford assays was used (B). MBP, maltose binding protein; NS3, non-structural protein 3; NS3(m), (mutant of) non-structural protein 3; S2, *Drosophila melanogaster* Schneider-2; RHBV, rice hoja blanca virus.

Materials and Methods

Plasmid constructs

Agrobacterium expression plasmids for MBP, MBP-NS3 and MBP-NS3m as well as the inducible insect expression vectors for Firefly and Renilla luciferase were described previously (Chapter 2, van Rij et al., 2006). The 3' UTR of the par6 gene was PCR amplified from the existing insect expression plasmid (Eulalio et al., 2007) to introduce *Sst*II and *Xba*I sites and allow feasible cloning behind the eGFP coding sequence. This reporter (eGFP-3'UTR) was cloned into the binary expression vector pK2GW7 (Karimi et al., 2002) using Gateway technology. In a similar fashion the par 6 gene 3'UTR was cloned behind the Firefly luciferase coding sequence (Fluc-3'UTR) in pMT-Fluc (van Rij et al., 2006). The binary vector expressing primary-miRNA-1 was PCR amplified using the previously described insect expression vector (Eulalio et al., 2007) as template, followed by Gateway technology recombination with the binary pK2GW7 vector (Karimi et al., 2002).

The insect expression vectors of MBP, MBP-NS3, MBP-NS3m and CIRV P19 were constructed by Gateway technology using the already described Entry vectors (Chapter 3) into pIB-GW (Invitrogen). Expression vectors encoding short hairpin RNA were constructed by annealing previously reported (Wakiyama et al., 2005) DNA oligos either against Firefly luciferase or eGFP and cloning them as *Kpn*I and *Xba*I fragment in pMT-B (Invitrogen). Inducible expression vectors encoding pri-miRNA1 or pri-miR12 were excised and subsequently cloned as either *Not*I-*Xba*I or *Kpn*I-*Xba*I fragment from the existing vectors (tub-miRNA1 and pAc-miR12, (Eulalio et al., 2007)) into pMT-B (Invitrogen).

Sensor vectors for the endo-siRNA assay were constructed using DNA oligos described by Czech (Czech et al., 2008) and cloned as *Mlu*I fragment behind the Firefly luciferase coding sequence of pMT-Fluc (van Rij et al., 2006).

Agrobacterium tumefaciens transient transformation assay (ATTA)

Agrobacterium infiltration was performed as previously described (Bucher et al., 2003). RDR6 silenced *N. benthamiana* leaves (Schwach et al., 2005) were co-infiltrated with *Agrobacterium* (at an OD₆₀₀=0.1) harbouring binary vectors encoding eGFP-3'UTR, pri-miRNA1 and either MBP, MBP-NS3 or MBP-NS3m. The eGFP expression in the leaves was visualized 5 days post infiltration with a Leica binocular microscope (Type S) and the GFP plus Fluorescence module 10446143.

Northern blot analysis

RNA extraction was performed as described previously (Bucher et al., 2004) and for the small RNA detection (miRNA/miRNA* and siRNAs), five µg RNA enriched for small RNAs as described previously (Hamilton & Baulcombe, 1999) was separated on a 20%, 0.5x TBE denaturing acrylamide gel. Following separation, the RNA was electroblotted onto Hybond-N⁺ (Pharmacia-Biotech) and crosslinked by UV-light. Hybridization was performed overnight at 48°C in modified church buffer (0.36 M Na₂HPO₄, 0.14 M NaH₂PO₄, 7% SDS and 1 mM

EDTA) with either an eGFP or pri-miRNA1 specific DIG-labelled DNA probe. The blots were washed briefly for three times with 2x SSC and three times for 15 minutes with 2xSSC and 0.2% SDS at 48°C. The labelled probe was detected by Western blot analysis using a DIG-specific antibody conjugated to alkaline phosphatase in blocking buffer (maleic acid buffer + 1% blocking reagent) and CSPD substrate (Roche) according to the manufacturer's recommendations.

Cell culture and transfection

Schneider (S)-2 cells were grown in Schneider medium (Invitrogen) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco) at 28°C. To reach a confluence of 60-70% at the time of transfection, cells were seeded 24 hours pre-transfection in a 96 well plate at a concentration of 5×10^4 cells per well. Transfections were performed using Cellfectine II (Invitrogen) according to the manufacturers' instructions.

For the shRNA suppressor assays, cells were co-transfected with luciferase-expressing plasmids, (15ng pMT-Fluc and 6ng pMT-Rluc) and 50 ng of sh-expressing vectors, either non-specific or Fluc specific. Next to this, cells were additionally co-transfected with 100 ng of the RSS expressing plasmid (MBP, MBP-NS3, MBP-NS3m or CIRV P19).

For the miRNA based suppressor assays, cells were co-transfected with 100 ng RSS expression plasmid (MBP, MBP-NS3, MBP-NS3m or CIRV P19), 12.5 ng pMT-Fluc-3'UTR, 3 ng pMT-Rluc and 2.5 ng pMT-miRNA, either pri-miRNA1 or pri-miRNA12.

For the endo-siRNA based assay, cells were co-transfected with 3.5 ng Fluc expression constructs (pMT-Fluc-esi2.1 or pMT-Fluc-non target), 2 ng pMT-Renilla luciferase and 190 ng RSS expressing construct (pIB-MBP, - MBP-NS3, or -CIRV P19).

Expression of the inducible constructs was induced 48 hours post transfection by 5 μ M CuSO₄ and assayed 24 hours post induction.

Luciferase assays and expression analysis

Cells were lysed 72 hours post transfection and luciferase expression was determined using self made buffers for the Dual luciferase reporter assay (Dyer et al., 2000).

Expression of MBP-tagged proteins was analysed by western blot analysis. Protein concentration of the lysate prepared for the Dual luciferase assay was determined by Bradford assay (Biorad), as described by the manufacturers' procedures. Two μ g total protein was resolved on a SDS-page gel, semi-dry transferred to Immobilon-P (Millipore) and MBP was subsequently detected using a MBP-specific rabbit primary- and goat alkaline phosphatase conjugated secondary antibody. Proteins were visualized with NBT-BCIP as substrate (Roche) according to the manufacturers' protocol. As loading control proteins were stained by commasie brilliant blue.

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Chapter 6

Diverging affinity of tospovirus RNA silencing suppressor proteins, NSs, for various RNA duplex molecules

Abstract

The tospovirus NSs protein was previously shown to suppress the antiviral RNA silencing mechanism in plants. Here the biochemical analysis of NSs proteins from different tospoviruses, using purified NSs or NSs containing cell extracts, is described. The results showed that all tospoviral NSs proteins analyzed exhibited affinity to small double stranded RNA molecules, i.e. siRNAs and miRNA/miRNA* duplexes. Interestingly, the NSs proteins from *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Groundnut ringspot virus* (GRSV) also showed affinity to long dsRNA, whereas *Tomato yellow ring virus* (TYRV) NSs did not. The TSWV NSs protein was shown to be capable to inhibit Dicer-mediated cleavage of long dsRNA *in vitro*. In addition, it suppressed the accumulation of GFP-specific siRNAs during co-infiltration with an inverted repeat-GFP RNA construct in *Nicotiana benthamiana*. *In vivo* interference of TSWV NSs on the miRNA pathway was shown by suppression of an eGFP miRNA-sensor construct. The ability to stabilize miRNA/miRNA* by different tospovirus NSs proteins *in vivo* was demonstrated by increased accumulation and detection of both miRNA171c and miRNA171c* in tospovirus-infected *N. benthamiana*. All together, these data suggest that tospoviruses interfere in the RNA silencing pathway by sequestering siRNA and miRNA/miRNA* molecules before they are uploaded into their respective RNA induced silencing complexes. The observed affinity to long dsRNA for only a subset of the tospoviruses studied is discussed in light of evolutionary divergence and their ancestral relation to the animal-infecting members of the *Bunyaviridae*.

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Introduction

Over recent years, RNA silencing has become known as one of the major defense mechanisms acting against viruses in plants and insects (Ding & Voinnet, 2007, Galiana-Arnoux et al., 2006, Galiana-Arnoux & Imler, 2006, van Rij et al., 2006). During a virus infection double stranded (ds)RNA molecules arise as replicative intermediates or due to the formation of secondary RNA structures. These are recognized by specific Dicer-like proteins (Dicer in insects and predominantly DCL4 in plants) and processed into 21 nt siRNA molecules (Bernstein et al., 2001, Deleris et al., 2006, Hamilton & Baulcombe, 1999). One strand of this molecule, the guide-strand, is incorporated in RISC and enables its to recognize and degrade complementary (viral) target RNA molecules through the action of the core AGO protein (Hammond et al., 2000, Nykanen et al., 2001). In plants, these 21 nt primary siRNAs have been shown to serve as primers for the host encoded RDR to convert RNA target sequences into new long dsRNAs. These in turn are processed into secondary siRNAs. In this way, silencing is not only being amplified but also spread along the entire RNA target sequence (transitive silencing) (Vaistij et al., 2002).

Two other plant DCL proteins (DCL-2 and DCL-3) also play a role in processing dsRNA, but these generally lead to 22-26 nts sized siRNAs which are suggested to function in a range of other purposes unrelated to viral defence (Aliyari & Ding, 2009). DCL-1 produces miRNAs that are structurally similar to siRNA molecules, but originate after processing of long host encoded RNA transcripts called pri-miRNA, via pre-miRNA into miRNA/miRNA* duplexes. After unwinding of miRNA/miRNA* duplexes one strand is incorporated into RISC whereas the other strand, miRNA*, is rapidly degraded after cleavage by an AGO protein (Wang et al., 2008). MicroRNAs are regulatory factors that are loaded into RISC to silence host encoded genes by either RNA degradation or translational inhibition of their target (Voinnet, 2009). In insects, Dicer-2 is required for the siRNA and Dicer-1 for the miRNA processing steps that are divided over multiple DCLs in plants (Aliyari & Ding, 2009).

As a response to antiviral RNA silencing, many plant and insect viruses have been shown to express RSS proteins (Alvarado & Scholthof, 2009, Li & Ding, 2006). Most of these RSS proteins specifically bind either siRNAs or long dsRNA (Deleris et al., 2006, Lakatos et al., 2006, Merai et al., 2006), whereas some RSS proteins, like the polioviral p0 (Bortolamiol et al., 2008, Pfeffer et al., 2002, Zhang et al., 2006), interacts with key proteins of the RNA silencing pathway such as DCL or AGO. A few RSS proteins are able to interact at multiple points in the RNA silencing pathway. One such example is CMV 2b, which not only binds RNA molecules but also interacts with AGO (Goto et al., 2007, Zhang et al., 2006). Besides their interference in the antiviral siRNA pathway viral RSS proteins also effect the miRNA pathway, likely by sequestering miRNA/miRNA* duplexes, and thereby cause developmental defects in Arabidopsis after transgenic expression (Chapman et al., 2004, Dunoyer et al., 2004).

TSWV, is the type species of the *Tospovirus* genus within the family of arthropod-borne *Bunyaviridae*. In contrast to all other mammalian infecting members of the *Bunyaviridae*,

Tospoviruses specifically infect plants and are transmitted in a propagative manner by thrips (Falk & Tsai, 1998, Wijkamp et al., 1993). Tospoviruses are therefore a likely target of antiviral RNA silencing in both plant and insect hosts. Previously, the TSWV NSs protein has been shown to suppress RNA silencing in plants and insects (Bucher et al., 2003, Garcia et al., 2006, Reavy et al., 2004, Takeda et al., 2002). Accumulation of this protein, at least in plants coincides with increased virulence of the virus (Kormelink et al., 1991). It is hypothesized that the RSS mode of action of Tospovirus NSs proteins is accomplished by interacting with a component of the antiviral RNA silencing pathway that is shared between plants and insects, i.e. dsRNA. To test this hypothesis, the affinity of TSWV NSs to a range of dsRNA molecules of the si- and miRNA pathway was analyzed *in vitro* and verified by reporter-based assays *in vivo*. The analysis was broadened to other tospoviral NSs proteins in order to determine if the observed mode of action is a general feature for tospoviral NSs proteins.

Results

TSWV NSs binds long and short dsRNA *in vitro*

Previous results showed that from the TSWV proteins tested, only the NSs protein was able to suppress RNA silencing in *Agrobacterium*-infiltration assays (Bucher et al., 2003, Takeda et al., 2002). Furthermore, silencing suppression by NSs revealed significantly lower levels of target siRNA in comparison to suppression by tombusviral P19 and RHBV NS3, both known to specifically bind only small dsRNAs (i.e. siRNAs) (Bucher et al., 2003, Hemmes et al., 2007, Lakatos et al., 2006, Takeda et al., 2002). These results implied that TSWV NSs interferes in the RNA silencing pathway upstream of siRNA synthesis, e.g. by binding to long dsRNA and thereby preventing these from becoming processed into siRNAs by Dicer (like) proteins.

To analyze whether TSWV NSs exerts its suppressor function by sequestering dsRNA, the affinity to various dsRNA molecules was analyzed. *In vivo*, NSs protein tends to form large insoluble aggregates (Kormelink et al., 1991), and earlier attempts to express and purify NSs from *E. coli* failed due to solubility problems. Thioredoxin has been reported to increase translation efficiency and solubility of eukaryotic proteins expressed in *E.coli* (LaVallie et al., 1993). For these reasons and because N-terminal fusions to NSs were shown not to hamper RSS activity (data not shown), NSs was expressed in *E. coli*, fused at its N-terminus to his-tagged (HP-) Thioredoxin. After purification, the HP-thioredoxin-NSs fusion protein was incubated with radio-labelled 114 nt dsRNA or 21 nt siRNA molecules and subsequently analyzed by EMSA on native acryl-amide gels (Lakatos et al., 2004). These analyses revealed that upon increasing NSs concentration both siRNA (Figure 6-1A) and 114 nt dsRNA (Figure 6-1C) showed a retardation in electrophoretic mobility. Altogether this indicates that TSWV NSs was able to bind both siRNA and long dsRNA. As negative controls, dsRNA or siRNAs were incubated with purified HP-thioredoxin N-terminal fused to the inert MBP and in both cases, even at the highest concentrations tested, no complex formation was observed

(Figure 6-1B and 6-1D). While the mobility of siRNAs was still retarded when incubated in the presence of rather low concentration (14.8 nM) of NSs, only binding to 114 nt dsRNA was observed at significantly higher concentrations of NSs (237.5 nM). These results indicated a higher affinity of TSWV NSs for siRNA molecules compared to long dsRNA.

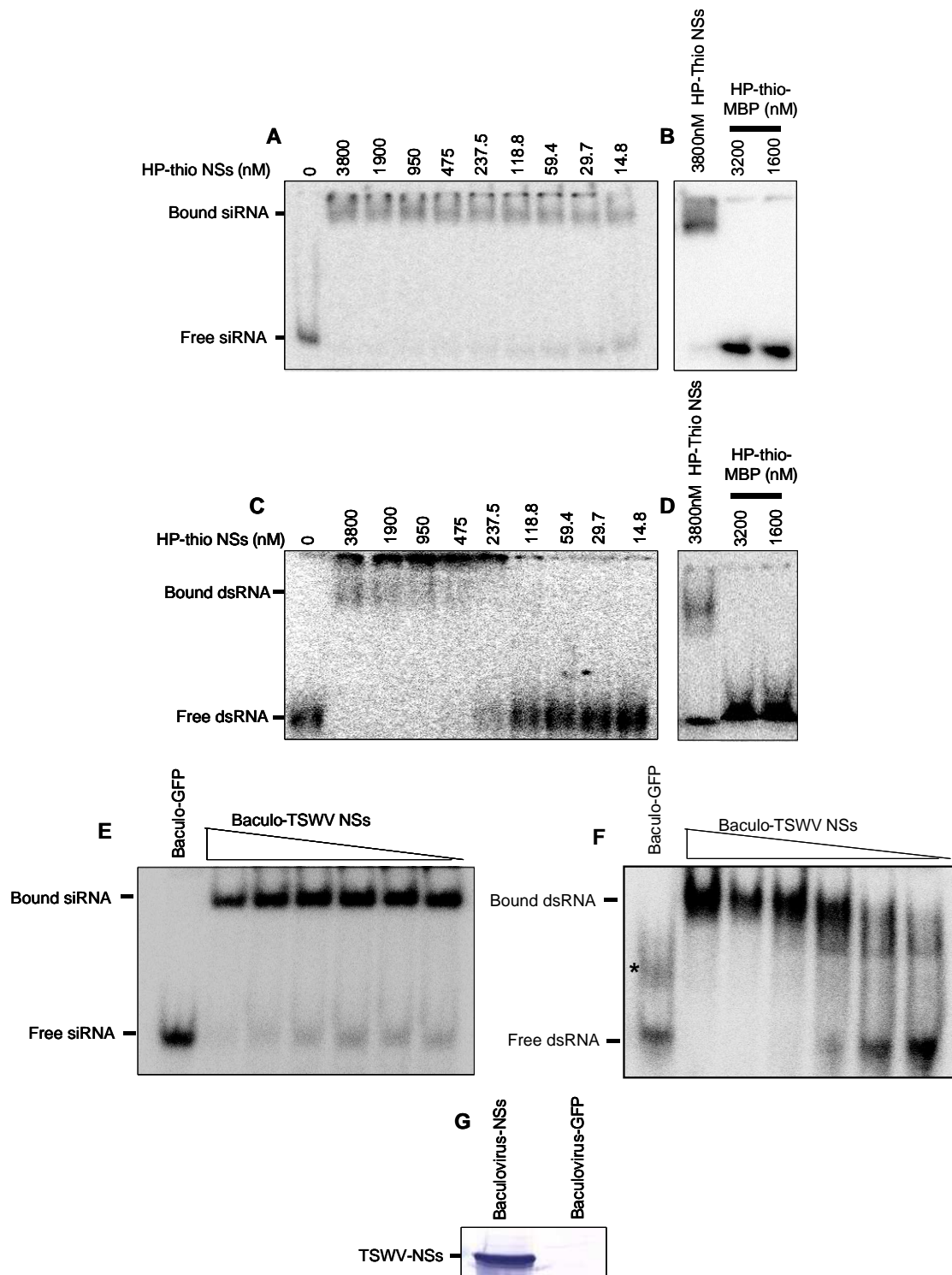


Figure 6-1: Affinity of TSWV NSs for 21 nt siRNAs and 114 nt dsRNA.

Different concentrations of either bacterially purified HP-thioredoxin-NSs protein or insect cell extract infected with a recombinant baculovirus expressing NSs or GFP, were incubated for 20 minutes at room temperature with 100 pM of 32 P-labelled siRNA (panels A, E) or 114 nt dsRNA (panels C, F). RNA-protein complexes were separated on a native polyacrylamide gel and a representative picture is shown from at least two independent experiments. As negative controls, RNA was incubated in the presence of elution buffer (panels A and C, first lane), GFP containing insect cell extracts (panel E and F, first lane) or HP-thioredoxin-MBP (panel B and D). In case of HP-thioredoxin-MBP (panel B and D), HP-thioredoxin-NSs was used as positive control. Expression of NSs in infected insect cell extracts was verified by western blot analysis using a NSs specific polyclonal antibody (panel G). TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment; nt, nucleotide; dsRNA, Double stranded RNA; GFP, green fluorescent protein; siRNA, small-interfering RNA; HP-, His-tagged; MBP, maltose binding protein; Thio, Thioredoxin.

To verify these results, experiments were repeated using native NSs protein produced from the eukaryotic baculovirus-insect cell expression system. Due to the lack of a tag for convenient purification purposes, entire insect cell extracts containing the expressed NSs were used. To determine the binding affinity of NSs, extracts were prepared and incubated with radio-labelled 21 nt siRNAs (Figure 6-1E) or long (114 nt) dsRNA molecules (Figure 6-1F). Western blot analysis demonstrated the expression and presence of NSs in the soluble fraction of the used insect cell extract (Figure 6-1G). The EMSA analyses (Figure 6-1E & F) showed that the NSs containing insect cell extracts exhibited dsRNA affinity profiles similar to the results obtained with purified HP-thioredoxin NSs. Similar binding affinities were also observed with longer, 400 nt dsRNA molecules (data not shown). Although, complex formation with 114 nt dsRNA (and 400 nt dsRNA) was also observed in the negative control, insect cell extracts infected with a recombinant baculovirus expressing GFP (Figure 6-1F, asterisk), the complex clearly showed a different mobility from the NSs-RNA complex. Due to the absence of this complex when using siRNA in EMSA (Figure 6-1E), the origin of this complex was not investigated further.

To confirm the higher affinity of NSs for siRNAs versus long dsRNA, an *in vitro* affinity competition was performed. To this end, a fixed amount of baculovirus-infected cell extract was mixed with radio-labelled siRNAs in the presence of an increasing concentration of non-labelled long dsRNA competitor (Figure 6-2A) and vice versa (Figure 6-2B). For easier comparison the percentage of bound versus unbound labelled RNA molecules was quantified and presented in a graph (Figure 6-2C). Results show that whereas the amount of siRNAs present in a NSs complex (approx. 91%) hardly changed upon addition of long dsRNA (Figure 6-2A), in the reciprocal situation the amount of NSs-long dsRNA complexes readily reduced (from an approx. 100% bound status in the absence of siRNA competitor to an approx. 58% bound status at 100x molar excess of siRNA competitor) by the addition of siRNA competitors (Figure 2B & C). The latter was confirmed by an increased signal of unbound dsRNA (Figure 6-2) (from 0% to 90%). These results further supported a higher affinity of TSWV NSs for siRNA molecules over long dsRNA. As described earlier above (Figure 6-1F, asterisk) again a distinct mobility shift was observed when, as a negative control, the long dsRNA molecules were incubated with insect cell extracts infected with a recombinant baculovirus expressing GFP (Figure 6-2B).

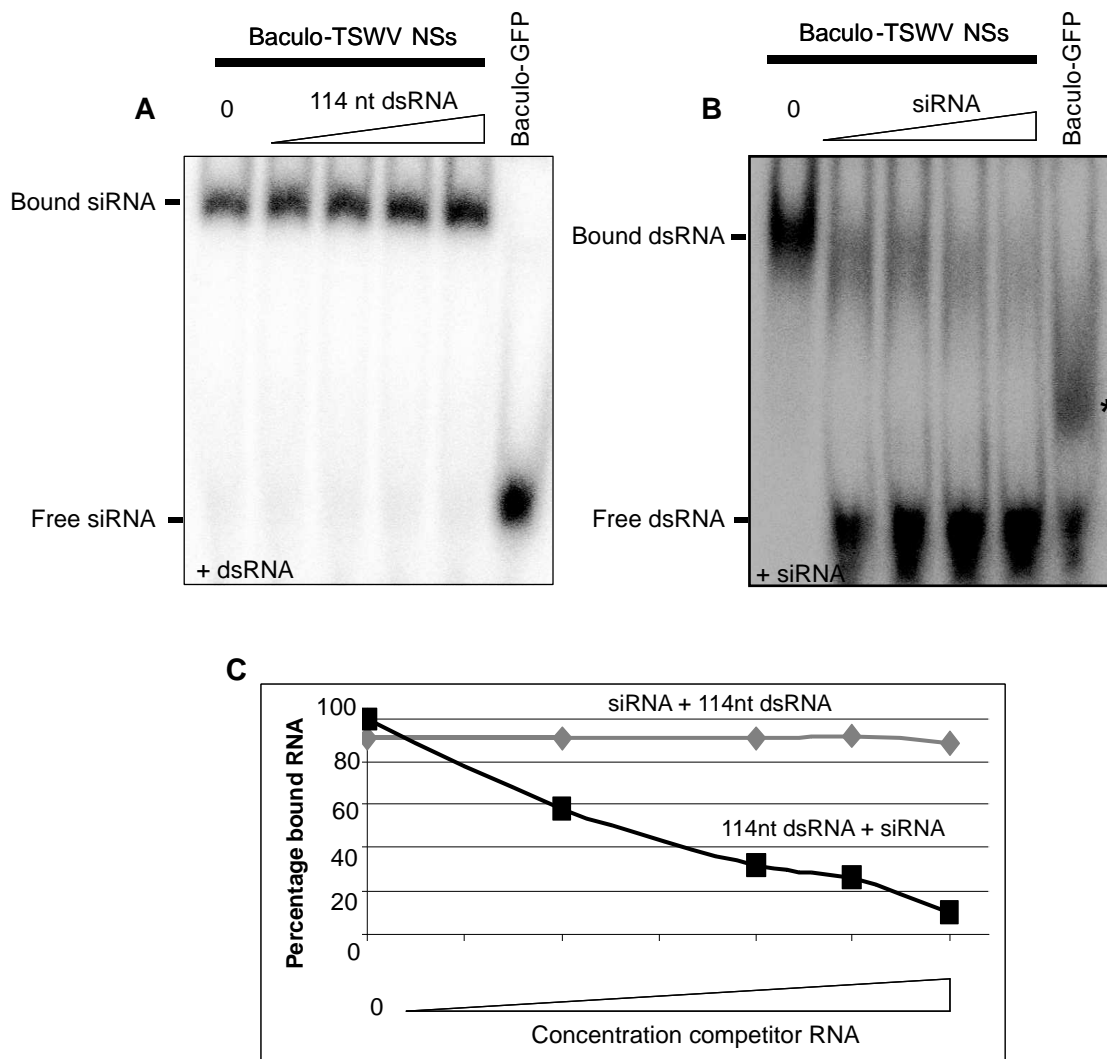


Figure 6-2: Competition experiments with recombinant baculovirus-NSs-infected cell extracts for siRNAs and 114 nt dsRNA.

Fixed concentrations of insect cell extracts infected with a baculovirus expressing TSWV NSs were incubated with ^{32}P -labelled siRNA and increasing amounts (0, 100x, 200x, 250x, 300x molar excess) of unlabeled 114 nt dsRNA competitor molecules (panel A) or ^{32}P -labelled 114 nt dsRNA and increasing amounts (0, 100x, 200x, 250x, 300x molar excess) of unlabeled siRNA competitor molecules (panel B). Samples were loaded and resolved on a native acrylamide gel. As negative control, extracts from cells infected with a GFP expressing baculovirus were used. In case of 114 nt dsRNA, a lower retardation complex is formed for the negative control (*). The percentage of bound RNA was quantified by GeneTools (SynGene) and represented versus the concentration of competitor (114 nt dsRNA or 21 nt siRNA in case of the diamond or quadrat line respectively) concentration (panel C). TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment; nt, nucleotide; dsRNA, Double stranded RNA; GFP, green fluorescent protein; siRNA, small-interfering RNA.

TSWV NSs inhibits Dicer-mediated dsRNA processing

Since TSWV NSs exhibited affinity to long dsRNA (Fig 1C & 1F), and lower levels of target siRNAs from silenced genes were observed in previous experiments (Bucher et al., 2003), it was tempting to assume that TSWV NSs prevents long dsRNA from becoming cleaved by DCL proteins. To test whether TSWV NSs indeed was able to interfere with Dicer-mediated dsRNA processing, Dicer cleavage assays using *Drosophila* embryo extracts were performed. As substrate 114 nt dsRNA was used and the production of 21 nt siRNAs was monitored in

the presence of increasing amounts of insect cell extracts containing recombinant baculovirus-expressed TSWV NSs. As a negative control, extract of insect cells infected with a recombinant baculovirus-GFP was included. Only when extracts harbouring TSWV NSs were added, the formation of siRNAs was significantly reduced (Figure 6-3A), indicating that TSWV NSs interfered with Dicer cleavage of dsRNA *in vitro*. For comparative purposes, the relative percentage of processed dsRNA was quantified for three independent Dicer cleavage reactions (Figure 6-3B).

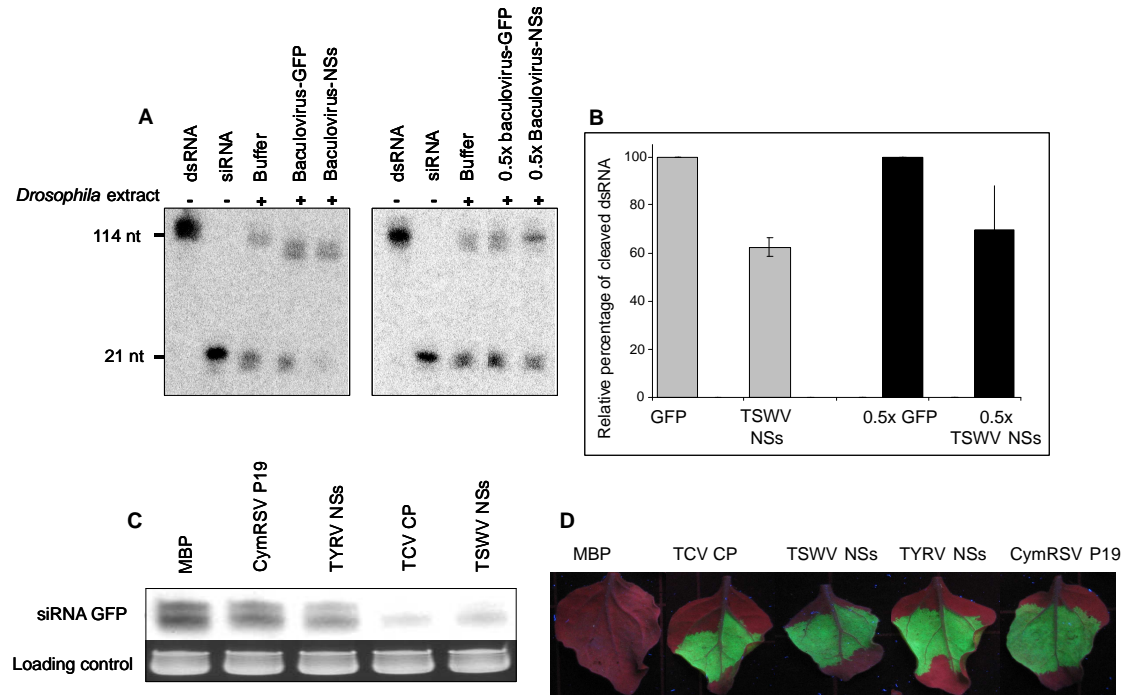


Figure 6-3: Analysis of Dicer-mediated dsRNA cleavage in the presence of TSWV-NSs.

Radioactively labelled dsRNA was cleaved into siRNA using *Drosophila* embryo extract in lysis buffer, but inhibited in the presence of TSWV NSs expressing baculovirus infected insect cell extracts. As controls, GFP expressing baculovirus-infected insect cell extracts were used as negative control and undiluted and 0.5x diluted ("0.5xGFP, 0.5x TSWV NSs") extracts. A representative picture of at least two independent experiments is presented (panel A). The fraction of cleaved dsRNA was quantified by GeneTools (SynGene) and represented as relative percentage, by setting the amount of cleaved dsRNA in GFP extract as 100% (panel B), for undiluted (grey bars) and 0.5x diluted extracts (black bars; panel B).

Agrobacterium strains harbouring vectors encoding IR-GFP were co-infiltrated in *N. benthamiana* leaves with MBP (negative control), TCV CP (positive control), CymRSV P19, TSWV NSs or TYRV NSs constructs. The corresponding GFP siRNAs levels were detected by northern blot analysis (panel C). Ethidium bromide-stained RNA was used as loading control. To ensure silencing suppressor activity of the tested RSS proteins, their gene constructs were co-infiltrated with a GFP construct in *N. benthamiana* leaves and monitored for GFP expression 5 days post-infiltration (panel D). TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment: nt, nucleotide; dsRNA, Double stranded RNA; GFP, green fluorescent protein; siRNA, small-interfering RNA; MBP, maltose binding protein; TCV CP, Turnip crinkle virus coat protein; CymRSV, Cymbidium ringspot virus; TYRV, Tomato yellow ring virus.

To verify whether the *in vitro* observed inhibition of Dicer cleavage by TSWV NSs also occurred *in vivo*, plants were co-infiltrated with *Agrobacterium* harbouring a plasmid encoding an inverted repeat of GFP (IR-GFP) and /or GFP and either TSWV NSs, *Tomato yellow ring virus* (TYRV) NSs or *Cymbidium ring spot virus* (CymRSV) P19, the latter as a negative control. *Turnip crinkle virus* (TCV) coat protein (CP), previously shown to bind long dsRNA and to inhibit Dicer cleavage (Merai et al., 2006), was used as a positive control. An *Agrobacterium* strain expressing the Maltose binding protein (MBP) was included as negative

control. The presence of green fluorescence (Figure 6-3D) during all co-infiltrations, except for the negative control MBP, indicated that all RSS proteins analyzed (TSWV NSs, TYRV NSs, CymRSV P19 and TCV CP) were able to inhibit RNA silencing induced by IR-GFP. To analyze whether this was due to inhibition of Dicer cleavage of the IR-GFP RNA, leaves were harvested three days post infiltration, RNA was isolated, enriched for small RNA and investigated by northern blot analysis for the presence of GFP siRNA (Bucher et al., 2003). Relative to the loading controls, GFP specific siRNAs were observed in significant lower amounts in samples from leaves co-infiltrated with TSWV NSs or TCV CP (Figure 6-3C), compared to those from leaves co-infiltrated with TYRV NSs, CymRSV P19 or MBP. These findings supported the idea that TSWV NSs, like TCV CP, was able to associate with long dsRNA and thereby inhibited Dicer cleavage of these long dsRNA. As CymRSV P19 is known to exclusively bind siRNAs and suppresses RNA silencing downstream of Dicer, the similar levels of GFP siRNAs observed for TYRV NSs and CymRSV P19 suggested that TYRV NSs was not able to inhibit Dicer cleavage of long dsRNA.

Binding dsRNA is a common feature of tospoviral NSs proteins

The observation that TYRV NSs was not able to inhibit Dicer cleavage suggested a very low affinity of TYRV NSs for long dsRNA and is therefore distinct from TSWV NSs. To test whether the affinity for differently sized dsRNA molecules, as was demonstrated for TSWV NSs (Figure 1), is shared among other tospoviruses a comparative EMSA analysis was performed with several tospoviruses. To this end, infected plant extracts from a range of different Tospovirus species (TSWV, *Groundnut ringspot virus* (GRSV) and *Impatiens necrotic spot virus* (INSV) from the *American* clade and TYRV from the *Eurasian* clade (Hassani-Mehraban et al., 2005, Pappu et al., 2009) were incubated with radio-labelled molecules (siRNA or 114 nt dsRNA) and analyzed by EMSA (Meraï et al., 2006). The results showed that NSs containing protein extracts of all tospoviruses were able to shift, and thus bind siRNA molecules (Figure 6-4A). Surprisingly, the extracts from INSV and GRSV showed an additional strong affinity for longer dsRNA molecules (Figure 6-4C), while the extract from TYRV NSs did not (Figure 6-4C). The latter was in agreement with the earlier observed lack of the indirect inhibition on Dicer cleavage by TYRV NSs (Figure 6-3C). Surprisingly, nearly no affinity to 114 nt dsRNA could be observed for TSWV NSs in this assay (Figure 6-4C), in contrast to previous results with *E.coli* and baculo-virus expressed TSWV NSs (Figure 6-1). As already mentioned for the baculovirus infected extract (Figure 6-1F, asterik) a lower complex was observed in case of TSWV and TYRV with 114 nt dsRNA. This retardation was not due to NSs since a similar retardation complex was being observed with uninfected plant extracts as negative control (Figure 6-4C, asterik) and therefore not further investigated.

To quantify the affinity to longer and shorter dsRNA a serial dilution series of INSV- and GRSV-infected plant material was made and used in dsRNA binding assays. Results from this analysis showed that binding to long dsRNA was lost at dilutions of INSV- and GRSV-infected plant extracts that contained ~0.25 µg total protein per 10 µl whereas binding to 21 nt

siRNA molecules was not observed below $\sim 0.06 \mu\text{g}$ total protein per $10 \mu\text{l}$ (compare Figure 6-4E & F). This indicated a slightly higher affinity to siRNA molecules of GRSV NSs and INSV NSs (data not shown), as similarly observed for baculovirus and *E.coli* expressed TSWV NSs (Figure 6-1A-D).

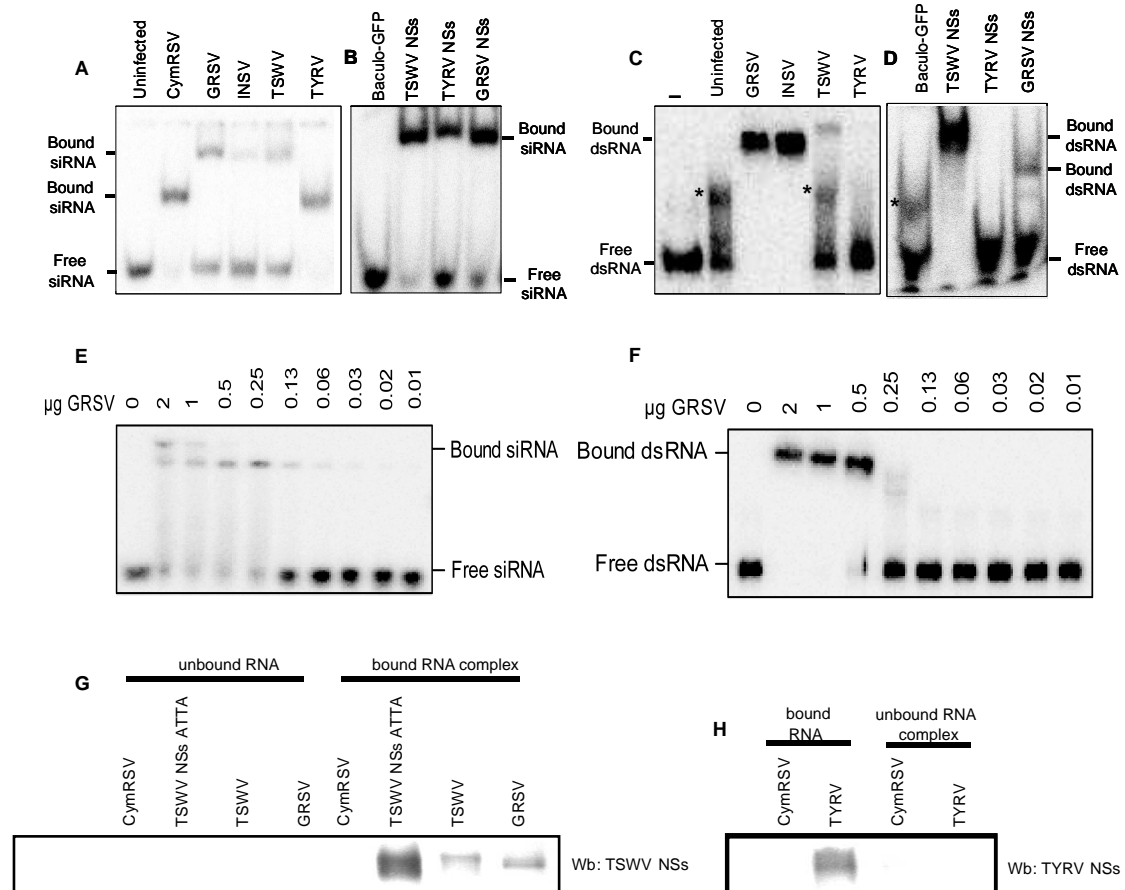


Figure 6-4: Affinity analysis of NSs from Tospovirus-infected plant extracts for dsRNA molecules.

Electrophoretic mobility shift assays were performed using systemically infected *N. benthamiana* leaf extracts containing CymRSV, GRSV, INSV, TSWV or TYRV, incubated with radioactively labelled 21 nt siRNA and subsequently resolved onto a 8% native gel (A). Except for CymRSV the experiment was repeated with radioactively labelled 114 nt dsRNA and resolved on a 5% native gel (C). Uninfected plant extract (uninfected) and RNA only (-) were included as negative controls (panels A and C, first two lanes). Experiments of A and C were repeated using recombinant baculovirus-infected extract containing GRSV NSs, TYRV NSs, TSWV NSs or GFP (siRNAs panel B, 114 nt dsRNA panel D). Complexes formed in the negative controls (uninfected or GFP expressing) are indicated (*) (panel C, lane 2 and panel D, lane 1). A serial dilution of GRSV infected *N. benthamiana* extracts ($2 \mu\text{g}$ - 15.6 ng protein content) was tested for the affinity to 21 nt siRNA (panel E) or 114 nt dsRNA (panel F). As negative control, the RNA duplex in extraction buffer was included (panels E and F, first lane). Western blot analysis was performed on excised and denatured siRNA-protein complexes as observed in panel A using either an antibody against TSWV NSs (G) or a monoclonal NSs antibody detecting Asian tospoviral NSs (H). The siRNA-protein complex of TSWV NSs expressed in *Agrobacterium* transient transformation assay (ATTA) infiltrated leaf extract (TSWV NSs ATTA) was used as positive control. As negative control gel slices of unbound siRNAs were used (unbound RNA complex). TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment: nt, nucleotide; dsRNA, double stranded RNA; GFP, green fluorescent protein; siRNA, small-interfering RNA; TCV CP, Turnip crinkle virus coat protein; CymRSV, Cymbidium ringspot virus; TYRV, Tomato yellow ring virus; GRSV, Groundnut ringspot virus; INSV, Impatiens necrotic spot virus.

It was assumed that the tospoviral NSs proteins provided the dsRNA binding activity in the infected plant extract. Due to the lack of any NSs specific antibody (INSV) or the incapacity of

the present antibodies to detect native NSs proteins efficiently (GRSV, TSWV and TYRV), supershift and immuno-precipitation experiments (data not shown) showed negative results. Therefore, the presence of NSs in the retarded dsRNA complexes formed in tospoviral plant infected extract was demonstrated. TSWV-, GRSV-, TYRV- and CymRSV- (negative control) infected plant extracts and *Agrobacterium* infiltrated leave extract expressing TSWV NSs (positive control) were incubated with radiolabeled siRNA molecules, EMSA analyzed and shifted siRNA complexes excised from gel. These were subsequently resolved on SDS-PAGE, using excised unbound siRNA molecules as negative control, and after western blotting screened for the presence of NSs. The mobility shifted siRNA complexes from TSWV-, GRSV- and TYRV-infected or TSWV-NSs agro-infiltrated extracts clearly showed the presence of NSs (Figure 6-4G & H) which was not detected in the unbound siRNA or CymRSV infected samples. This supports the hypothesis that the retarded complexes consist of the tospoviral NSs proteins that provide the dsRNA binding activity in the infected plant extracts.

To further substantiate the observed differences in binding affinities of tospovirus NSs proteins to long dsRNA and to strengthen the idea that the NSs proteins were binding the dsRNA molecules, another expression system was used. EMSA analyses were performed using extracts from recombinant baculovirus-infected insect cells expressing NSs from TSWV, GRSV (*American* clade) and TYRV (*Eurasian* clade). As expected, all NSs extracts showed retardation of siRNA molecules (Figure 6-4B) but not of single stranded RNA molecules (data not shown). A retardation of long, either 114 nt or 400 nt dsRNA was again observed in the presence of NSs from TSWV and GRSV, but not with TYRV NSs containing extracts (Figure 6-4D, data not shown). This is in agreement at least with the results obtained with infected plant extract containing GRSV and TYRV NSs. In dilution series and competition EMSA analysis, baculovirus expressed GRSV NSs revealed again higher affinities for siRNA than for long dsRNA molecules (data not shown), as already observed in infected plant extract (Figure 6-4E & F) and for baculovirus expressed TSWV NSs (Figure 6-1 and 6-2).

Tospoviral NSs proteins interfere with the miRNA pathway

The reported loss of leaf polarity and interference with the formation of plant reproduction organs after constitutive expression of plant viral RSS in *Arabidopsis* has been explained as a result from suppression of miRNA-mediated gene regulation (Chapman et al., 2004, Dunoyer et al., 2004). Since siRNAs and miRNA/miRNA* duplexes share structural similarities, it was not surprising that the RHBV NS3 and tombusvirus P19 RSS proteins, both able to bind siRNAs, exhibited similar affinity to miRNA/miRNA* duplexes (Dunoyer et al., 2004, Hemmes et al., 2007). To investigate whether the TSWV NSs protein was also able to interfere with miRNA-mediated gene regulation, the affinity to either miRNA/miRNA* duplexes or the longer pre-miRNA was analyzed using infected insect cell extracts containing baculovirus expressed TSWV NSs. EMSA assays with increasing amounts of NSs containing cell extracts showed

retardation of the miRNA/miRNA* duplex slightly lower than to that observed for siRNAs (Figure 6-5A and Figure 6-1A). For the pre-miRNA molecules no shift was observed even at the highest concentration of cell extracts used (Figure 6-5B), indicating that in this assay TSWV NSs only shows affinity for miRNA/miRNA* duplexes.

The affinity of TSWV NSs for miRNA/miRNA* duplexes *in vitro* implied that NSs could potentially interfere with the miRNA pathway in plants by sequestering miRNA/miRNA* molecules. To test this hypothesis, a miRNA-based sensor construct encoding eGFP harbouring a 3'UTR with target sites for miRNA1 (eGFP-3'UTR) was agro-infiltrated in RDR6 silenced *N. benthamiana* together with either TSWV NSs or the negative control MBP. Using the miRNA1 dependent sensor construct, instead of previous reported ones (Parizotto et al., 2004), ensured that the observed miRNA/miRNA* duplex binding by NSs is not sequence specific for miRNA171 (chapter 5, Figure 5-1A).

Normally, during *Agrobacterium* infiltration of this eGFP-miRNA1 sensor construct, the host encoded RDR6 converts functional RNA transcripts into dsRNA, resulting into silencing of eGFP and production of eGFP specific siRNAs (Figure 6-5D). In RDR6 knock down plants, no silencing of this construct occurs unless it is co-infiltrated with pri-miRNA1 (Dalmay et al., 2000, Parizotto et al., 2004). As expected, in the absence of pri-miRNA1, RDR6 knock down plants showed similar eGFP fluorescence in the presence or absence of RSS protein (Figure 6-5C upper panels), while a drastic decrease in eGFP fluorescence level was observed when these plants were co- infiltrated with eGFP-3'UTR and pri-miRNA1 (Figure 6-5C lower left panel). Enhanced GFP fluorescence was restored by the addition of TSWV NSs (Figure 6-5C lower right panel) and not when using MBP as negative control (Figure 6-5C lower left panel), demonstrating that TSWV NSs was able to suppress miRNA-induced silencing. Whereas no eGFP specific siRNAs were observed in the absence of miRNA1 (Figure 6-5D left panel, lane 2 & 3), only a slight amount of siRNAs was observed in the presence of miRNA1 (Figure 6-5D left panel, lane 4 & 5). In contrast, elevated levels of eGFP specific siRNAs were produced in case the sensor construct was infiltrated into wild type *N. benthamiana* plants (Figure 6-5D, lane 6 left panel). This strongly indicates that eGFP silencing was most likely the result of translational repression.

Similar results were obtained in insect cells expressing TSWV NSs and a Firefly luciferase based miRNA1 sensor construct (Figure 6-5E). This organism- independent suppressor effect supported the idea that the observed interference with the miRNA pathway is possibly due to miRNA/miRNA* sequestering and not to a protein specific interaction.

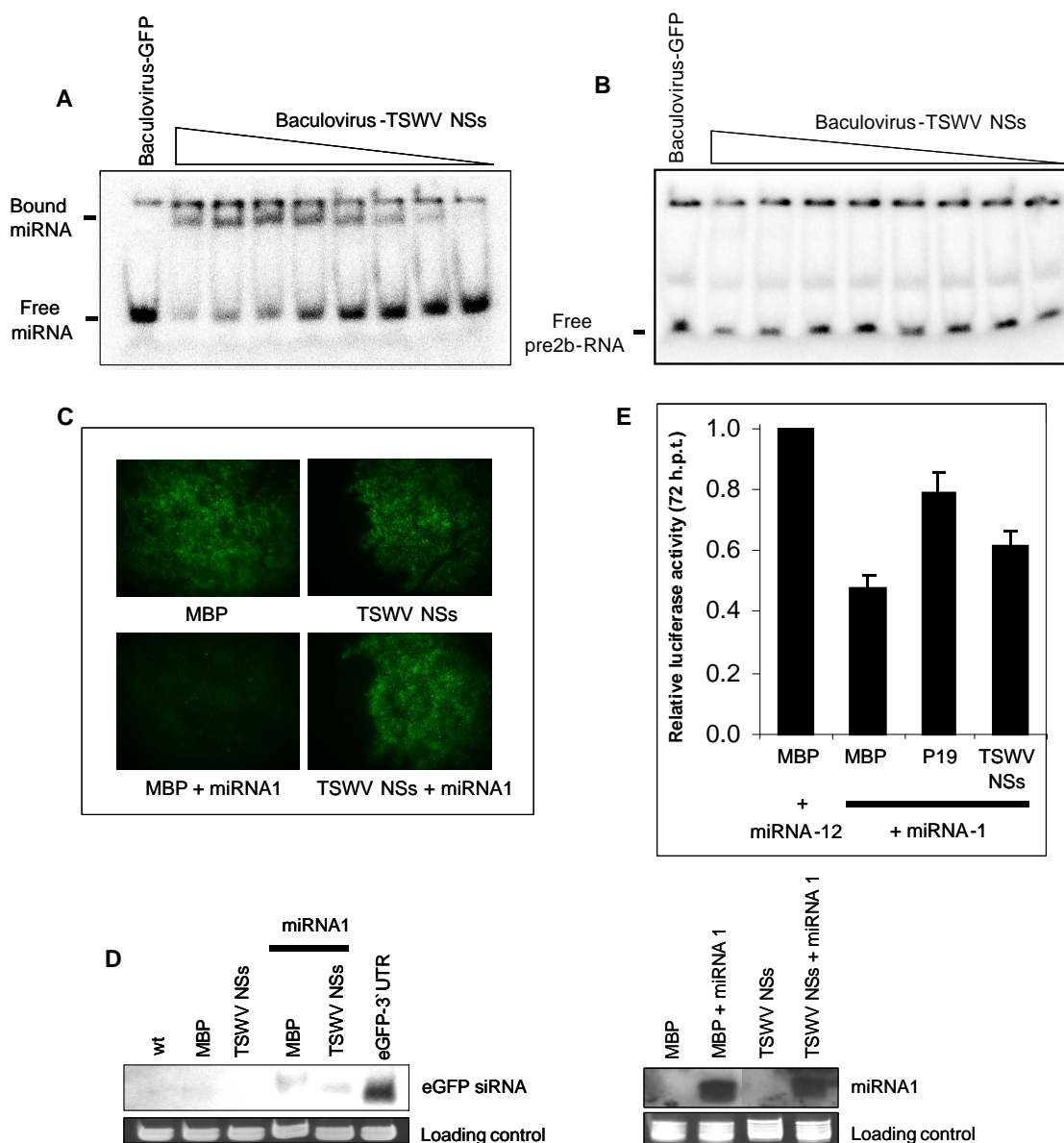


Figure 6-5: Analysis of TSWV NSs interference with the miRNA pathway.

Electrophoretic mobility shift analysis of radioactively labelled miRNA171/miRNA171* duplex (panel A) or pre-miRNA171/miRNA171* duplex (panel B) in the presence of increasing amounts of recombinant baculovirus-TSWV NSs infected insect cell extracts. A representative picture of at least three independent repetitions is shown. TSWV NSs interference on the miRNA pathway in RDR6 knockdown *N. benthamiana* plants as visualized by eGFP fluorescence from an eGFP-miRNA sensor construct (eGFP-3' UTR) 5 days post co-infiltration (Panel C). As controls, leaves were infiltrated with *Agrobacterium* harbouring vectors for eGFP-3' UTR and MBP (Panel C upper left) or eGFP-3' UTR and TSWV NSs (Panel C upper right). Silencing was induced by co-infiltration of pri-miRNA1 (Panel C lower left and right), and suppressed in the presence of TSWV NSs (Panel C, lower right). Levels of eGFP siRNA (D, left panel) and processed miRNA1 (Panel D right panel) were detected by northern blot hybridization. RNA of wildtype *N. benthamiana* plant infiltrated with *Agrobacterium* harbouring the eGFP-3' UTR sensor was used as positive control (Panel D, last lane top panel). Non-infiltrated *N. benthamiana* was used as negative control (Panel D, first lane top panel; wt). As loading control, RNA was stained by ethidium bromide. Suppression of miRNA1-induced silencing of a Firefly luciferase-miRNA1 sensor construct was investigated in insect cells (panel E). *Drosophila* S2 cells were co-transfected with a pMT-Renilla luciferase (RLuc), pMT-Firefly luciferase (Fluc)-miRNA1 sensor construct, either specific (miRNA1) or unspecific (miRNA12) primary miRNA in concert with either pIB-MBP, -TSWV NSs or - Carnation Italian ringspot virus (CIRV) P19. After induction at 48 hours post transfection (hpt), relative luciferase expression (Firefly/Renilla) was determined 72 hpt and the mean of at least two independent experiments is shown with standard error.

TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment; (e)GFP, (enhanced) green fluorescent protein; MBP, maltose binding protein; mRNA, messenger RNA; miRNA, microRNA; pre-miRNA, precursor microRNA; hpt, hours post transfection; wt, wild type

To determine if the capacity to suppress the miRNA pathway was shared among tospoviral NSs proteins, the EMSA assays using miRNA/miRNA* duplex or pre-miRNA were repeated for the other tospoviruses. Next to the positive control CymRSV, miRNA/miRNA* retardation was only observed when infected plant extracts containing TYRV were used, and not with GRSV, INSV and TSWV (Figure 6-6A). In contrast, retardation of pre-miRNA complexes was only observed with infected leaf extracts containing GRSV and INSV and not with TSWV and TYRV (Figure 6B). Strikingly, no affinity to miRNA/miRNA* duplexes was observed for TSWV NSs when using crude extracts of virus-infected plants (Figure 6-6A), whereas a binding was observed when using recombinant baculovirus- TSWV NSs infected cell extracts (Figure 6-6C) or plant extracts agro-infiltrated with TSWV NSs (data not shown, Figure 6-5A & 6-6C). Similar results were obtained for *Agrobacterium*-infiltrated leaf extracts and baculovirus-infected cell extract of GRSV NSs and TYRV NSs (data not shown, Figure 6-6C).

These results indicated that the NSs proteins of the tospoviruses analyzed interfered with the miRNA pathway. However their mode of action seemed to differ depending on the expression system used. To substantiate these findings with evidence from the natural situation, RNA was isolated from tospovirus-infected plant material and assayed for the presence of miRNA171c and miRNA171c* molecules. If the tospovirus NSs protein binds miRNA/miRNA* duplexes it would prevent RISC loading of the miRNA guide strand, the subsequent target cleavage in plants and degradation of the miRNA* strand (Chapman et al., 2004, Dunoyer et al., 2004). Detection of miRNA/miRNA* duplexes and specifically of the miRNA* strand in plants containing NSs thus would be indicative for direct association of the RSS with miRNA/miRNA* duplexes. Indeed, both miR171c and miRNA171c* were readily detected in RNA samples from TSWV and TYRV-infected plant material (Figure 6-6D), whereas in uninfected plants only the miR171c strand could be detected under the same conditions. The miRNA* strand was also readily detected in RNA samples of GRSV and INSV-infected plant material (Figure 6-6D). This indicates that their NSs proteins also interfere with the RISC loading step, most likely by sequestering the miRNA/miRNA* duplexes. This would assume that GRSV and INSV NSs exhibit affinity for miRNA/miRNA* duplexes *in vivo*, in contrast to the *in vitro* data with infected plant extract (Figure 6-6A).

Discussion

A common strategy employed by many plant viruses to counteract antiviral RNA silencing is by sequestering and inactivation of (antiviral) siRNAs through their viral RSS protein. Size-selective binding of siRNAs has been described in literature for several plant viral suppressors like RHBV NS3, TEV HC-Pro, *Beet yellows virus* (BYV) P21, P19 of several toombusviruses, *Peanut clump virus* P15 and *Barley stripe mosaic virus* γB (Hemmes et al., 2007, Lakatos et al., 2006, Merai et al., 2006). TSWV NSs has been described previously to act as RSS in plants and insect cells (Bucher et al., 2003, Garcia et al., 2006, Reavy et al., 2004), however its mode of action remained unclear. Results shown in this work demonstrate that *E.coli*- and recombinant baculovirus-expressed TSWV NSs can bind both siRNA and

long dsRNA molecules. This enables TSWV to block antiviral RNA silencing at two stages, i.e. before and after Dicer-mediated dsRNA cleavage. Similar results were obtained for NSs from GRSV and INSV, two other tospovirus species that together with TSWV belong to the *American clade* of tospoviruses (Pappu et al., 2009) and share 49.7 – 82.2% amino acid sequence similarity. In contrast, the NSs protein of the more distantly related *Eurasian* clade tospovirus TYRV (15-22% protein similarity) (Hassani-Mehraban et al., 2005) only revealed affinity to small dsRNA molecules in all used extracts. The observed lack of TYRV NSs to bind long dsRNA is unclear. The same is true for the low affinity to long dsRNA binding in TSWV infected plant extract, which is in contrast to the results of baculovirus or *E.coli* expressed TSWV NSs. Considering the large quantities of dsRNA, at least, in infected plants, a significant part of NSs could be pre-loaded with dsRNA and a difference in the remaining soluble and free NSs protein levels within the infected-plant extract cannot be excluded. It is not known, if all used tospoviruses produce similar amounts of viral siRNAs during infections, possibly resulting in differences in NSs pre-loading. On the other hand, the fact that no Dicer inhibition could be observed for TYRV NSs, in contrast to TSWV NSs in an *Agrobacterium*-infiltration assay, strengthens the observation that TYRV NSs does not bind long dsRNA molecules in contrast to TSWV NSs.

Whereas all tospovirus NSs proteins analyzed did exhibit a clear affinity for siRNAs, the EMSA assays showed a clear difference in siRNA-NSs complex mobility between those from GRSV, INSV and TSWV versus TYRV. However, this was only in case plant extracts were used and not with baculovirus-infected cell extract (Figure 6-4A). In plant extracts all tested tospovirus NSs proteins (GRSV, TSWV and TYRV) showed, in addition to the monomeric form, higher molecular weight bands corresponding in size to dimers, trimers and multimers (data not shown). This was irrespective of (non-)denaturing conditions and independent of the presence of RNA. Similar multimers were observed under semi-denaturing conditions in recombinant baculovirus NSs-infected cell extracts, in the presence or absence of RNA (data not shown). Therefore, the discrepancy in stoichiometry of siRNA-RSS complexes between these two groups of tospoviruses observed only with NSs from plant extracts was likely not to be attributed to differences between oligomerization in plant versus insect cells. Instead, differences in post translational modification (e.g. phosphorylation) and/or interaction with host proteins or viral proteins may account for the observed differences of siRNA-NSs complex mobility. Predictions for post-translational modifications revealed several potential phosphorylation sides. To our knowledge no viral or host encoded interacting partner has yet been identified for tospovirus NSs.

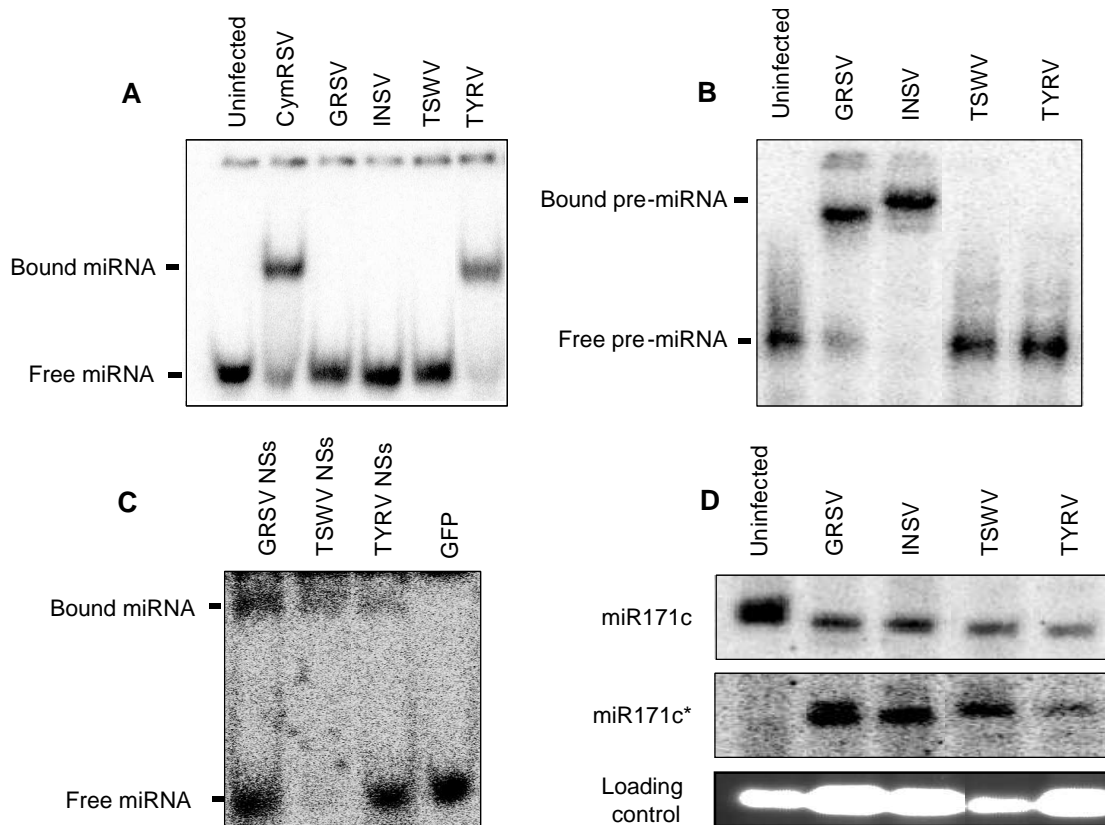


Figure 6-6: Affinity of Tospovirus NSs for duplex RNA molecules from the miRNA pathway.

Electrophoretic mobility shift assay analysis using leaf extracts from *N. benthamiana* systemically infected with TSWV, TYRV, CymRSV, GRSV or INSV and incubated with radioactively labelled miRNA171/miRNA171* duplexes (panel A) or pre-miRNA2b (panel B). Baculo-virus infected cell extract expressing GFP, TSWV NSs, TYRV NSs or GRSV NSs were incubated for 20 minutes with radioactively labelled miRNA171/miRNA171* and loaded on an 8% native gel (panel C). As negative controls, RNA was incubated with extracts from uninfected plants (Panel A first lane) or GFP expressing baculo-virus infected extract (Panel C second lane). Northern blot detection of miRNA171c and miRNA171c* (after stripping) in RNA samples from *N. benthamiana* leaves systemically infected with GRSV, TSWV, INSV and TYRV. (panel D). As negative control, RNA from uninfected leaves was included (panel D, first lane).

TSWV, Tomato spotted wilt virus; NSs, non-structural protein of the S-segment; miRNA, microRNA; pre-miRNA, precursor microRNA; CymRSV, Cymbidium ringspot virus; TYRV, Tomato yellow ring virus; GRSV, Groundnut ringspot virus; INSV, Impatiens necrotic spot virus.

The reason for the additional affinity to long dsRNA to counter defend against RNA silencing for TSWV, GRSV and INSV remains intriguing. The question remains if this affinity to long dsRNA molecules represents an ancestral activity, lost by TYRV NSs during time or newly gained by the *American* tospoviral clade. Recently, long dsRNA has been described to induce an antiviral response in *Drosophila* diverse of RNA silencing (Kemp & Imler, 2009). Thereby, it is tempting to speculate that tospoviruses from the *American* clade benefit from their long dsRNA affinity in order to counteract two different antiviral pathways in insects. If a similar antiviral response is present in the thrips insect vector is still unknown. Hitherto, a similar divergence in the affinity of RSS to longer and shorter dsRNAs has been only observed in one other well studied but completely unrelated family of viruses - the *Tombusviridae* (Meraï et al., 2006, Meraï et al., 2005).

Although binding of TSWV NSs to longer dsRNA molecules has not or only to a low amount been observed in all used cell extracts, since it is nearly lacking in infected plant extracts, this

binding property is supported by its *in vitro* inhibitory effect on Dicer mediated processing of dsRNA molecules into siRNAs. A similar inhibitory effect on Dicer cleavage of an inverted repeat of GFP has also been observed in plants that transiently expressed TSWV NSs. Previous research reported that TSWV NSs is not able to suppress IR-induced RNA silencing (Takeda et al., 2002), suggesting a mode of action upstream of DCL in the RNA silencing pathway. However, in our hands analyses have consistently shown that TSWV NSs was perfectly capable to suppress IR-induced silencing in *Agrobacterium* infiltrated plants.

Among plant viruses, binding of viral RSS proteins to longer dsRNA and subsequent inhibition of Dicer mediated dsRNA cleavage so far has only been observed for two members of the positive-stranded *Tombusviridae*, i.e. *Turnip crinkle virus* (TCV) CP and aureusvirus p14 (Merai et al., 2006, Merai et al., 2005). This property, though, is more common to RSS proteins of insect and mammalian infecting viruses like *Flock house virus* B2, *Drosophila C virus* 1A and *Ebola virus* VP35, that all have been shown to exhibit a high affinity to long dsRNA (Kimberlin et al., 2009, Lingel et al., 2005, Merai et al., 2006, Merai et al., 2005, van Rij et al., 2006).

Tospoviruses are the only plant-infecting members of the *Bunyaviridae* family and are transmitted by thrips in which they also replicate (Wijkamp et al., 1993). As a result, tospoviruses are targeted by antiviral RNA silencing in plants as well as insects. By interacting with both long and short dsRNA molecules tospoviruses are able to interfere with multiple steps in the antiviral RNA silencing machinery, notably Dicer-mediated processes, assembly of active RISC complexes and possibly the amplification of the silencing signal in plants. The NSs protein of the animal-infecting bunyavirus La Crosse virus (LACV) has been shown to be an active suppressor of RNA silencing in human cells by interfering with siRNA mediated RNA silencing (Soldan et al., 2005). Recently, however, contradictory results have been published (Blakqori et al., 2007). Since vertebrates possess an effective antiviral defense system based on interferon induction that also involves dsRNA species, the antiviral activity of the ubiquitous RNA silencing machinery in vertebrate systems is still being debated. For the same reason, the biological relevance of RSS activity of some proteins from animal-infecting viruses is being disputed, as many of these proteins also have been shown interferon antagonistic properties (Basler & Garcia-Sastre, 2002, Haller & Weber, 2009). Besides being an inducer of RNA silencing and a substrate for Dicer, long dsRNA molecules of cellular or viral origin also activate the dsRNA-dependent protein kinase (PKR), and thereby trigger the interferon-induced antiviral defence mechanism in mammals (Gantier & Williams, 2007). Sequestering long dsRNA molecules would therefore be an effective way for vertebrate viruses to simultaneously suppress antiviral RNA silencing and interferon induction. The capacity of NSs proteins of some Tospoviruses to bind long dsRNA could thus reflect a property inherited from a common ancestor shared between the plant- and mammalian-infecting bunyaviruses (de Haan et al., 1991, Kormelink et al., 1992). Whether this long dsRNA binding is redundant or still of biological relevance to tospovirus infections in plants or insects remains to be investigated. Comparison of mutated NSs proteins lacking

long dsRNA binding ability with wildtype NSs proteins could shed light on the question if siRNA binding is sufficient for the RSS activity of tospoviral NSs proteins. For this more information regarding the RNA binding domain(s) in the tospoviral NSs proteins would be required.

The property of tospovirus NSs proteins to bind miRNA/miRNA* duplexes and thereby interfere in the regulation of host gene expression has previously been shown for a few other plant viral RSS proteins like potyviral HC-Pro and tombusviral P19 (Chapman et al., 2004, Dunoyer et al., 2004). The affinity of TSWV, like TYRV, for miRNA/miRNA* but not for pre-miRNA molecules, as observed for GRSV and INSV, is somewhat intriguing in light of earlier mentioned similarities and differences between the NSs proteins studied and needs further analysis. The observed lack of affinity of TSWV, GRSV and INSV infected plant extracts for miRNA/miRNA* duplexes is likely due to reasons discussed for the observed low binding affinity of infected TSWV plant extract to long dsRNA; being that a significant part of NSs is already being loaded with (viral) dsRNA molecules arising during a viral infection. This is supported by the ability of all tested NSs proteins (TSWV, GRSV and TYRV) to bind miRNA/miRNA* duplexes in *Agrobacterium*-infiltrated plant extracts (data not shown) and baculovirus infected cell extracts (Figure 6-6C). The low amount of NSs-siRNA complexes observed for TSWV, INSV and GRSV infected plant extracts compared to baculovirus-NSs infected cell extracts (Figure 6-4A & B) and *Agrobacterium*-infiltrated plant extract (data not shown) strengthens this explanation. Furthermore, in light of the slightly lower affinity to miRNA/miRNA* duplexes (appr. 5% bound at lowest used concentration) compared to siRNAs (appr. 90% bound at lowest used concentration) in case of baculo-virus infected extract expressing TSWV NSs, it is maybe not that surprisingly that no miRNA/miRNA* retardation was observed for TSWV, GRSV and INSV infected plant extract.

Despite these observations, the accumulation of miRNA171c/miRNA171c* duplexes in leaf material infected with any tospovirus strengthens the idea that all tospoviral NSs proteins stabilize the miRNA/miRNA* duplexes and prevent their uploading into RISC. This possibly occurs by sequestering and, thereby interfering with miRNA-mediated gene regulation *in planta*. Since miRNA171c is predicted to target transcripts for the SCARECROW-like transcription factor (Sunkar & Zhu, 2004, Xie et al., 2005) and a beneficial effect of this gene on virus replication is not evident, it is tempting to speculate that NSs interference with the miRNA pathway is most likely due to the high structural similarity of miRNA/miRNA* molecules to antiviral siRNA molecules,. Further support for this idea comes from the observed silencing suppression effect of TSWV NSs on a “randomly” selected miRNA1 sensor construct during *Agrobacterium* infiltrations on *N. benthamiana* leaves. Whereas the interference of plant viral RSS in the miRNA pathway might reflect an aberrancy due to structural similarities between siRNA and miRNA/miRNA*s, the interference by RSS proteins of human infecting viruses (e.g. HIV-1) with miRNA silencing has been proposed as genuine to down or up-regulate genes involved in antiviral (defence) responses (Berkhout & Jeang, 2007, Triboulet et al., 2007). This is elegantly exemplified by the higher expression level of

the miRNA regulated histone acetylase p300/ CBP-associated factor (PCAF) during HIV infections, a host factor that has been shown to be required as cofactor for the HIV transactivator of transcription (Tat) protein. Until recently, no such case has been reported for virus infections in plants and insects. In plants, interference of TCV CP with AGO1 has been reported, resulting in changes of miRNA levels that in turn create a virus-favourable environment in the plant (Azevedo et al., 2010). Whether this interaction is TCV specific or a global plant viral characteristic is not yet known. The here reported interaction of tospoviral NSs proteins with the miRNA pathway leaves the possibility that interference with the plant miRNA pathway could be a characteristic shared by all plant viruses. Whether this interaction with the NSs protein and the miRNA pathway occurs during tospovirus infections and results in a viral beneficial environment remains to be investigated.

Materials and Methods

Plasmid constructs

Agrobacterium expression plasmids for MBP, TSWV NSs, IR-GFP and GFP were described previously (Bucher et al., 2003, Hemmes et al., 2007, Merai et al., 2006).

The coding sequence for *Tomato yellow ring virus* tomato strain (TYRV-t) NSs and *Groundnut ring spot virus* (GRSV) NSs was PCR amplified and cloned into the binary pK2GW7 (Karimi et al., 2002) vector using Gateway technology. Baculovirus expressing TSWV NSs or GFP have been described previously (Kaba et al., 2002, Kormelink et al., 1991). Recombinant baculovirus expressing GRSV NSs and TYRV NSs were constructed by Gateway technology and the Bac-to-Bac system (Invitrogen) following the manufacturers' protocol. The insect expression vectors of TSWV NSs were constructed by Gateway technology into pIB-GW (Invitrogen) and the other expression vectors (MBP and CIRV P19) have been described previously (Chapter 5). The miRNA reporter constructs (eGFP-3'UTR and Fluc-par6), pri-miRNA1 and pri-miRNA12 expression plasmids have been described previously (Chapter 5).

Cell culture and transfection

Schneider (S)-2 cells were grown, transfected and expression induced as described in chapter 5. Luciferase expression was determined using self made buffers for the Dual luciferase reporter assay (Dyer et al., 2000).

Bacterial expression and purification of Thioredoxin-TSWV NSs and -MBP

The coding sequence of TSWV NSs or MBP was PCR amplified to introduce Gateway specific recombination sites and cloned into pDONR207 (Invitrogen). For expression and subsequent purification, the NSs or MBP coding sequence was cloned in frame with HP-Thioredoxin, in pDest49-BAD (Invitrogen) by Gateway reaction. Proteins were expressed in DH10beta cells (Qiagen) according to the manufacturer's recommendations. After induction for 6h at 37°C with 0.2% w/v L-arabinose, cells were harvested by centrifugation for 15 min at 4000 rpm (Sorvall GSA rotor) at 4°C. Cells were lysed by sonification on ice with 30 sec. intervals for 3 times 30 sec. in lysis buffer (50 mM K₂PO₄, 400 mM NaCl, 100 mM KCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and EDTA-free protease inhibitor cocktail (Roche)). The soluble fraction was recovered by centrifugation at 4000 rpm (Sorvall GSA rotor) for 30 min at 4°C. Recombinant protein was purified using a Talon metal affinity resin column (Clontech) and eluted with 2.5 packed bed volumes (PBV) elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 200 mM imidazole, 10% (v/v) glycerol) after washing with 15 PBV lysis buffer. Protein fractions were instantly frozen in aliquots in liquid nitrogen and stored at -80°C until use. Protein concentrations of eluted fractions were determined using the standard procedure of the Bio-Rad protein assay according to the manufacturer's recommendations and the purification process was analyzed by SDS-PAGE and subsequent staining with Coomassie brilliant blue.

Preparation of virus-infected plant extracts, baculovirus-infected insect cell extracts and *Agrobacterium*-infiltrated leaf extracts

Groundnut ringspot virus (GRSV), Impatiens necrotic spot virus (INSV), Tomato yellow ring virus tomato strain (TYRV-t), Tomato spotted wilt virus (TSWV) and Cymbidium ring spot virus (CymRSV) were mechanically inoculated on *Nicotiana benthamiana* and extracts prepared from systemically infected leaves essentially as described previously (Merai et al., 2006) with minor modifications. Virus accumulation was verified either by ELISA or NSs specific western blot analysis prior to preparation of virus-infected plant extracts. To prepare infected or *Agrobacterium*-infiltrated extracts, 0.6 g leaf tissue was ground in liquid nitrogen and resuspended in 1.5 ml buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 50 mM KCl, 1 mM DTT). The total protein concentration was determined using the standard procedure of the Bio-Rad protein assay according to the manufacturer's recommendations. Crude extracts were centrifuged twice at 14000 rpm for 15 min at 4°C. Extracts were immediately frozen in liquid nitrogen and stored at -80°C until use.

TSWV NSs, TYRV NSs, GRSV NSs and GFP were expressed in *Trichoplusia ni* Hi5 cells, using baculovirus expression vectors expressing the genes under control of the polyhedrin promoter.

Hi5 cells were infected with baculoviruses at a multiplicity of infection (MOI) 10 and incubated for 48 hours at 28°C. Cells were detached, harvested by low speed centrifugation (1500 rpm) and washed with PBS prior to lysis by sonification during 3 intervals of 30 sec. in lysis buffer (100 mM NaCl, 20 mM Tris 7.4, 2 mM MgCl₂, 1 mM DTT, 10% (v/v) glycerol). The infection was monitored either by GFP-fluorescence or SDS-PAGE and Western immunoblot analysis for TSWV NSs. The total protein concentration was determined by the Bio-Rad protein assay according to the manufacturer's protocol.

Expression analysis

Expression of different NSs proteins was monitored by western immunoblot analysis. Samples of the extracts were mixed with 2x SDS-loading buffer, heated for 5 minutes at 95°C and centrifuged for 3 minutes at 14000 rpm. For complex formation 2 x SDS-loading buffers was used lacking the β -mercaptoethanol and samples were loaded on the gel without previous heating. Crosslinking was performed either directly or after 30 min incubation of the extract with siRNA molecules by the addition of 0.25% paraformaldehyde, incubation for 30 min at room temperature followed by the addition of 2x SDS-loading buffer and 10 min at 65 degree. For the reversion, samples were treated in the same way, but incubated for 10 min at 95 degree. Proteins were separated by SDS-PAGE and transferred to Immobilon-P (Millipore) by semi-dry blotting. TSWV and GRSV NSs protein were detected using a NSs-specific polyclonal antibody. TYRV NSs protein was detected using a monoclonal antibody (kindly provided by Dr. S-D. Yeh). Protein-antibody complexes were detected by an alkaline phosphatase conjugated secondary antibody, and visualized with NBT-BCIP as substrate (Roche) according to the manufacturer's recommendations.

dsRNA preparation

A 114 nt dsRNA molecule was generated by T7 RNA polymerase (Promega) transcription on a gel purified (High Pure PCR purification kit, Roche) eGFP template in the presence of alpha³²P-CTP (Perkin Elmer). The latter template was provided with T7 RNA polymerase promoter sequences at both ends by PCR amplification using DNA oligos T7_dsRNA114 F (5' GTA ATA CGA CTC ACT ATA GGG GGC GTG CAG TGC TTC AGC CGC 3') and T7_ds114 R (5' GTA ATA CGA CTC ACT ATA GGG GCC GTC GTC CTT GAA GAA GAT GG 3'). Precursor miRNA 2b was generated by T7 RNA polymerase transcription in the presence of alpha³²P-CTP (Perkin Elmer) on a template obtained after annealing of two long primers: 5' GTA ATA CGA CTC ACT ATA GGC GTT GCG AGG AGT TTC GAC CGA CAC TAT ACT TAT AAC AAC TGT TGT ACA GTG ACG GTG AAA CTT CTG TCA ACT TC 3' and 5' GAA GTT GAC AGA AGT TTC ACC GTC ACT GTA CAA CAG TTG TTA TAA GTA TAG TGT CGG TCG AAA CTC CTC GCA ACG CCT ATA GTG AGT CGT ATT AC 3'. Following T7 transcription, reaction mixtures were incubated at 70°C for 10 min and cooled down to room temperature. Template DNA was removed by treatment with DNase I and dsRNA was gel purified from an 8% PAGE, 0.5x TBE native gel. Labelling of custom made RNA oligos targeting the GFP sequence or corresponding to the *A. thaliana* microRNA 171a sequence was performed by end labeling of the GFP siRNA guide strand or miRNA171a strand using gamma³²P-ATP (Perkin Elmer) and T4 polynucleotide kinase. These radio-labelled strands were annealed to the RNA oligo corresponding to the respective GFP siRNA passenger or miRNA171* strand and PAGE purified essentially as described previously (Haley et al., 2003).

Electrophoretic mobility shift assay and Western blot analysis

In a binding reaction, radio-labelled RNA (0.5 nM) was incubated with ~2 µg total protein from virus-infected leaf or cell extracts per 10 µl reaction and incubated for 20 min. at room temperature as previously described (Hemmes et al., 2007, Merai et al., 2006). As controls, RNA was loaded without plant extracts, with healthy plant extracts or GFP-expressing baculovirus-infected insect cell extracts. The same reaction was performed with serial dilutions of the bacterial expressed HP-Thioredoxin-NSs or -MBP proteins. The complexes were separated on a 0.5x TBE native PAGE gel. For 114 nt dsRNA and pre-miRNA 2b a 5% gel was used and an 8% gel for siRNA and miRNA/miRNA* molecules. Following electrophoresis gels were dried, overnight exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphor imager, Amersham Biosciences). A representative picture of at least three independent experiments was shown.

To determine the presence of NSs in the RNA-protein complex, the excised gel slices were grinded in 2x SDS-loading buffer and PBS. After denaturation, the solution was loaded on a SDS-PAGE, blotted and a western blot analysis was performed using either polyclonal TSWV NSs or monoclonal antibody detecting Asian tospoviral NSs (supplied by Dr. S.D. Yeh).

Dicer cleavage reactions

Drosophila embryo extract preparation was described previously (Haley et al., 2003). In Dicer-mediated cleavage reactions embryo extracts were incubated for 3 hrs at 25°C in reaction mixtures as described previously (Haley et al., 2003) where KCl was omitted from the reaction mixture. In a typical 10 µl reaction 2 µl *Drosophila* embryo extract, 0.5 ng dsRNA and 2 µg virus-infected extract were mixed. Samples were deproteinized and RNA was analyzed on a 12% denaturing gel. After electrophoresis gels were dried, exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

Agrobacterium tumefaciens transient transformation assay (ATTA)

Agrobacterium infiltration was performed as previously described (Bucher et al., 2003). *N. benthamiana* leaves were co-infiltrated with *Agrobacterium* (at an OD₆₀₀=0.25) harboring binary vectors encoding IR-GFP, GFP and different constructs coding for MBP, CymRSV P19, TCV CP, TYRV NSs, TSWV NSs or GRSV NSs. Expression of GFP in the leaves was monitored 3 days post infiltration (dpi) with a hand-handled UV lamp and photos taken with a Canon Power shot A710IS digital camera, using the high fluorescent setting. For the miRNA based sensor constructs, experiments were performed as previously described (Chapter 5).

Northern blot analysis

RNA extraction was performed as described previously (Bucher et al., 2004), and 7 µg of total RNA was mixed with formaldehyde loading buffer, heated for 5 minutes at 70°C and separated on an 1% agarose gel. The RNA was transferred onto a Hybond-N membrane (Pharmacia-Biotech) followed by UV-cross linking.

For the miRNA1 and siRNA detection, 5 µg RNA enriched for small RNAs (Hamilton & Baulcombe, 1999) was separated on a 20%, 0.5x TBE denaturing acrylamide gel. Following separation, the RNA was electro-blotted onto Hybond-N⁺ (Pharmacia-Biotech) and cross-linked by UV-light. Hybridization was performed overnight at 48°C in modified church buffer (0.36 M Na₂HPO₄, 0.14 M NaH₂PO₄, 7% (w/v) SDS, 1 mM EDTA) with either a eGFP or miRNA1 specific DIG-labeled DNA probe. The blots were washed briefly for three times with 2x SSC and three times for 15 minutes with 2x SSC supplemented with 0.2% (w/v) SDS at 48°C. The labeled probe was detected by Western blot analysis using a DIG-specific antibody conjugated to alkaline phosphatase in blocking buffer (maleic acid buffer + 1% blocking reagent) and CSPD as substrate (Roche) according to the manufacturer's recommendations. For the detection of miRNA171 in tospoviral-infected extracts 5-15 µg small RNA was loaded onto an 12%, 1x TBE denaturing gel, electro-blotted onto Hybond-N⁺ (Pharmacia-Biotech) and hybridized overnight at 50°C in hybridization buffer (1 mM EDTA, 0.36 M Na₂HPO₄, 0.14 M NaH₂PO₄, 7% (w/v) SDS) using locked nucleic acid probes (2 µg). Probes specific for miRNA171c or miRNA171c* were labeled using polynucleotide kinase and gamma³²P-ATP. Following hybridization, blots were washed briefly with 2x SSC, 0.2% (w/v) SDS, 2x 20 min with 2x SSC, 0.2% (w/v) SDS and 1x 20 min with 1x SSC, 0.1% (w/v) SDS at 50°C. Blots

were exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences). Stripping of blots was performed at 85°C using 200 ml buffer containing 1 mM EDTA and 0.1% (w/v) SDS for 15 min and used for subsequent hybridization experiments.

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Chapter 7

General Discussion

Over recent years it has become clear that RNA silencing is present in a variety of organisms and involved in a number of essential cellular processes. These include developmental gene regulation, silencing of transposons and antiviral responses. To counteract antiviral RNA silencing, viruses have evolved RSS proteins, which specifically interfere with the established antiviral RNA silencing pathways in plants and insects. A large proportion of plant and insect viruses have been found to encode at least for one RSS protein (reviewed by Csorba et al. 2010). In this thesis the interaction between the RSS proteins of the tenuivirus RHBV and the tospovirus TSWV, respectively NS3 and NSs, with distinct RNA silencing pathways in different organisms are investigated. Using *in vitro* RNA affinity studies and various reporter-based RNA silencing assays for distinct RNA silencing pathways (notably the siRNA pathway, the miRNA pathway and the endo-siRNA pathway), the targets of RHBV NS3 and tospovirus NSs interference have been identified. Figure 7-1 summarize this. Moreover, the results obtained with the plant-infecting tospovirus (*Bunyaviridae*) will be discussed in this chapter in light of the evolutionary relation between plant- and animal-infecting viruses within the family *Bunyaviridae*, and the possible role of the RNA silencing response as antiviral defense in vertebrates, more specifically mammals.

Tenuiviral NS3 and tospoviral NSs proteins act as RSS by binding dsRNA

The RHBV NS3 and TSWV NSs exhibit RSS activity due to their affinity to small dsRNA molecules, i.e. siRNA and miRNA/miRNA* duplexes (Chapter 2, 4, 6: Hemmes et al. 2007), and for TSWV to some extent also to long dsRNA. Since long and short RNA duplex molecules play highly conserved key roles in the RNA silencing pathways in different organisms (Deleris et al., 2006, Gasciolli et al., 2005, Lee et al., 2004a) both viruses are potentially capable to counteract antiviral RNA silencing both in plants and the insect vector in which they replicate (Falk & Tsai, 1998, Ramirez et al., 1993, Ramirez et al., 1992, Wijkamp et al., 1993). This has been observed using transient reporter based assays for their respective RSS proteins in both types of organisms (Bucher et al., 2003, Hemmes et al., 2007, Reavy et al., 2004, Chapter 5 & 6).

Recent reports suggest that siRNAs can act as systemic signal molecule in plants (Dunoyer & Voinnet, 2009) and in insects (Fragkoudis et al., 2009). This potentially enables NS3 and NSs to interfere at three different steps in the RNA silencing pathway by sequestering a single key molecule: siRNA. These effects include local silencing in the infected cell, systemic silencing and primer dependent amplification of the silencing signal (Baulcombe, 2004, Cogoni & Macino, 1999a, Dunoyer & Voinnet, 2009, Sijen et al., 2001, Vaistij et al., 2002).

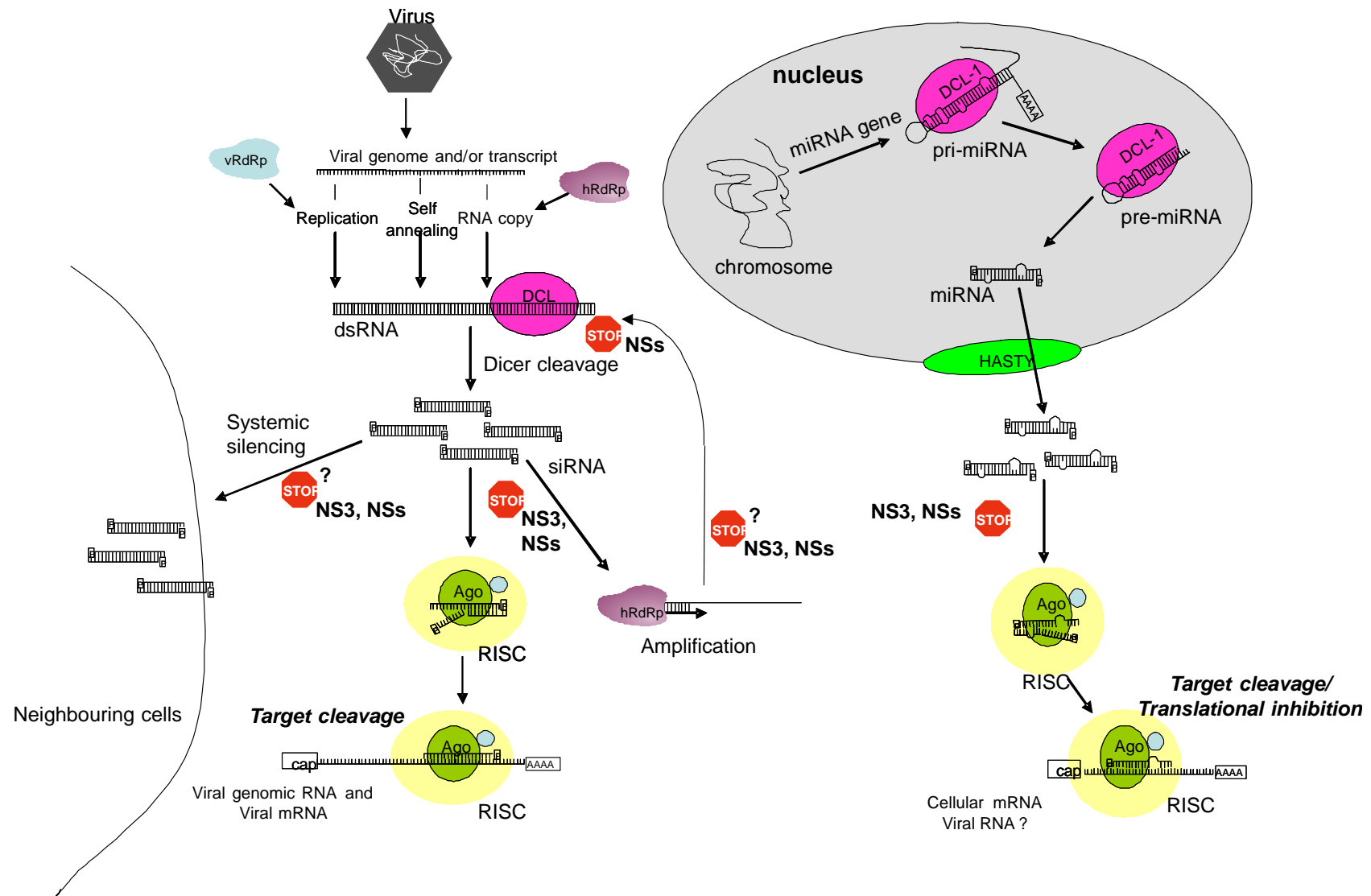


Figure 7-1: Schematic representation of the siRNA and miRNA pathway in plants and the interference (indicated by "stop") of rice hoja blanca virus NS3 and tomato spotted wilt virus NSs with these pathways. Question marks indicate possible points of interference. RISC, RNA induced silencing complex; DCL, Dicer-like protein; Ago, Argonaute protein; vRdRp, viral RNA-dependent RNA polymerase; hRdRp, host-encoded RNA-dependent RNA-polymerase; NS3; non structural protein 3; NSs, non structural protein - S RNA segment encoded.

Whereas RHBV NS3 and tospoviral NSs have not yet been reported to suppress systemic silencing, the tenuivirus RSV NS3 protein has been shown to efficiently suppress systemic and local RNA silencing. The ¹⁷³KKR¹⁷⁵ region in RSV NS3 (Xiong et al., 2009) is thereby critical and coincides with the position of a highly conserved triple lysine cluster in most other tenuivirus NS3 proteins, including RHBV. For RHBV, this region had earlier been shown to be required for small dsRNA (siRNA and miRNA/miRNA*) binding and crucial for its local RSS activity (Chapters 2-5). RSV NS3 is the only tenuivirus not encoding this triple lysine cluster, but instead contains two lysines and an arginine (¹⁷³KKR¹⁷⁵) (Xiong et al., 2009). These provide again three polar amino acids predicted to be a canonical RNA binding domain (online tool BindN). In analogy to RSV, RHBV NS3 is also expected to suppress systemic silencing and amplification of the silencing signal, due to its siRNA binding capacity. The ability to suppress systemic silencing is important for a successful viral infection, as previously demonstrated and described for the RSS proteins of several plant viruses like P25 of PVX and 2b of CMV unrelated to tenuivirus and tospovirus (Voinnet et al., 2000, Ye et al., 2009).

In contrast to NS3, TSWV NSs showed affinity to both long and short dsRNA molecules (Chapter 6). This enables the protein to interfere at an additional distinct step of the antiviral RNA silencing pathway: Dicer cleavage of long dsRNA into siRNA and like NS3, preventing siRNA incorporation into RISC and interfering with the systemic, transitive and local RNA silencing. Such a size-independent RNA binding has hitherto only been reported for two other plant viral RSS proteins, CP (P38) of TCV and P14 of Aureusvirus (Merai et al., 2006).

Tospoviruses, like TSWV, belong to the family *Bunyaviridae* and represent the only genus within this family containing members that infect plants instead of mammals. It is not clear why TSWV and some other tospoviruses (GRSV and INSV) bind both long and short dsRNA whereas another tospovirus strain, TYRV, only revealed affinity to siRNAs (Chapter 6). In mammals, long dsRNA is not only a potential Dicer substrate but also activates the protein-K response (PKR), resulting in IFN production and an activated antiviral state in the cell (reviewed by Gantier & Williams, 2007). Recent results have suggested a similar role of dsRNA in *Drosophila*, interacting with Dicer to not only induce the RNA silencing response but also other antiviral innate immune responses like the JAK-STAT pathway, mediated via the Vago protein (reviewed by Kemp & Imler, 2009). Tospoviruses thereby might benefit from their long dsRNA affinity and counteract two different antiviral pathways in insects (RNA silencing and e.g. Jak-STAT). The fact that all tested tospovirus NSs proteins revealed a higher affinity to siRNA molecules than to long dsRNA raised the question of a biological relevance of long dsRNA binding during a natural infection in plants and insects. This relevance is supported by out-competition of long dsRNA by siRNA molecules in *in vitro* competition experiments (Chapter 6). It is possible that the affinity to long dsRNA is a remnant activity derived from a viral ancestor, where binding of long dsRNA possibly had another biological function.

A growing number of RSS proteins and their modes of action have been identified over the last years (Csorba et al., 2009) and the picture emerges that most of these, like RHBV NS3,

act by size-specific siRNA binding (Merai et al., 2006). A small proportion of the RSS proteins acts by specific interactions with proteins of the RNA silencing pathway, like CMV 2b that interacts with AGO1 and AGO4, polerovirus P0 that induces degradation of AGO1 or TCV CP interacting with AGO1 through its GW domains (Azevedo et al., 2010, Csorba et al., 2010, Gonzalez et al., 2010). Mostly, however, these protein interactions are properties in addition to the binding of dsRNA molecules (e.g. siRNA in case of CMV 2b) and not essential for the local RSS activity, as recently shown for CMV 2b (Gonzalez et al., 2010). Although speculative, it is possible that protein-protein interactions are needed for additional roles of the RSS protein during viral infection, like interactions with other antiviral pathways (e.g. salicylic acid) (Ji & Ding, 2001). Besides, viruses that rely for their RSS protein activity solely on protein-protein interactions may faster generate host escape mutants. However, despite overlapping functions no sequence homology between RSS proteins of different virus families was found. Although often similar amino acids (polar and positive) play an important role in binding, the mechanism and requirements to bind to the dsRNA can be rather different. For those RSS proteins with a known crystal structure, i.e. tombusviral P19 and closteroviral P21 binding occurs in a different fashion, i.e. P19 acts as a dimer whereas P21 forms octomeric rings. Furthermore, both proteins recognize different parts of the dsRNA molecule: P19 requires the typical two nucleotide single stranded overhangs of siRNAs, whereas P21 requires an overall alpha-helical structure of dsRNA (Vargason et al., 2003, Ye & Patel, 2005). Even RSS proteins within the same taxon, e.g. those from the *Tombusviridae*, appear to have adopted different strategies to suppress RNA silencing (Figure 1-3). A similar observation has been made for the tospoviral NSs proteins, which have shown affinity to long and short dsRNA in case of TSWV, GRSV and INSV but only a size-specific, siRNA binding for TYRV (Chapter 6). This discrepancy in RSS activity, even for closely related viruses is unexpected but interesting in light of the fact that on the one hand the RNA silencing pathway is considered to be evolutionary ancient and on the other hand these viruses have an ancestral relation to the animal-infecting bunyaviruses.

This brings up the question of how RSS proteins have evolved; by independent (convergent) evolution or by co-evolution? Like many proteins encoded by the condensed small genomes of plant viruses, RSS proteins are often multifunctional proteins and may already harbor an affinity to nucleic acids as a requirement for functions in e.g. viral replication, movement or encapsulation. In such a case only slight adaptations may lead to the involvement of a RSS protein that acts by binding dsRNAs (reviewed by Li & Ding, 2006). This would be called independent (convergent) evolution of RSS proteins. In case of RHBV NS3 thus far no additional functions have been reported, whereas for the TSWV NSs protein it has been speculated that it acts as poly-A-binding protein (PABP) analogue during viral infection (van Knippenberg, 2005). Overall, the data obtained on tospoviruses seem to support the idea that RSS proteins arose by co-evolution. Tospoviruses represent the only plant infecting genus within the family of animal-infecting *Bunyaviridae*.

All their members have membrane-bound viral particles with two glycoproteins. Their viral RNA genomes are all tripartite and share significant sequence homologies between the viral RdRps and glycoproteins, which suggest that these viruses likely evolved from an ancestral virus originally replicating in mammals or (vector) insects (de Haan et al., 1991, Kormelink et al., 1991). In light of the fact that long dsRNA can also induce antiviral pathways distinct of RNA silencing (Gantier & Williams, 2007, Kemp & Imler, 2009) in mammals and possibly even in insects, the affinity to long dsRNA observed for the NSs protein of only a subset of the tospoviruses may reflect an ancient relic retained in some members, but lost in others.

Do NSs proteins of mammal-infecting viruses of *Bunyaviridae* act as RNA silencing suppressor in their vertebrate and insect hosts?

The presence of RSS activity for tospoviral NSs proteins raised the question whether animal infecting counterparts from the *Bunyaviridae* would also encode RSS proteins. Most of these viruses are arthropod-borne and transmitted by mosquitoes, ticks and phlebotomine flies, and replicate in both the mammalian host and insect vector. As a consequence, they encounter (at least) antiviral RNA silencing in the insect vector and the antiviral interferon response in mammals upon infection. Until now, no RSS proteins have been identified in arboviruses, although several viral proteins have shown interference with the innate immune response in mammals (e.g. JAK-STAT and IFN pathway) (reviewed by Fragkoudis et al., 2009; Fros et al., 2010 In Press).

Members of only two of the four genera of animal-infecting *Bunyaviridae* encode a paralogue of the NSs protein (Orthobunya- and Phlebo-virus) while only those of the phleboviruses are coded at a genomic position (Figure 7-2) analogous to those from the tospoviruses. However, plant based RSS assays of representatives, i.e. Rift valley fever virus (RVFV) (Phlebovirus), La Crosse virus (LACV) or Bunyamwera virus (Orthobunyavirus) did not reveal any RNA silencing suppressor activity for their NSs protein paralogues. Since their expression could not be verified due to the lack of corresponding antibodies (data not shown; Bucher, 2006), these data still have to be regarded as preliminary.

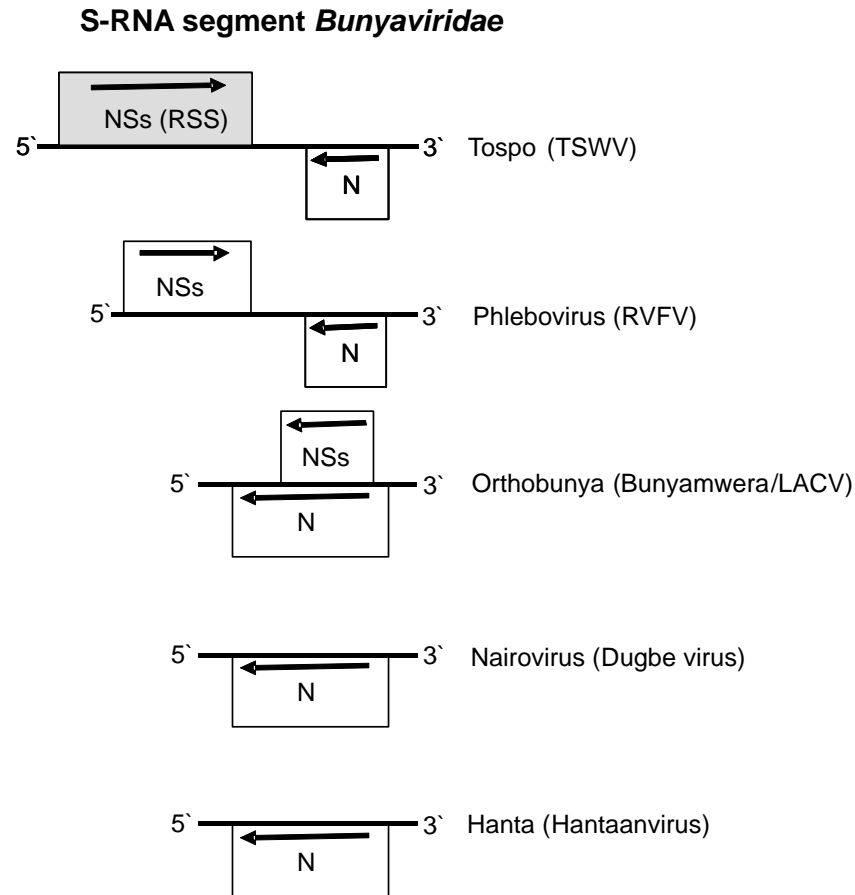


Figure 7-2: Schematic overview of the viral S-RNA segments of the type species of the five genera of the *Bunyaviridae*.

The RNA silencing suppressor (RSS) protein, NSs of Tomato spotted wilt virus (TSWV) is indicated as grey box. NS, non-structural protein; N, nucleocapsid protein; RVFV, Rift valley fever virus; LACV, La Crosse virus.

While for LACV and Bunyamwera NSs no significant RSS activity was observed in insect cells, using either short hairpin or *in vitro* transcribed long dsRNA as inducer, an overall reduction in reporter protein expression appeared (Figure 7-3). This is possibly due to a NSs induced host shutoff. Earlier published results on RSS activity assays for RVFV NSs using various cell systems (plant, insects and mammals) showed similar results. In which NSs was only demonstrated to act as “true” IFN antagonist by suppressing type I interferon induction, via modulation of the basal host transcriptional machinery (Blakqori et al., 2007, Bucher, 2006, Garcia et al., 2006). Surprisingly, a strong RSS activity of RVFV NSs was observed if shRNA was used as an inducer molecule, but lacking in case of *in vitro* transcribed dsRNA (Figure 7-3). In agreement with earlier reports, this suggested that the RSS activity was merely an effect of down regulation of host transcription.

In contrast, the NSs protein of the shrimp infecting Mourilyan virus (MoV), a recently discovered and as yet unclassified virus with strong structural similarities to the *Bunyaviridae*, tested positive for RSS activity in insects cells similar to TSWV-NSs (Figure 7-3).

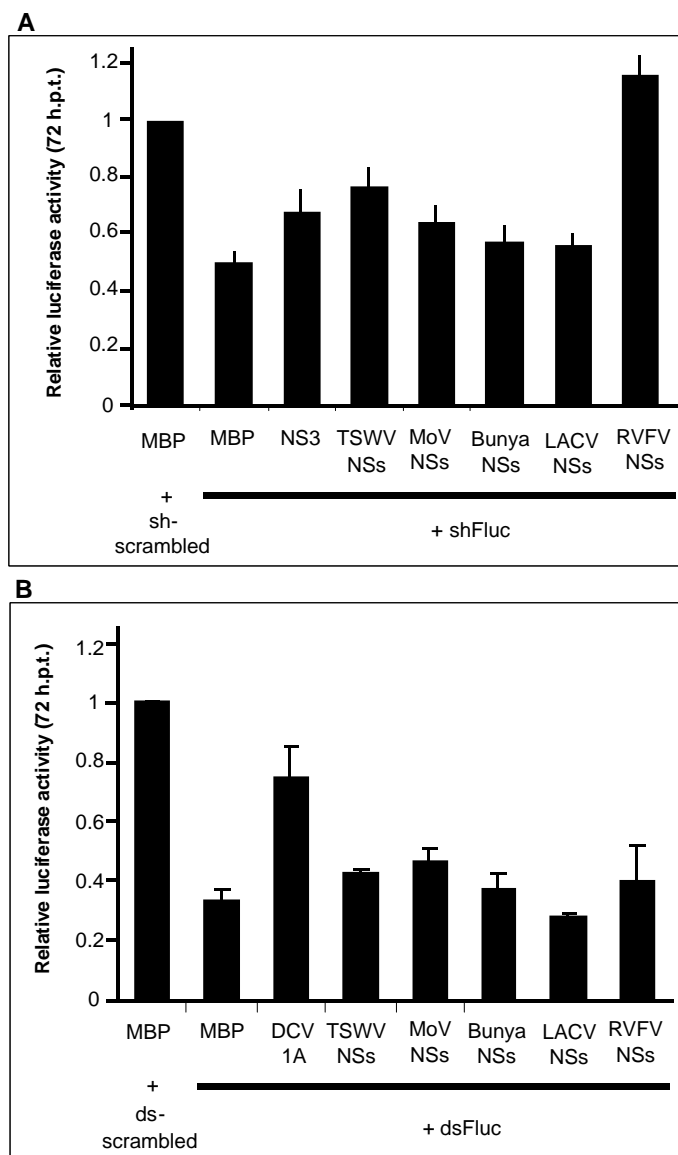


Figure 7-3: RNA silencing suppressor activity of bunya virus(-like) NSs proteins in insect cells

To measure the effect of different NSs proteins (TSWV, MoV, Bunyamwera, LACV and RVFV) of viruses established or tentatively belonging to the *Bunyaviridae*, on the siRNA pathway, S2 cells were co-transfected with a pMT-Renilla, pMT-Firefly (Fluc), and either specific (shFluc) or unspecific (sh-scrambled) short hairpin RNA (panel A) in concert with either pIB-MBP, -RHBV NS3, TSWV NSs, MoV NSs, Bunyamwera NSs, LACV NSs, RVFV NSs or DCV 1A. After induction at 48 hpt, relative luciferase expression (Firefly/Renilla) was determined 72 hpt and the mean of at least two independent experiments is shown with standard error (panel A). The effect on dsRNA induced silencing was determined by repeating experiments from panel A, but this time adding *in vitro* transcribed dsRNA as inducer molecule, either specific (dsFluc) or unspecific (ds-scrambled) to the medium at 48 hpt. Expression was induced 55 hpt, relative luciferase expression (Firefly/Renilla) was determined 72 hpt and the mean of at least two independent experiments is shown with standard error bars (panel B). TSWV, Tomato spotted wilt virus; MoV, Mourilyan virus; LACV, La Crosse virus; RVFV, Rift valley fever virus; NSs, non-structural protein encoded by the s-RNA segment; MBP, maltose binding protein; RHBV, rice hoja blanca virus; hpt, hours post transfection; DCV, Drosophila C virus; dsRNA, double stranded RNA; hpt, hours post transfection

Collectively, these results did not only show the sensitivity of many commonly used reporter based assays, but additionally stressed the importance to be cautious while interpreting results from such assays. This is being strengthened by the initial report on RSS activity of LACV NSs during siRNA-induced silencing in mammalian cells that recently became disputed due to a study that only reported interferon antagonistic activity (Blakqori et al., 2007). In light

of this observation it is interesting to note that seemingly contradictory results have been observed for RHBV NS3 on the miRNA pathway in insect cells (Chapter 5), but which finally appeared to be due to a concentration dependent effect. This concentration dependency likely explains also the observed lack of RSS activity of RHBV NS3 and tombusvirus P19 when (an excess amount of) *in vitro* transcribed dsRNA was used as inducer instead of plasmid encoded shRNA (data not shown). The sensitivity and contradictory results observed with reporter based assays, stresses the importance of experiments in a viral context to resolve the RSS activity of bunyaviral NSs proteins. Attempts are currently made on this point (in collaboration with Prof. Dr. R. Elliot, St. Andrews, UK) to exchange Bunyamwera NSs for RHBV NS3 or TSWV NSs, but these experiments have thus far not been successful.

Antiviral RNA silencing and suppression in mammals

A hallmark of antiviral RNA silencing in plants and insects is the accumulation of virus-derived siRNAs (Hamilton & Baulcombe, 1999, Li et al., 2002). These molecules have not yet been identified in mammalian cells infected with a wide range of human viruses (Pfeffer et al., 2004). Recently such molecules were described in low concentrations for several endogenous and exogenous viruses using deep sequencing (Bennasser et al., 2005, Parameswaran et al., 2010, Soifer et al., 2005, Yang & Kazazian, 2006), but the significance and biological activity of these findings is still being debated (Lin & Cullen, 2007).

Accumulating evidence exists in support of antiviral RNA silencing in mammalian cells, but most probably not involving siRNA molecules. In case of HIV-1, a positive effect of knockdown of specific RNA silencing proteins (e.g. Dicer and Drosha) on the virus production has been demonstrated (Triboulet et al., 2007). The observed trans-complementation of HIV-1 Tat protein by NS3, but not the NS3 mutant, regarding virus production support the targeting of HIV-1 by the RNA silencing pathway. The fact that NS3 and HIV-1 Tat could also interfere with the miRNA pathway in mammalian cells (Chapter 4) supports a possible role of the miRNA pathway in antiviral defense. This idea is more and more supported by experimental evidence reported over the years. Firstly, virus-associated (VA) RNAs encoded by the adenovirus DNA genome are exported out of the nucleus and compete with pre-miRNAs for Exportin-5 dependent nuclear transport (Lu & Cullen, 2004). In addition, their subsequent cleavage by Dicer into small dsRNA molecules, and uploading into RISC leads to oversaturation of RISC and interference in the RNA silencing pathway (Andersson et al., 2005). Next to this, VA1 RNA also inhibits PKR activation by binding to it. As such VA RNAs function dually in two different pathways (Mathews & Shenk, 1991). Interestingly, the significant decrease in replication of mutant adenoviruses lacking these RNAs could be restored *in trans* by two other small RNAs coded by Eppstein-Barr-virus, strengthening the importance of structure rather than sequence of these VA RNAs (Bhat & Thimmappaya, 1983). Secondly, HIV-1 is indirectly inhibited by the expression of certain miRNAs cluster that down-regulate host proteins regarded as essential for a successful viral life cycle. On this point differences in miRNA expression patterns have been observed in HIV-1 infected cells

compared to non-infected cells (Triboulet et al., 2007, Yeung et al., 2005). Application of specific miRNA inhibitors to these cells reversed HIV-1 latency (Zhang, 2009). Until now, similar antiviral effects of cellular miRNAs, either directly targeting viral RNA or indirectly by down regulating important host proteins have only been reported for viruses able to persistently infect mammalian cells (e.g. retroviruses and herpes viruses) (reviewed by Ouellet & Provost 2010). The question if miRNAs also target other viruses, including plant and insect viruses, still remains to be answered. Computer prediction has identified several viral RNAs with potential human miRNAs binding sides, e.g. miR-507 and miR-136 targeting Influenza A virus Polymerase B2 and hemagglutinin genes, respectively (Scaria et al., 2006). Another potential of the miRNA pathway could be the possibility of a systemic (antiviral) signal in mammals. Recent studies on this point have revealed the secretion of miRNAs, either viral or host encoded, via exosomes and multivesicular bodies (Bennasser et al., 2005, Gibbings et al., 2009, Jopling et al., 2005, Omoto et al., 2004, Pegtel et al. 2010).

Evidence against the idea of antiviral miRNA activity comes from HCV which depends on the presence of the liver specific miR-122 for successful viral replication but also from HIV-1 which has been shown to encode several miRNAs itself (Bennasser et al., 2005, Jopling et al., 2005, Omoto et al., 2004). Although it does not rule out the possibility of an antiviral miRNA pathway, in both cases the virus would not benefit from suppression of the miRNA pathway by viral encoded RSS proteins. More detailed experiments show that the HIV-1 encoded miRNAs suppress the Nef function, resulting in a lower HIV-1 virulence (Bennasser et al., 2005). This, together with the differences in host encoded miRNA expression profile in HIV-1 infected cells, would indicate that miRNAs are an important regulator for a persistent infection of HIV-1. The low expression of the proposed RSS, Tat, in persistently infected cells supports this (Triboulet & Benkirane, 2007, Triboulet et al., 2007, Yeung et al., 2005). Other viruses producing persistent infections and belonging to the Herpesvirus group have been reported to encode several viral miRNAs, possibly targeting cellular and viral RNA, which are suggest to play also a role in their persistent infections (reviewed by Ouellet & Provost 2010). Overall it is likely that mammalian viruses have to cope with multiple (antiviral) pathways (innate immune response by IFN, siRNA and miRNA-mediated pathway), some which have a primary antiviral function while others due to e.g. structural similarities overlap and side effects appear to end up antiviral too.

Recent studies suggest links between the IFN response and the RNA silencing machinery, specifically with the miRNA pathway. In one study, host miRNAs were shown to positively regulate IFN-beta production (Witwer et al., 2010) whereas IFN-beta induction in HCV-infected cells triggered the expression of host miRNAs that negatively-regulated viral replication (Pedersen et al., 2007). In a second study, IFN-induced RNA editing enzymes (ADARs) altered the miRNA target-specificity (Ohman, 2007). In a third study, several proteins were identified to have an important role in the RNA silencing pathway, e.g. TAR binding protein (TRBP) interacting with Dicer, have also been described as IFN-effector (Bennasser et al., 2006, Gatinol et al., 2005). A similar interaction has recently been

reported in insects between Dicer and Vago, a protein that is involved in the induction of an antiviral state (Kemp & Imler, 2009). Regarding the interplay between miRNA pathway and IFN response it may not be surprising that many RSS proteins from mammalian viruses are reported to antagonize both. Thus, it seems that the miRNA pathway or at least some miRNA/miRNA* duplexes have the potential to act antiviral, but how this acts precisely remains enigmatic. It is unknown whether the biological relevance of this interplay occurs for all mammalian infecting viruses or only for a hand full.

Are there antiviral miRNAs in plants and insects?

Whereas the idea of an antiviral miRNA pathway or at least miRNAs acting antiviral against mammalian virus infections is slowly becoming accepted, nothing is known on antiviral miRNA activity against plant and insect viruses. RHBV NS3 was shown to harbor a similar high affinity for miRNA/miRNA* duplexes as for siRNAs and interfered with miRNA regulatory pathway in plants, insect and mammalian cells (Chapters 3, 4 & 5). Whether these interactions are a specific response to counteract antiviral miRNAs or are just due to structural similarities between siRNA and miRNA/miRNA* duplexes is still not known. Several findings support interplay between the miRNA pathway and the antiviral 21 nt siRNA pathway in plants, giving a possible advantage for viruses to interfere with the miRNA pathway.

For example, in healthy plants AGO1 is normally loaded with mature miRNAs, however in virus infected plants it is known as important factor for the antiviral RNA silencing response. The observed interaction between two RSS proteins (CMV 2b and polerovirus P0) and plant AGO1 (Csorba et al., 2010, Gonzalez et al. 2010) further supports this interplay between siRNA and miRNA pathway. In addition, some findings point to a possible antiviral activity of the miRNA pathway or at least some miRNA molecules itself. Computer analysis revealed the presence of several 20-25 nt long sequences, probably expressed as miRNA molecules, in *Arabidopsis* with complementarities to plant viral genomes as potential interactive sequences. Several rice miRNAs, shown to be DCL1 dependent and detected by Northern blot analysis in different rice tissues, have been predicted to target parts of the RHBV RNA (Hemmes, 2007, Liu et al., 2005), i.e. the miRanda program (Enright et al., 2003) showed a possible target for the miR-528 in the nucleocapsid (N) gene of RHBV. If this viral sequence is targeted *in vivo* during a natural RHBV infection of rice, miRNA/miRNA* duplex binding by NS3 could prevent the RHBV N-gene transcript from becoming translationally arrested or degraded. After all a translational arrest would lead to lower expression of the N gene, and thereby in a decrease of viral replication activity, because RNA polymerases of negative strand RNA viruses are dependent on the concentration of nucleocapsid proteins (Meyer et al., 2002). Targeting the RHBV N gene would thus enable rice plants to resist RHBV infections. Until now, no miRNAs in planthoppers have been published. Whether antiviral miRNA activity against RHBV in plants and insects exists, thus still remains to be analyzed.

The same applies to the analyses on tospovirus NSs protein, which was shown to potentially interfere with the miRNA pathway in plants and insect cells (Chapter 6). Due to limited reports

on identified miRNAs in tomato only a few possible hits between tomato miRNAs and TSWV RNA transcripts could be predicted (Gu et al., 2010, Pilcher et al., 2007; Sanger miBase). One prediction identified the Sly-miR399 to target the TSWV movement protein transcript (NSm). This miRNA was of special interest because of its high degree of complementarities at the 5' end (seed region) with the NSm target (energy < -20 kCal/Mol) and expression, in *A. thaliana*, in all tissues and in tomato at least in the roots and leaves (Gu et al., 2010, Sunkar & Zhu, 2004, Zhang et al., 2008). Targeting the NSm transcript could block viral spread and thereby diminish or localize viral infection (Kormelink et al., 1992, Prins et al., 1997, Storms et al., 1995). Another hit of interest is Sly-miR169c and as predicted target the TSWV glycoprotein precursor transcript (5' end seed region & energy < 20 kCal/Mol). Although targeting the glycoprotein precursor transcript would not abrogate nor decrease viral infection and spread, it would result in a lower titer of mature virus particles and as a consequence to lower transmission rates. Research in *A. thaliana* showed the expression of the homologues miRNA molecule (Ath-miR169) in seeds, stem and flowers of 4 week old plants and seedlings, but nearly undetectable in leaves (Moxon et al., 2008, Reinhart et al., 2002). Whether the same expression profile applies to tomato is unknown, although recent data reports expression of miRNA 169 in roots of tomato and at least miRNA 169g is expressed in the leaves (Gu et al., 2010). Similarly to the situation for plant hoppers, the same applies to the thrips, insect vector of TSWV, for which no information on miRNAs is yet available (Sanger miBase).

Besides a direct antiviral role of miRNAs during RHBV or TSWV infection, additional indirect effects of miRNAs via host gene regulation, important for virus replication are possible. To date there is no evidence to substantiate or disprove this assumption. However, if so, RHBV and TSWV would benefit from binding siRNA and miRNA/miRNA* duplexes in multiple ways. The fact that for RHBV NS3 the same amino acids are important for binding siRNA and miRNA/miRNA* duplexes (Chapter 2) and required to maintain RSS activity would suggest that NS3 interferes in the same way with several RNA silencing pathways during viral infection. This is in contrast to several other RSS proteins, where a more distinct interaction with several (antiviral) RNA silencing pathways has been reported, e.g. for potyvirus Hc-Pro.

Interplay between other small dsRNA pathways and viral infections in plants?

For most RSS proteins, no interacting is known with other RNA silencing pathways or if established, the resulting effect on viral infection is lacking. In case of CMV 2b interactions with AGO1, AGO4 and siRNAs, involved in different RNA silencing pathways, have been demonstrated, but for which only the interaction with siRNAs was important for its RSS activity on local silencing (Gonzalez et al. 2010). The NLS in CMV 2b is not important for the local RSS activity but could be related to induction of severe viral symptoms in plants (Gonzalez et al. 2010). This observation could possibly be explained by the fact that DCL3-dependent 24 nt siRNAs, in combination with AGO4 and normally important in host DNA methylation, are located in the nucleus and CMV 2b needs nuclear localization to interact with

AGO4. These AGO4/DCL3 dependent 24 nt siRNAs, have been shown to inhibit viral transduction in case of R-gene mediated plant disease resistance, affecting viruses not carrying an avirulent gene and linking RNA silencing and R-gene mediated responses (Bhattacharjee et al., 2009). These findings, give a possible explanation for the interaction of RSS proteins and different RNA silencing pathways, distinct of the known antiviral 21 nt siRNA pathway.

Whether RHBV NS3 or TSWV NSs also interfere with other pathways, besides the 21 nt siRNA and miRNA pathway and whether there is an effect of this possible interaction on the viral infection, is not known. However, RSV NS3 has been reported to encode a NLS and at the same ¹⁷³KKR¹⁷⁵ region as is predicted for RNA binding (Xiong et al., 2009). For RHBV NS3, although predicted, no nuclear localization was observed in mammalian and insect cells (data not shown), but no information is present for a natural viral infection in plants.

Do endo-siRNAs act antiviral?

The endogenous siRNA pathway has been reported in insect and mammalian cells to be important in silencing of transposons in non-germline cells (Chung et al., 2008, Czech et al., 2008, Ghildiyal et al., 2008, Kawamura et al., 2008, Okamura et al., 2008a, Tam et al., 2008, Watanabe et al., 2008a, Watanabe et al., 2008b). Endo-siRNAs have the same biochemical properties as viral siRNAs molecules. Therefore it was not surprising that in *Drosophila*, either for homologous (FHV B2) or heterologous (tombusvirus P19) proteins RSS activity has been observed on the endo-siRNA pathway (Berry et al., 2009). However, no RSS activity on the endo-siRNA pathway could be observed for RHBV NS3 (Chapter 5), even though high binding affinity to endo-siRNAs was anticipated. It is likely that these analyses turned out to be negative in light of the fact that the concentration of endo-siRNAs in the assays performed was relatively high while NS3 expression may have been relatively low to cope with these endo-siRNA amounts. Since tombusvirus P19 (positive control) also revealed a lack of RSS activity in the assay performed with RHBV NS3, the presence of RSS activity on endo-siRNAs can not be excluded yet. Until now, no antiviral activity has been linked to the endo-siRNA pathway and it is likely that the interaction between RSS and endo-siRNAs is due to the high structural similarities between viral- and endo-siRNAs. Next to their role in the cytoplasm, endo-siRNAs have recently been shown to be involved in TGS in the nucleus resulting in heterochromatin formation in somatic tissue in *Drosophila*. FHV-B2 and nuclear localized tombus-virus P19 interfered with this TGS by binding either the precursor molecules of endo-siRNAs (FHV-B2) or mature endo-siRNAs (tombusvirus P19) (Fagegaltier et al., 2009). It is not yet known if TGS in insects or plants has a potential antiviral activity for viruses replicating in the cytoplasm like TSWV and RHBV. The observed interaction of CMV, which replicates in the cytoplasm, with parts of the TGS machinery (AGO 4) leading to abolishment of DNA methylation (Gonzalez et al. 2010), suggests a possible role in antiviral defense, either direct or indirect (e.g. affecting host gene expression). A direct antiviral effect would make sense for DNA viruses replicating in the nucleus like the insect baculoviruses. Small dsRNA derived

from overlapping baculovirus gene transcripts could theoretically act antiviral, either in the cytoplasm or exported back into the nucleus targeting the baculovirus DNA genome in a similar way as reported for the endo-siRNAs and the heterochromatin formation. No antiviral RNA silencing response, either post transcriptional or transcriptional, has yet been reported for baculovirus infection in insects. In plants antiviral activity of, resulting in TGS has been reported (reviewed by Raja et al. 2010).

Whether the endo-siRNAs have a potential to play a role in antiviral defense in mammalian cells remains to be elucidated. In light of this it is interesting to note that during retrovirus infections integration of the viral genome into the host genome occurs. As retro-transposon elements are suggested to originate from retrovirus infections and silenced by endo-siRNAs, an antiviral activity of the endo-siRNA pathway is not unlikely.

Concluding remarks and outlook

During this course of the work described in this thesis, the mode of action of the RSS proteins NS3 and NSs from two plant-infecting negative stranded RNA viruses, RHBV and TSWV respectively have been investigated by means of biochemical analysis and reporter-based assays. In conclusion, both RSS proteins, RHBV NS3 and tospovirus NSs, interact in a similar way with different RNA silencing pathways in several organisms by interaction with dsRNA, key molecules of all these pathways (Chapter 2 - 6). Interestingly, NSs proteins of different tospovirus species showed slight divergence in affinities for dsRNA molecules, pointing to co-evolution of these RSS proteins (Chapter 6).

This information about the mode of action of RSS proteins and their point of interference in the RNA silencing pathways could give more detailed insight into the different small dsRNA pathways in the hosts. Furthermore, detailed information about the RNA silencing pathways can be extrapolated to use them in other applications, e.g. transgenic virus resistant organisms using engineered dsRNA molecules and detailed information between vector/host - virus interaction. Biochemically well-characterized RSS proteins could be further used as tools to gather more information about RNA silencing pathways in other organisms (e.g. humans) as described for RHBV NS3 (Chapter 4). Another application could be the increase in e.g. protein or virus particle production normally restricted by RNA silencing in different organisms. The information presented in this thesis, that both RSS proteins bind dsRNA molecules *in vitro* and at least for RHBV NS3 this affinity was crucial for its RSS activity represent important insight in their mode of action as RSS proteins (Chapters 2 - 6). To give a final answer about their activity and interaction with the vector/host pathways during a natural infection more research is needed. This section will focus on the direct follow up experiments resulting from the findings obtained during this thesis research. One of the major questions would be to determine the relevance of the reported results in real life: in virus infection and transmission in nature.

First, the observed *in vitro* binding affinity of NS3 and NSs to the different RNA molecules should be proven during a natural infection to support their biological relevance. Comparison

of the bound RNA in insect vector versus plant host can even give information if the RNA silencing response targets the same part of the virus in insects and plants. Information would be obtained to answer the question what is the source (secondary structures in genomic/transcript RNA or dsRNA replication intermediates) and frequency of viral siRNAs of negative stranded RNA viruses in insects and plants. By using mutant viruses lacking the corresponding RSS protein (NS3 or NSs) during this analysis, the importance of the RSS protein and their biological activity would be further elucidated. These results could be placed in the overall picture of RNA silencing (induction), using data already known for other RNA viruses (Aliyari et al., 2008, Yan et al., 2010).

Second, the observed interference of NS3 and NSs with the (induced-) miRNA pathway in plants and insects should be first confirmed in whole organisms and later during a natural viral infection to understand the biological relevance.

At the moment the relevance of the interaction between RSS proteins and the miRNA pathway is undecided. Therefore, the efficiency of RHBV and TSWV viral infections in plants and insects deficient in the miRNA pathway (i.e. knockdown of Dicer-1, Drosha or DCL-1) compared to wildtype should be determined. To investigate the relevance of the predicted antiviral activity of certain miRNAs (e.g. Os-miR-528 for RHBV and Sly-miR-399 or Sly-miR-169c for TSWV), specific miRNAs inhibitors could be applied and the effect on either transient expressed viral RNA transcript level or RSS negative virus could be observed in e.g. protoplasts.

Third, more biochemical analysis of NS3 and NSs is needed to point out the importance of certain interaction with the host/vector, regarding the outcome of the infection. Attempts have been made to characterize domains important for the reported RSS activity and at least for NS3 a triple lysine cluster has been shown to be important for small dsRNA binding and RSS activity (Chapters 2 - 4). More analysis should be performed to determine other possible host/vector and viral interaction partners, e.g. by co-immunoprecipitation and mass spectrometry. For domain characterization either unbiased alanine scan mutation analysis could be performed or directed mutagenesis after solving the crystal structure of NS3 and NSs.

In conclusion, it is clear that the research of RNA silencing and silencing suppression is a still growing field and will be the focus of many further research projects. It becomes obvious that the different RNA silencing pathways are connected and interference with one will influence the others (Azevedo et al., 2010). In light of this, the mode of action of RSS proteins should be investigated in a broader context than only in the well established antiviral 21 nt siRNA pathway. The results shown in this thesis pointing to an antiviral response by the miRNA pathway in insects and plants are novel and contribute to our understanding of antiviral responses in general.

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List of Abbreviations

dsRNA,	double stranded RNA
siRNA,	small interfering RNA
miRNA,	microRNA
mRNA,	messenger RNA
RSS,	RNA silencing suppressor
RISC,	RNA induced silencing complex
PTGS,	post transcriptional gene silencing
RNAi,	RNA interference
CP,	coat protein
PDR,	pathogen derived resistance
DCL,	dicer-like protein
AGO,	Argonaut
RDR6,	RNA dependent RNA polymerase 6
RdRp,	RNA dependent RNA polymerase
Pri-miRNA,	primary microRNA
Pre-miRNA,	precursor microRNA
UTR,	untranslated region
Ta-siRNA,	transactivating small interfering RNA
TGS,	transcriptional gene silencing
RdDM,	RNA dependent DNA methylation
Endo-siRNA,	endogenous small interfering RNA
LTR,	long terminal repeat
dsRBD,	double stranded RNA binding domain
PAZ domain,	PIWI/ Argonaut/ Zwilli domain
piRNA,	PIWI-interacting RNA
nat-siRNA,	natural small interfering RNA
ra-siRNA,	repeat-associated small interfering RNA

Summary

The research described in this thesis focuses on the mode of action of RNA silencing suppressor (RSS) proteins encoded by negative-strand RNA plant viruses. RNA silencing is an important antiviral defense mechanism in plants and insects acting by sequence specific RNA degradation. A characteristic of RNA silencing is the recognition of double stranded (ds)RNA and its processing into small dsRNA of 21-30 (nt) nucleotides by enzymes of the Dicer family. These small RNA molecules are subsequently incorporated into the RNA induced silencing complex (RISC) and guide this complex to complementary target RNAs that are subsequently cleaved or inactivated by the Argonaut proteins in RISC. As counter defense, plant and insect viruses encode RSS proteins that interfere with the RNA silencing pathway. In the last years a wide range of RSS proteins have been identified and for some of these proteins the mode of action has been determined. At the onset of this thesis research, little was known on RSS proteins of negative-stranded RNA viruses. Negative-strand RNA viruses are unique in the ability to replicate in their insect vector as well as plant host and thereby are likely to encounter antiviral RNA silencing in two distinct organisms. RNA silencing has been discovered throughout eukaryotic life, including yeast, plants, insects and mammals. Next to antiviral defense, RNA silencing is involved in a range of other processes, such as gene regulation during development and genome protection against transposons. Key molecules of these pathways are always small RNAs and proteins of the Dicer and Argonaut class, supplemented by several co-factors.

At the start of this thesis research, RSS proteins of two plant infecting negative-stranded RNA viruses, *in casu* NS3 from Rice hoja blanca tenuivirus (RHBV) and NSs from Tomato spotted wilt tospovirus (TSWV) had been identified. Little was known on their mode of action in the antiviral RNA silencing pathway. Even less was known on a possible interaction with other RNA silencing pathways and the biological relevance of this. The high affinity of RHBV NS3 to 21 nt small interfering (si)RNAs *in vitro* suggested that NS3 exerted its RSS activity in plants and insects by sequestering siRNA molecules. Chapter 1 summarizes the state of the art of antiviral RNA silencing and the counteracting activity of RSS proteins.

Chapter 2 of this thesis describes the importance of siRNA binding for RSS activity of RHBV NS3 in plants. Alignments with NS3 orthologs from other tenuiviruses, resulted in the identification of two conserved regions, predicted to be possibly involved in RNA binding. Whereas deletion of these regions resulted in abrogation of RSS activity in plants, single alanine substitutions in these regions did not affect RSS activity nor siRNA binding. Further analysis revealed that substitution of a triple lysine (K173-K175) cluster in the carboxy-terminal conserved region of NS3 resulted in loss of siRNA binding and concomitantly of RSS activity. These results demonstrated the requirement of siRNA binding for NS3 RSS activity in plants. Small interfering RNAs are conserved key molecules of the RNA silencing pathway in all eukaryotes. Therefore, it was hypothesized that if NS3 acts as RSS solely by binding siRNA molecules, it would be able to show RSS activity even in non-host mammalian cells. In

chapter 3 it was shown that wild type NS3, but not NS3 mutant (mutated in the triple lysines K173-K175), was indeed able to act as RSS in mammalian cells when using either short hairpin RNA or synthetic siRNA molecules as RNA silencing inducer. However, in the case of siRNA-induced silencing, suppression could only be achieved upon *a priori* delivery of NS3. Altogether, this strengthened the idea that NS3 exerted its RSS activity by sequestering siRNAs.

In mammals, long dsRNA molecules induce the interferon response considered as major antiviral innate immune response. Because of this, the (additional) presence of an antiviral RNA silencing pathway in mammals has been an issue of strong debate over the last years. The biological relevance of RSS activity with proteins of several mammalian-infecting viruses (e.g. Tat of HIV-1), that earlier were already shown to contain interferon antagonistic properties, has not been widely accepted. Since NS3 was able to suppress RNA silencing in mammalian cells, it was questioned whether this protein could be used as a tool to investigate the presence of antiviral RNA silencing against mammalian viruses. Prior to this it was first shown (Chapter 4) that NS3 indeed did not have interferon antagonistic activities. In a following experiment a Tat-negative HIV-1 mutant, normally showing reduced virus titers due to the lack of Tat (a transcription activator of HIV-1 and protein with RSS activity), was successfully trans-complemented with NS3, but not with the NS3 mutant. Meanwhile, reports verified interplay between viral infections (e.g. HIV-1) and the miRNA pathway, a branch of the RNA silencing pathway involved in host gene regulation. This suggested a possible role of miRNAs in antiviral defense, at least in mammals. NS3, but not NS3 mutant, exhibited an affinity to miRNA/miRNA* similar as to siRNAs (Chapter 4), probably due to structural similarities of these molecules. Using a miRNA-based reporter assay both NS3 and HIV-1 Tat were demonstrated to interfere on the endogenous miRNA pathway in mammalian cells. In addition to the successful trans-complementation of a Tat-negative HIV-1 mutant with NS3, this demonstrated that HIV-1 is being targeted by antiviral small dsRNA molecules (i.e. siRNA or miRNA/miRNA*). The biological relevance of Tat's RSS activity is thus likely explained to counteract this antiviral RNA silencing response.

Chapter 5, verifies the *in vivo* RSS activity of NS3 on the miRNA pathway in plant and insect cells using miRNA-based sensor constructs. The observation that NS3 mutant was not able to suppress siRNA- and miRNA-mediated silencing proved again the importance of the small dsRNA binding affinity of NS3 in RSS activity. The appearance of a leaf curling phenotype of *Arabidopsis* transgenically expressing NS3 was, thus, likely due to NS3 interference in endogenous miRNA-mediated host gene regulation. The question, if this interaction with the miRNA pathway is a side effect, due to structural similarities with the antiviral 21 nt siRNA molecules or intended is not yet known.

In Chapter 6, the mode of action of the TSWV RSS protein, NSs, on different RNA silencing pathways was analyzed in order to determine if the observed properties of NS3 generally applied to the RSS protein of other negative-strand RNA plant viruses. In contrast to NS3, TSWV NSs exhibited a size-independent binding of dsRNA molecules, i.e. it bound small and

long RNA duplex molecules. Its ability to bind long dsRNA was further supported by (partial) inhibition of Dicer-cleavage of dsRNA into siRNA, *in vitro* and *in planta*. NSs protein of a few other tospoviruses belonging to the *American* clade also showed the size-independent affinity. In contrast, only siRNA-specific binding was observed for Tomato yellow ring virus, a tospovirus belonging to the *Eurasian* clade. For TSWV NSs an additional binding and interference with the miRNA pathway was demonstrated *in planta* and in insect cells. Since tospoviruses are the plant-infecting members of the *Bunyaviridae* family, whose members primarily infect mammals, the binding affinity to long dsRNA by tospoviral NSs proteins most likely reflects an ancestral relation to the animal-infecting members of the *Bunyaviridae*.

In chapter 7 all findings of this thesis are discussed in light of the current knowledge on other RSS proteins, their interference in different RNA silencing pathways and the potential of these, besides the antiviral 21 nt siRNA pathway, to act antiviral during a natural infection of the plant and insect vector host.

Samenvatting

Het in dit proefschrift beschreven onderzoek richt zich op de werking van RNA-silencing-suppressor (RSS)-eiwitten van min-streng RNA-plantenvirussen. RNA-silencing is een sequentie-specifiek RNA-afbraakmechanisme, dat voor verschillende doeleinden wordt gebruikt. Bij planten en insecten is één van de belangrijkste hiervan de inzet als antiviraal afweermechanisme. RNA-silencing wordt geïnduceerd door lang, dubbelstrengs (ds) RNA. Deze RNA-moleculen worden door het enzym Dicer of “Dicer-like” (DCL) in kleine stukjes van 21 nucleotiden (nt) geknipt, de zogenaamde “small interfering RNAs” (siRNAs). Deze siRNA-moleculen worden opgenomen in een “RNA-induced silencing complex” (RISC), dat vervolgens in staat is (virale) RNA-moleculen met complementariteit aan de siRNA-sequentie te detecteren en deze aansluitend af te breken. Op deze wijze kunnen planten en insecten virusinfecties te lijf gaan en de eventuele schade als gevolg daarvan beperken. Echter, planten- en insectenvirussen coderen, als reactie hierop, voor RSS-eiwitten die dit proces remmen. De laatste jaren is, van voornamelijk plus-streng RNA-plantenvirussen een groot aantal RSS-eiwitten geïdentificeerd en van enkele is het werkingsmechanisme verder ontrafeld.

Tot nu toe is RNA-silencing in elk onderzocht eukaryotisch organisme geconstateerd (bv gist, plant, insect, mens), alwaar het een belangrijke rol speelt in diverse cellulaire processen zoals genregulatie en het in actie komt als afweersysteem tegen transposons, mobiele DNA-elementen. Het mechanisme van RNA-silencing kent een aantal verschillende verschijningsvormen, die sommige aspecten met elkaar delen. Hiertoe behoren ondermeer het gebruik van korte, meestal dubbel-strengs, RNA-moleculen. In Hoofdstuk 1 is een overzicht van RNA-silencing gegeven met nadruk op de rol in antivirale afweer en de interactie met (plant-) virale RSS-eiwitten.

Voorafgaand aan het hier beschreven onderzoek waren de RSS-eiwitten van twee min-streng RNA-plantenvirussen geïdentificeerd, nl. het NSs-eiwit van het tomatenbronsvlekkenvirus (Engels: “Tomato spotted wilt virus” ((TSWV)) en het NS3-eiwit van het “Rice hoja blanca virus” (RHBV), maar er was slechts weinig bekend over hun werkingsmechanisme. Deze virussen zijn vrij uniek onder plantenvirussen, omdat ze zich zowel in planten als insecten (vector) kunnen vermeerderen. Hierdoor dienen ze zich te verweren tegen RNA-silencing in twee fundamenteel verschillende organismen. De vraag naar het werkingsmechanisme van deze RSS-eiwitten in planten en dieren stond daarom centraal. Vooraf was alleen van RHBV NS3 bekend dat dit eiwit *in vitro* affiniteit had voor kleine (21 nt) dsRNA-moleculen en hoogstwaarschijnlijk *in vivo* door binding van deze moleculen via de siRNA-route met de afweer in planten en insecten interfereert. Of het NS3 eiwit daarnaast ook met andere RNA-silencing-routes interfereerde en over het belang daarvan voor virusinfectie, was niets bekend.

Het belang van de binding van siRNAs door NS3 voor RSS-activiteit in planten, is in hoofdstuk 2 verder onderzocht. Met behulp van computeranalyses van een aantal tenuivirus NS3-orthologen werden twee geconserveerde gebieden in het RSS-eiwit geïdentificeerd, die mogelijk een rol spelen in RNA binding. Terwijl het vervangen in deze gebieden van enkele aminozuren door alanine geen effect had, resulteerden deleties van beide gebieden, dan wel een vervanging van drie lysines (K173-K175) door alanines in het verlies van siRNA-binding en gelijktijdig verlies van RSS-activiteit in planten. Hiermee was het belang van siRNA binding voor de NS3 RSS-activiteit in planten duidelijk aangetoond.

Vanwege de geconserveerdheid van siRNA-moleculen werd verwacht dat NS3 ook in staat zou zijn om deze moleculen in andere eukaryoten te binden, zoals bijv. zoogdiercellen, ook al zijn deze geen natuurlijke gastheer van RHBV. In hoofdstuk 3 is aangetoond dat NS3, maar niet de NS3-mutant (met alaninevervangingen van K173-K175), RNA silencing in zoogdiercellen kan tegengaan wanneer silencing werd geïnduceerd door zogenaamde “short hairpin” (sh-) of synthetische siRNA-moleculen. Opvallend was dat de RSS-activiteit bij de inductie door siRNA-moleculen alleen werd waargenomen wanneer NS3 van tevoren tot expressie werd gebracht. Tezamen met voorgaande resultaten onderstreepte dit het belang van siRNA-binding voor de RSS-functionaliteit van NS3.

Terwijl RNA silencing algemeen wordt geaccepteerd als hét antiviraal afweersysteem in planten en insecten, staat de rol van dit proces bij zoogdieren nog steeds ter discussie. Bij zoogdieren wordt de interferon-geïnduceerde antivirale afweer gezien als voornaamste initiële reactie tegen virusinfecties. Deze afweer wordt echter, net als bij RNA silencing, door lange dsRNA-moleculen geïnduceerd en dat bemoeilijkt het beantwoorden van de vraag of RNA-silencing, naast de interferon-geïnduceerde afweer, actief is als antiviraal afweersysteem in zoogdieren. Antwoord op deze vraag zou tevens uitsluitsel kunnen geven over de biologische relevantie van eerder geïdentificeerde RSS-eiwitten van zoogdiervirussen (bijv. Tat van HIV-1), omdat vele van deze eiwitten voorheen al geïdentificeerd waren als interferonantagonisten. Vanwege de RSS-activiteit van NS3 in zoogdiercellen en de afwezigheid van affiniteit voor lange dsRNA-moleculen is onderzocht of NS3 gebruikt zou kunnen worden om de aanwezigheid van antiviraal RNA silencing in zoogdieren aan te tonen. In hoofdstuk 4 werd geverifieerd dat NS3 geen interferon-antagonistische activiteiten vertoont. Tevens werd vastgesteld dat het wild-type NS3, maar niet de NS3-mutant, in staat is om de lage virustiter van een Tat-negatieve HIV-1 mutant terug te brengen tot op het niveau van het wild-type virus. Hiermee werd bewezen dat NS3 in staat is tot transcomplementatie van HIV-1 Tat en dat hiervoor binding met kleine dsRNA-moleculen essentieel is. Ondertussen werden interacties tussen virussen en de miRNA-route gerapporteerd, wat de mogelijkheid van een antivirale functie van miRNA-moleculen openlaat. Met behulp van biochemische analyses werd aangetoond dat het NS3-eiwit, maar niet de NS3-mutant (K173-K175), een vergelijkbare affiniteit voor zowel miRNA/miRNA*-duplexes *in vitro* als voor siRNA moleculen heeft. Dit is hoogstwaarschijnlijk vanwege de grote structurele overeenkomsten tussen beide moleculen. Door gebruik te maken van

miRNA-sensorconstructen kon RSS-activiteit van zowel NS3 als van HIV-1 Tat op de endogene miRNA-route in zoogdiercellen worden aangetoond (hoofdstuk 4). Tezamen versterkten deze resultaten het beeld dat HIV-1 doelwit is van antivirale RNA-silencing, waarbij kleine dsRNA-moleculen (siRNAs of miRNA/miRNA*s) een rol spelen en dat de RSS-activiteit van HIV-1 Tat nodig is als verweer hiertegen.

In hoofdstuk 5 is de *in vivo* RSS-activiteit van NS3 op de miRNA-route in planten en insecten aangetoond met behulp van miRNA-sensorconstructen. De afwezigheid van RSS-activiteit van de NS3 mutant tijdens alle analyses ondersteunde nogmaals de conclusie dat binding van kleine dsRNA-moleculen (siRNA en miRNA/miRNA*) belangrijk is voor de RSS-activiteit van NS3. Deze resultaten suggereerden tevens dat de aanwezigheid van misvormde bladeren (verkrulling) in transgene Arabidopsis-planten die NS3 tot expressie brengen, hoogstwaarschijnlijk het gevolg is van interferentie van NS3 op de endogene miRNA-route in planten.

In hoofdstuk 6 is de RSS-activiteit van het TSWV NSs-eiwit bestudeerd om te bekijken of de resultaten van NS3 algemeen van toepassing zijn op RSS-eiwitten van min-streng RNA-plantenvirussen. In tegenstelling tot NS3 bond TSWV NSs zowel korte als lange dsRNA-moleculen, en dus niet lengtespecifiek. De affiniteit voor lange dsRNA-moleculen werd verder ondersteund door de *in vitro* en *in vivo* (planten) vastgestelde (gedeeltelijke) remming van de Dicer-knip van lange dsRNA- in kleine siRNA-moleculen door NSs. Een lengte-onafhankelijke binding van dsRNA door NSs werd tevens voor een aantal andere tospovirussen (van het Amerikaanse cluster van deze groep) aangetoond, maar niet voor het Tomato yellow ring virus NSs (Euraziatische cluster). Deze laatste bond, net als NS3, alleen kleine dsRNA-moleculen. Voor het TSWV NSs werd tevens een interactie met de miRNA-weg in planten en insecten aangetoond.

De binding van lange dsRNA-moleculen door NSs van een aantal tospovirussen is hoogstwaarschijnlijk terug te voeren op de relatie van deze plantenvirussen met de zoogdier-infecterende *Bunyaviridae*.

In hoofdstuk 7 zijn de behaalde resultaten vergeleken met het werkingsmechanisme van andere inmiddels gekarakteriseerde RSS-eiwitten en zijn de interacties met overige RNA-silencing-routes, naast de antivirale siRNA-route, besproken in het kader van een additionele antivirale afweer tijdens een natuurlijke infectie in plant en insect met deze min-streng virussen.

Acknowledgments

This part of my thesis booklet will probably been read by most people. The last four and a half years have been rushed away quite fast and a lot of people have contributed to make it a great time to remember. Therefore, I would like to spend some words to thank those who have contributed to either small or big parts of this thesis and the associated research.

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I also want to express my gratitude to Rob for the time being my promoter. He always had time to discuss results, new experiments, ideas and even topics not related to science (e.g. how to get water pipes best inside our holiday house in Sweden) as well as checking the manuscripts, if needed in a short time, or being the 'critical reviewer' to ensure that the manuscript and presentations were well-thought out. I will never forget his typical answer to the question if he had a few minutes time: "Hmm, not really but come in I can spare 5 minutes" and you could be sure that it would at least take half an hour. The support he gave me was really great. I still remember when I had to give my first talk in front of a big audience in Nürnberg (immediately after Bryan Cullen). He was there the whole time, just to make sure that I was fine. Thanks for everything.

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Ronald, thanks for sharing your knowledge, expertise and reagents around *Drosophila* with me and the time you and Joël spent to produce good working *Drosophila* embryo extracts. Furthermore, a special thanks to the other collaborators; Frank, Bernadette, Peter, Rob, Martijn, Richard and Julian. It was great to work with all of you, even though the results were not always as we hoped for.

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Curriculum Vitae



Esther Schnettler was born on the 18th of June 1980 in Düsseldorf, Germany. She completed here secondary education in June 1999 at the Gymnasium Gerresheim Am Poth in Düsseldorf, Germany. After having spent some time in Ireland as au-pair, she did a three months internship at the research group of Janssen-Cilag (belonging to the Johnson and Johnson group) in Neuss, Germany. The aim of her research was to develop and test a new drug against diarrhea. Afterwards Esther followed a three-month combination of Dutch and Physics courses at the James Boswell Institute in Utrecht, The Netherlands. These were obligatory for her further bachelor and master study in Biotechnology at Wageningen University, which she finished in March 2006. As part of her MSc, she carried out a thesis project at the Laboratory of Virology (Wageningen University), supervised by Dr. Marcel Prins, to investigate the potential RNA silencing suppressor activity of the mammalian-infecting Influenza A virus NS1 protein in mammalian cells. Subsequently she started a seven-months internship at the University of Erlangen-Nürnberg, supervised by Prof. Bernhard Fleckenstein. In his laboratory she detected and analyzed several complexes, formed between the oncoprotein (Tio) of the *Herpesvirus ateles* and different host proteins, proposed to be critical for carcinogenesis. After her graduation Esther continued her career at Wageningen University by undertaking a PhD at the Laboratory of Virology, initially under supervision of the late Prof. Rob Goldbach, about the dual role of several viral proteins, known as RNA silencing suppressor proteins, in virulence, as described in this thesis. In July 2010, Esther was granted a Rubicon fellowship from the Netherlands Organisation for Scientific Research (NWO) to work for two years in the group of Dr. Alain Kohl at the The Roslin Institute, University of Edinburgh, on the RNA silencing response of ticks against arbovirus infection. She will start with this work in November 2010.

Account

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** first shared author*

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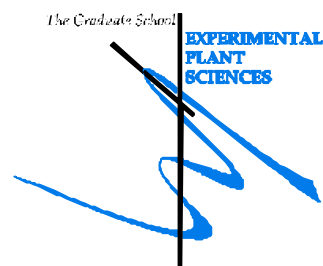
Date: **27 September 2010**

Group: **Laboratory of Virology, Wageningen University**

1) Start-up phase		<u>date</u>
► First presentation of your project The dual activity of RNAi suppressor proteins		Jul 03, 2006
► Writing or rewriting a project proposal		
► Writing a review or book chapter Plant Pathogens as Suppressors of Host Defense, Advances Botanical Research, Vol 51, pg 39 -88 (2009)		2009
► MSc courses		
► Laboratory use of isotopes Stralingscursus 'Veilig werken met radioactieve stoffen en stralingsbronnen'		Apr 2006
Subtotal Start-up Phase		6.0 credits*
2) Scientific Exposure		<u>date</u>
► EPS PhD student days EPS PhD Student Day, Wageningen University EPS PhD Student Day, Wageningen University EPS PhD Student Day, Leiden University		Sep 19, 2006 Sep 13, 2007 Feb 26, 2009
► EPS theme symposia EPS theme 2 Symposium 'Interactions between plants and biotic agents', University of Amsterdam EPS theme 2 Symposium 'Interactions between plants and biotic agents', Utrecht University		Feb 02, 2007 Jan 22, 2009
► NWO Lunteren days and other National Platforms ALW -meeting in Lunteren ALW -meeting in Lunteren ALW -meeting in Lunteren CW -meeting in Lunteren CW -meeting in Veldhofen Dutch association for plant virology (NKPV)		Apr 03, 2006 Apr 01 & 02, 2007 Apr 07 & 08, 2008 Dec 06, 2006 Dec 08-10, 2008 Feb 28, 2008
► Seminars (series), workshops and symposia Dutch Annual Virology Symposium (DAVS) Dutch Annual Virology Symposium (DAVS) Dutch Annual Virology Symposium (DAVS) Dutch Annual Virology Symposium (DAVS) Dutch Annual Virology Symposium (DAVS) EPS Seminar, Jim Carrington Farewell Symposium Plasterk "The small RNA revolution" Symposium (Alchimica: Viruses -Exploiting nature's own Nanoparticles) Symposium (RNomics: Rediscovering RNA and its Multiple Functions)		Mar 10, 2006 Mar 09, 2007 Mar 07, 2008 Mar 06, 2009 Mar 05, 2010 Mar 26, 2007 Sep 08, 2007 May 12, 2006 Aug 07, 2008
► Seminar plus Jim Carrington		Mar 26, 2007
► International symposia and congresses European Congress of Virology (Nürnberg, Germany) International conference of virology (Istanbul, Turkey) Keystone symposium: The Biology of RNA silencing (Victoria, British Columbia, Canada) Dutch-German plant virology meeting (Hamburg, Germany) EMBO meeting 2009, Amsterdam European Congress of Virology (Milano, Italy) ESF-EMBO symposium: Antiviral Applications of RNA Interference, Sant Feliu de Guixols, Spain		Sep 01-05, 2007 Aug 10-15, 2008 Apr 25-30, 2009 Apr 08 & 09, 2009 Aug 29 - Sep 01, 2009 Apr 07-11, 2010 May 30-Jun 04, 2010
► Presentations Presentation at Lunteren (RNAi suppressor activity of the Rice Hoja Blanca virus (RHBV) NS3 protein) Presentation at European Congress of virology (The NS3 protein of Rice hoja blanca tenuivirus suppresses RNA silencing by efficiently binding to siRNAs) Lecture RNAi and Application for the course Fundamental and Applied virology Dutch association for plant virology (NKP) (How plant viruses counteract antiviral RNAi) DAVS (The NS3 protein of Rice hoja blanca tenuivirus suppresses RNA silencing by efficiently binding to siRNAs) Presentation at Lunteren (Antiviral RNAi counter defence by segmented (-)ssRNA plant viruses) Poster: International conference of virology (Istanbul, Turkey) Lecture RNAi and Application for the course Fundamental and Applied virology Poster: EPS-PhD day (Leiden 26-2-09); dutch-german plant virology meeting (Hamburg 8-4-09) Presentation at dutch german plant virology meeting (Hamburg 9-4-09): Tenuiviral RNAi suppressor terminates a longstanding enigma: Are human viruses targets of RNAi? Poster: Keystone: The Biology of RNA silencing (Victoria, British Columbia, Canada) Poster: EPS PhD Poster session, EPS Peer Review, Wageningen Poster: EMBO meeting 2009, Amsterdam Presentation at ECV 2010; Como, Italy (The NS3 protein of Rice hoja blanca tenuivirus suppresses RNA silencing by efficiently binding to siRNAs) Poster: ECV 2010; Como, Italy Presentation at Sigma symposium; Nijmegen Presentation at veiligheid congress Presentation at ESF-EMBO 2010; Spain (The NS3 protein of Rice hoja blanca tenuivirus suppresses RNA silencing by efficiently binding to siRNAs) Poster: ESF-EMBO 2010; Spain		Apr 02, 2007 Sep 03, 2007 Jan 16, 2008 Feb 28, 2008 Mar 07, 2008 Apr 08, 2008 Aug 11, 2008 Jan 14, 2009 Feb 26, 2009 and Apr 08, 2009 Apr 09, 2009 Apr 28, 2009 Jun 15, 2009 Aug 31, 2009 Apr 12, 2010 Apr 08, 2010 Mar 31, 2010 Apr 13, 2010 Jun 01, 2010 May 30-Jun 04, 2010
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3) In-Depth Studies		<u>date</u>
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	RNAi course	Apr 14-16, 2010
	Electron microscopy course	Jun 21-25, 2010
	▶ Journal club	
▶ Individual research training		
<i>Subtotal In-Depth Studies</i>		<i>4.4 credits*</i>
4) Personal development		<u>date</u>
▶ Skill training courses	Techniques for writing and presenting scientific papers	Oct 16-20, 2007
	Working with Endnote 9	Nov 13, 2007
	Project-and Time management	Jan 22-Mar 04, 2008
	Publish and perish (Ad Lagendijk, organized by KLV)	Sep 17, 2008
	Workshop: Scientific publishing	Nov 05, 2008
	Writing a Grant proposal	Oct 09-13, 2009
	College geven	Oct 26-Nov 09, 2009
	Workshop: Marie Curie individuel fellowships & grants	May 12, 2010
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	RNAi spring school	Apr 14-16, 2010
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<i>Subtotal Personal Development</i>		<i>7.6 credits*</i>
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* A credit represents a normative study load of 28 hours of study

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