Strategies underlying RNA silencing suppression by negative strand RNA viruses

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“We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for humanity”

Marie Curie (1867 - 1934), Lecture at Vassar College, May 14, 1921

Contents

Chapter 1

General introduction

This chapter will present an overview on the insights in the RNA silencing mechanism and its role in antiviral defence in plants and insects. In addition, it will be discussed how plant-pathogenic viruses counteract this host response by encoding specific proteins, the so-called suppressors of RNA silencing. As most of the experimental chapters (2, 3 and 4) will centre on the working mechanism of such suppressors as encoded by the tospoviruses and tenuiviruses, an introduction to the molecular biology of these viruses will be presented.

The discovery of RNA silencing

RNA silencing is an evolutionary conserved mechanism in many, if not all, eukaryotes to target and degrade aberrant endogenous or exogenous RNA molecules (Sontheimer, 2005; Tomari & Zamore, 2005; Voinnet, 2005). RNA silencing phenomena were first described in plants, where introduction of extra copies of the flower pigmentation gene chalcone synthase resulted in suppression of the transgene and the endogenous RNA. As a consequence, transgenic plants showed flowers with reduced pigmentation or even the complete absence of pigmentation (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). In another case, transgenic plants expressing untranslatable parts of plant viruses proved to be highly resistant to the homologous virus, but not to related viruses (de Haan *et al.*, 1992; Lindbo & Dougherty, 1992) Moreover, even non-transgenic plants that recovered from a viral infection showed resistance against an unrelated virus which carried a sequence insert from the first inoculated virus (Ratcliff *et al.*, 1999). These observations were described as ‘co-suppression’, ‘post-transcriptional gene silencing’ or ‘virus-induced gene silencing’. Transgenic plants expressing inverted repeats of viral transgenes were shown to greatly enhance the efficiency of resistance, implying a role for double stranded (ds)RNA in this antiviral activity (Smith *et al.*, 2000). Over time, similar observations had been described in *Neurospora crassa* where introduction of homologous RNA sequences caused sequence-specific RNA degradation or ‘quelling’ of the endogenous gene (Romano & Macino, 1992). In animals, introduction of sense or antisense RNA to endogenous mRNA also resulted in the degradation of the endogenous messenger in *Caenorhabditis elegans* (Guo & Kemphues, 1995). A breakthrough in the animal world came with the observation that injection of dsRNA in *C. elegans* resulted in degradation of endogenous mRNA and this was introduced as ‘RNA interference’ (RNAi) (Fire *et al.*, 1998). When dsRNA was injected into one region of *C. elegans* it caused systemic silencing, a phenomenon that is also observed in plants. This led to the hypothesis that RNA silencing was mediated by a stable silencing intermediate. This was further strengthened by experiments showing that gene silencing could be passed from parent to progeny in *C. elegans* (Grishok *et al.*, 2000). The presence of stable RNA silencing intermediates was first demonstrated in plants. It was generally thought that dsRNA had to be unwound in order for the antisense strand to bind the mRNA, although this full length antisense strand could never be detected. This led to the search for shorter forms of the antisense strand derived from longer dsRNA and the subsequent discovery of ~25 nucleotides (nt) small interfering (si)RNA molecules (Hamilton & Baulcombe, 1999), now considered as a hall mark of RNA silencing. A biochemical

approach using extracts of *Drosophila* cells demonstrated that dsRNA is converted by endonucleolytic cleavage into siRNA molecules (Hammond *et al.*, 2000; Zamore *et al.*, 2000). The identification of endogenous small RNA molecules, now known as micro (mi)RNAs, involved in development had also big impact on RNA silencing research. The miRNAs, *lin-4* and *let-7* miRNAs, were shown to be required for proper larval developments in *C. elegans* (Lee *et al.*, 1993; Moss *et al.*, 1997; Reinhart *et al.*, 2000; Wightman *et al.*, 1993). Cloning and computational approaches have identified hundreds of animal and plant miRNAs and potential target sites in mRNAs, which suggest that a large proportion of plant and animal transcripts are miRNA regulated (Brennecke *et al.*, 2005; Doench & Sharp, 2004; Lewis *et al.*, 2003; Llave *et al.*, 2002; Mette *et al.*, 2002; Reinhart *et al.*, 2002).

Functions of RNA silencing pathways

The biochemistry of RNA silencing has been best studied using *Drosophila* embryo extracts and can be regarded as a two-step reaction; initiation and effector phases (Fig. 1-1). The *initiation* step is characterised by the endonucleolytic cleavage of longer dsRNA molecules into siRNA or miRNA species. These small RNAs are 21-26 bp in size and contain 2 nt 3'-overhangs and 5'-phosphorylated termini, which are characteristic for their production by RNase-III-type enzymes from the Drosha/Dicer protein family (Bernstein *et al.*, 2001; Lee *et al.*, 2003). The siRNA and miRNA molecules differ in their origin and structure. While siRNAs originate from prefect complementary dsRNA duplexes, miRNAs contain mismatches, bulges or G:U wobble base pairs. miRNA molecules are host-encoded and derive from long non-coding single stranded (ss) RNAs (Bartel, 2004).

In the *effector* step of the RNA silencing pathway, one strand of the siRNA or miRNA duplex is loaded onto a ribonucleoprotein complex, the RNA induced silencing complex (RISC) for sequence specific identification of target RNAs (Khvorova *et al.*, 2003; Lee *et al.*, 2004b; Schwarz *et al.*, 2003; Tomari & Zamore, 2005). Enzymatic activity of members of the Argonaute (AGO) protein family enables the programmed RISC to slice complementary mRNAs or arrest their translation (Fagard *et al.*, 2000; Hammond *et al.*, 2000). Molecular and genetic analysis in plants and animals have revealed many RNA silencing pathways in which different small RNAs and RNA silencing proteins are key players. A schematic overview of these small RNA silencing pathways was recently reviewed (Vaucheret, 2006; Vazquez, 2006).

Antiviral RNA silencing in plants

As it acts as antiviral mechanism, the siRNA-mediated pathway is a most relevant RNA silencing pathway for plant virology. Antiviral silencing acts in the cytoplasm and is initiated predominantly by highly-structured viral ss RNAs, dsRNA replication intermediates of plant viruses, cytoplasmically replicating viruses or dsRNA produced by plant RNA dependent RNA polymerase (RDR) action (RDR1 or RDR6) (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). These dsRNA molecules are recognised by one of the four plant dicer enzymes; Dicer-like (DCL)4 and

subsequently processed into virus-specific siRNAs (Deleris *et al.*, 2006; Molnar *et al.*, 2005; Silhavy & Burgyan, 2004; Voinnet, 2001). In plants, RDR6 is also involved in a process called transitivity which can occur in both 5' and 3' direction of the cut initiated by the primary siRNA (Himber *et al.*, 2003; Vaistij *et al.*, 2002). This possibly reflects RDR6 primer-dependent 5' spreading and primer-independent 3' spreading, respectively. The newly synthesised dsRNAs are subsequently recognised by DCL4 to generate secondary siRNAs of exclusively 21 nt in size (Himber *et al.*, 2003), thereby amplifying silencing (Mourrain *et al.*, 2000). Interestingly, a second class of antiviral siRNAs (24-26 nt) can be generated specifically in plants, which seem to be involved in long-distance systemic silencing and are proposed to travel to different plant organs in advance of the invading virus (Mlotshwa *et al.*, 2002).

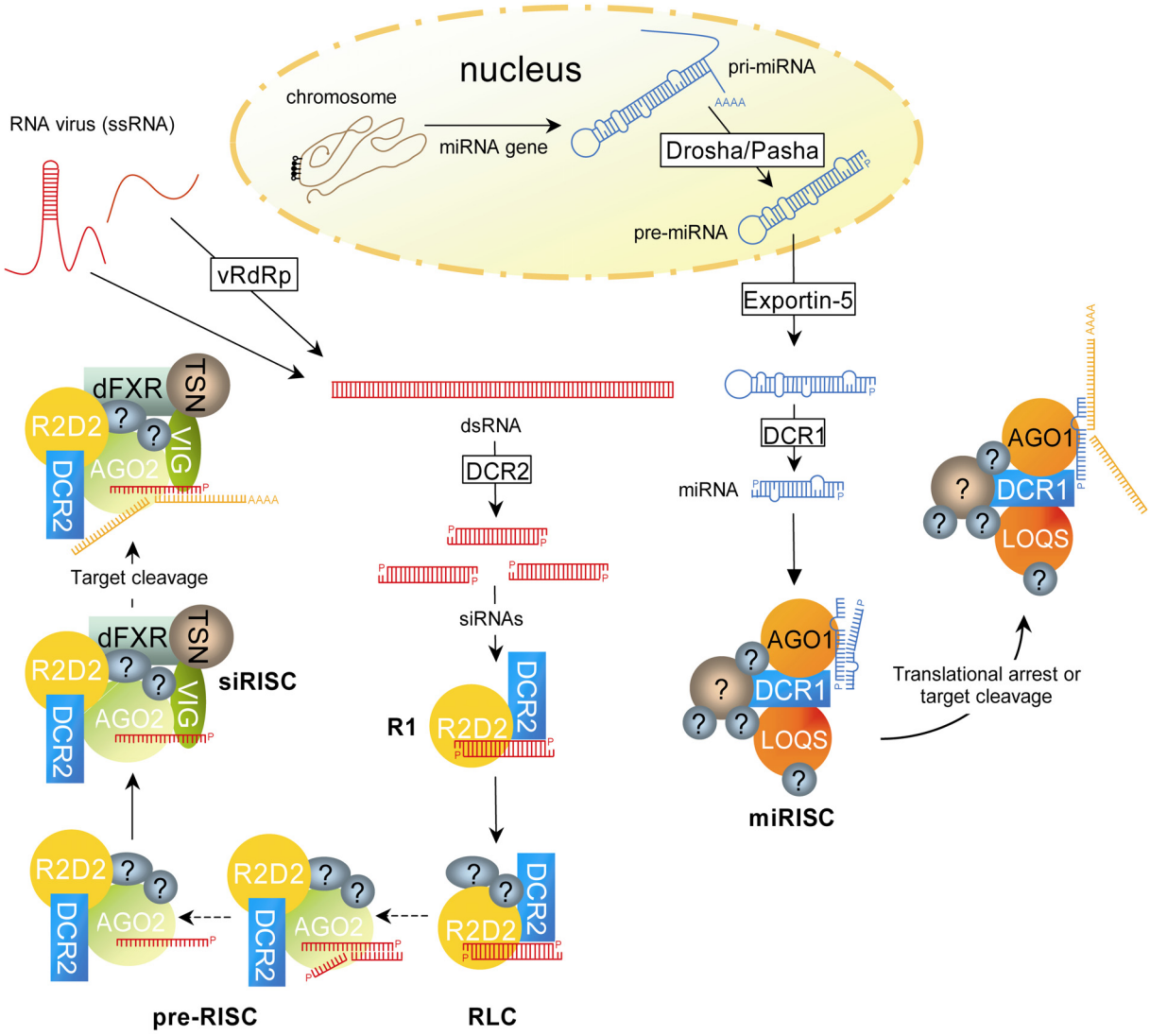

Figure 1-1: A simplified schematic overview of RNA silencing in *Drosophila melanogaster*. RNA silencing is initiated by the recognition of dsRNA by Dicer enzymes to produce ~21 nt small RNA molecules. One strand of the produced small RNA molecules is loaded into the RISC complex. The RISC complex matures and gives rise to an effector complex called holo-RISC (here denoted siRISC) for target cleavage of a homologous RNA target. The miRNAs find their origin in the nucleus as pri-miRNAs and mature miRNAs program RISC (miRISC) for translational arrest or target cleavage of a homologous mRNA molecule. Key proteins in RNA silencing are boxed or indicated in the various coloured shapes.

The miRNA pathway and plant development

In plants and animals, miRNAs are produced from endogenous partially folded transcripts. miRNAs act *in trans* by targeting cellular transcripts with small stretches of homology by either guiding their degradation or inhibiting their translation, the first being most common in plants, the latter in animals (Bartel, 2004). RNA polymerase II (Pol II) likely synthesises primary (pri)-miRNAs from *MIR* genes in plants (Lee *et al.*, 2004a; Xie *et al.*, 2005a). In animals, the pre-miRNA is recognised by the Drosha/Drosha protein heterodimer and cleaved into precursor (pre-)miRNAs. These are subsequently exported out of the nucleus into the cytoplasm by Exportin-5 and processed by Dicer into mature miRNA/miRNA* duplexes of 21 nt in size (Kim, 2005; Yi *et al.*, 2003). Because of the limited complementarity between the 5' end of the miRNA (the seed region) and its target, animal miRNAs guide translational repression of their targets (Bartel, 2004). In plants, the pri-miRNAs are recognised and cleaved by DCL1 that interacts with HYL1 to generate proper length miRNA/miRNA* duplexes (Park *et al.*, 2002). The miRNA/miRNA* duplexes are methylated by HEN1, which also methylates siRNAs. Methylation of these small RNA molecules most likely protects them from degradation and polyuridylation processes (Li *et al.*, 2005). The miRNA/miRNA* duplexes are subsequently exported out of the nucleus by HASTY, an ortholog of Exportin-5 (Park *et al.*, 2005) and the miRNA strand is incorporated into a RISC-like complex and the miRNA* strand degraded. In contrast to miRNA-mediated RNA silencing in animals, which mainly acts by translational inhibition, plant miRNAs most often guide AGO1-catalyzed mRNA cleavage (Baumberger & Baulcombe, 2005), likely due to the higher complementarity between miRNA and mRNA target sequence (Llave, 2004). Many of the hitherto studied plant miRNAs control the expression of transcription factors that regulate crucial steps during plant development (Jones-Rhoades & Bartel, 2004; Rhoades *et al.*, 2002), while animal miRNAs control a broad selection of phenomena by binding to 5' and 3' UTR sequences (Ambros, 2004). Interestingly, miR162 and miR168 guide specific cleavage of *DCL1* and *AGO1* mRNAs, respectively (Vaucheret *et al.*, 2006; Vaucheret *et al.*, 2004; Xie *et al.*, 2003). Since DCL1 is indispensable for miRNA production and AGO1 utilises these mature miRNAs for mRNA cleavage, this mechanism functions as a negative feedback mechanism and is probably essential for the maintenance of steady-state levels of miRNAs (Vaucheret, 2006).

More Gene regulation

In plants, two additional classes of endogenous small RNAs other than miRNAs are found to be active in gene regulation; transacting and natural siRNAs. The first class seems unique for plants and consists of *trans*-acting siRNAs (ta-siRNAs) that derive from *TAS* genes and are processed by DCL4 (Allen *et al.*, 2005; Gasciolli *et al.*, 2005; Xie *et al.*, 2005b). *TAS* primary transcripts are initially cleaved by a specific miRNA molecule and one of the two liberated strands is transformed into dsRNA by the action of RDR6 and SGS3 (Suppressor of gene silencing 3). These newly RDR6-synthesised *TAS* dsRNA molecules are processed by DCL4 in a phased fashion into 21 nt

ta-siRNAs which regulate expression by guiding cleavage of their target RNA (Allen *et al.*, 2005; Vazquez *et al.*, 2004). The second class of endogenous siRNAs derives from pairs of natural *cis*-antisense transcripts (nat-siRNAs). One RNA strand is constitutively expressed while the other strand is likely expressed under inducible conditions (Borsani *et al.*, 2005; Wang *et al.*, 2005). From the complementary region of the two single RNA strands 24 nt nat-siRNA are generated by DCL2 action. These 24 nt nat-siRNA direct the cleavage of the constitutive transcript and guide the sequential generation of 21 nt nat-siRNAs by DCL1. 4-20% of the genes in studied eukaryotes show a *cis*-antisense overlapping organisation and therefore nat-siRNAs could be a major mechanism for gene expression regulation (Borsani *et al.*, 2005).

Transcriptional gene silencing

siRNAs also play a key role in transcriptional gene silencing (TGS) that is active in the nucleus. TGS was discovered when tobacco plants were infected with Potato spindle tuber viroid. During this viroid infection, plant genome-integrated cDNA copies of the viroid became methylated *de novo*. This suggests that replication of viroids initiates methylation of the homologous DNA copies (Wassenegger *et al.*, 1994). This phenomenon was termed RNA-directed DNA methylation (RdDM). A role for RNA silencing in RdDM was indicated when methylation of promoters and subsequent TGS was observed when dsRNAs containing target promoter sequences were expressed in plants (Mette *et al.*, 2000). RdDM is limited to the region of homology between DNA and RNA and results in the methylation of cytosines (Wassenegger, 2000) by 'domain rearranged methyltransferases' (DRM1 and DRM2) and DNA methyltransferase MET1 (Cao & Jacobsen, 2002; Jones *et al.*, 2001). More recently, it has been observed that the siRNA-dependent *de novo* methylation of DNA in plants is maintained by histone modifications (Zilberman *et al.*, 2003). The RNA-independent maintenance of CG dinucleotide methylation requires the actions of MET1 and histone deactetylase HDA6 (Aufsatz *et al.*, 2004; Aufsatz *et al.*, 2002). The maintenance of the second type of nucleotide methylation, CNG, and histone H3 lysine 9 (H3K9) is depending on the action of DNA methyltransferase CMT3 and the H3K9 methyltransferase SUVH4 (Bartee *et al.*, 2001; Jackson *et al.*, 2002; Lindroth *et al.*, 2001; Malagnac *et al.*, 2002). Furthermore, the role of RNA silencing components (AGO4 and RDR2) in maintenance of CNG and H3K9 methylation was demonstrated. The accumulation of 24 nt repeat associated siRNAs (ra-siRNA) from transposons and centromeric repeats is dependent on DCL3 and RDR2 action (Chan *et al.*, 2005) and probably serves to protect the genome against damage caused by transposons (Xie *et al.*, 2004; Zilberman *et al.*, 2004).

Heterochromatin formation

Studies in *Schizosaccharomyces pombe* have shown that endogenous Pol II transcribed repeat elements that are highly present at centromeric regions, telomeres and mating-type loci (Cam *et al.*, 2005), are a source of dsRNA that is processed into siRNAs by DCR1. These siRNAs are incorporated into a RISC-like complex, the RNAi-induced transcriptional silencing (RITS)

complex. This complex contains proteins like chromodomain protein 1 (CHP1), TAS3 and AGO1. The siRNA-loaded RITS directs a methyltransferase (cryptic loci regulator 4; CLR4) to homologous DNA loci, which results in H3K9 methylation at these homologous regions (Hall *et al.*, 2002; Verdel *et al.*, 2004; Volpe *et al.*, 2002). H3K9 methylation also requires RDR and histone deacetylases actions. CLR4 serves as binding site for SWI6 (an ortholog of *Drosophila* heterochromatin protein 1; HP1), resulting in the recruitment of heterochromatin proteins and spreading of heterochromatin in upstream and downstream regions (further reviewed in Almeida & Allshire, 2005; Grewal & Elgin, 2007). The RDR action of RNA-directed polymerase 1 (RDP1), probably serves to amplify the RNAi-mediated heterochromatin assembly by binding the AGO1 cleaved nascent transcripts to generate dsRNA, which is then recognised by DCR1 for the generation of new siRNAs (Grewal & Elgin, 2007).

Plant DCL proteins; redundancy and hierarchy

The four DCL proteins being present in *Arabidopsis* generate different size classes of siRNAs from longer dsRNA molecules. DCL1 and DCL4 synthesise 21 nt miRNAs (Bartel, 2004) and viral siRNAs, respectively, whereas DCL2 and DCL3 serve for the processing of various long dsRNAs into 22 and 24 nt long RNAs respectively (Brodersen & Voinnet, 2006). From plant viral infection studies and individual mutations of the four DCL proteins, redundancy in DCL function has been observed (Deleris *et al.*, 2006). DCL2 can rescue antiviral silencing against positive (+) ss RNA viruses in the presence of dysfunctional DCL4 by producing 22 nt long viral specific siRNAs. DCL3, involved in chromatin modifications, is also able to generate 24 nt viral specific siRNA molecules in *Tobacco rattle virus* and *Cucumber mosaic virus* infections, although these are not functional in targeting homologous viral sequences (Ding & Voinnet, 2007).

In contrast to their disability to target (+) ss RNA viruses, DCL3 and DCL4 are involved in targeting plant DNA viruses. Additionally, DCL1 function stimulated the accumulation viral siRNA, whereas there are no indications for the contribution to antiviral RNA silencing for DCL1 in (+) ssRNA viral infections (Ding & Voinnet, 2007). Again the activity of DCL2 in antiviral RNA silencing was eminent when DCL4 was dysfunctional in DNA virus infections (Blevins *et al.*, 2006; Moissiard & Voinnet, 2006). The nature and origin of the DCL-generated small RNAs specify the involvement of these molecules in the various RNA silencing pathways. To date it is not clear what determines the affinity for various dsRNA molecules by DCL proteins. However, it can be envisaged that the subcellular localisation of dsRNA and DCL proteins and/or the involvement of specific dsRNA binding proteins guide the affinity for dsRNAs by DCL proteins (Ding & Voinnet, 2007; Vaucheret, 2006).

RNA silencing components and their biochemistry

Soon after the discovery of RNA silencing, *in vitro* systems were developed from cultured cells. Those originating from *Drosophila* cells and *Drosophila* embryos proved most successful to demonstrate the biochemistry of RNA silencing (Hammond *et al.*, 2000; Zamore *et al.*, 2000).

Most of the work in this field has focused on the characterisation of the components and activities of the 80S holo-RISC and intermediate complexes (Martinez *et al.*, 2002; Pham *et al.*, 2004; Sontheimer, 2005; Tomari & Zamore, 2005). The development of *Drosophila* cell or embryo extracts contributed significantly to the dissection of the RNA silencing machinery and identification of the different RNA silencing components. This will be described in more detail below.

Dicers and their partners

The first identified Dicer protein was isolated from *Drosophila* and proved to be a member of an RNase-III-like enzyme family (Bernstein *et al.*, 2001; Hammond *et al.*, 2000). Dicer proteins contain multiple domains (Fig. 1-2) and are characterised by the presence of an amino-terminal DexH RNA helicase/ATPase domain, followed by a domain of unknown function (DUF283), a PIWI/ARGONAUTE/ZWILLE (PAZ) domain, two neighbouring RNase III-like domains and a dsRNA-binding domain (dsRBD).

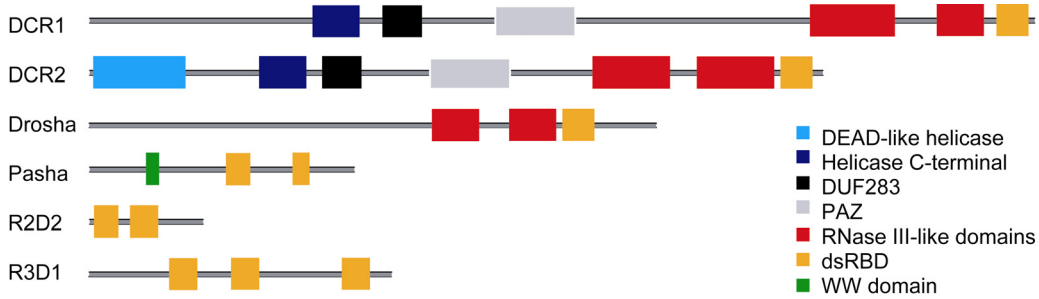

Figure 1-2: Schematic overview of RNase-III-like enzymes and their partners involved in *Drosophila* RNA silencing (Forstemann *et al.*, 2005).

Dicer proteins have been found in almost all eukaryotes studied and while most animals contain a single Dicer enzyme, the model plant *Arabidopsis thaliana* was shown to encode four Dicer-like enzymes (DCL1-4; see above) (Schauer *et al.*, 2002) and *Drosophila* two (DCR1 and DCR2). Both DCR1 and DCR2 contain tandem RNase-III-like domains that form an intramolecular dimer to create a single catalytic centre responsible for cleavage of dsRNA into ~21 nt siRNAs. Processing of dsRNA generates the characteristic 2 nt 3' overhang and 5' phosphate groups (Zhang *et al.*, 2004) and genetic studies in Drosophila have suggested that DCR1 and DCR2 act in miRNA and siRNA production, respectively (Lee *et al.*, 2004b).

Maturation of insect miRNAs occurs in a step-wise manner. In the nucleus long non-coding transcripts, primary (pri-)miRNAs are processed by the Drosha RNase-III endonuclease into precursor (pre)-miRNA of ~60-80 nt in length (Han *et al.*, 2004; Lee *et al.*, 2002; Zeng & Cullen, 2003). Drosha acts in concert with its partner Pasha that contains two dsRDBs and a putative WW domain (Denli *et al.*, 2004; Landthaler *et al.*, 2004). The latter is known to interact specifically with proline-rich sequences, but interaction with the proline-rich region of Drosha needs to be determined. The pre-miRNAs are actively exported to the cytoplasm by Exportin-5 (Lund & Dahlberg, 2006) and are processed into mature miRNAs by DCR1 (Kim, 2005; Lee *et al.*, 2002).

DCR1 is an ATP-independent enzyme and processes the stem-loop of pre-miRNA molecules while DCR2 preferentially cleaves dsRNA in an ATP-dependent manner (Jiang *et al.*, 2005; Liu *et al.*, 2003). In Drosophila, DCR1 forms a complex with its partner Loquacious (LOQS or R3D1) (Forstemann *et al.*, 2005; Jiang *et al.*, 2005; Saito *et al.*, 2005) and DCR2 forms a heterodimer with R2D2 (Liu *et al.*, 2003). Both R2D2 and R3D1 are putative dsRNA binding proteins and R3D1 greatly enhances the miRNA generation by DCR1, probably by increasing its affinity for pre-miRNA (Jiang *et al.*, 2005; Saito *et al.*, 2005). R2D2 preferentially binds to the more stable end of siRNA duplexes and interacts with the 5' phosphate on the siRNA strand that is going to be excluded from RISC (passenger strand). DCR2 is associated with the 5' end of the duplex with lower melting temperature and positioning of the R2D2-DCR2 complex could therefore serve as a sensor for thermodynamic stability of siRNA duplexes (Tomari *et al.*, 2004b).

RISC and intermediate complexes

Biochemical studies have mainly focused on siRISC formation. Active siRISC or holo-RISC is assembled in an organised sequential manner (Fig. 1-1) which can be visualised by native gel electrophoresis (Pham *et al.*, 2004; Tomari *et al.*, 2004a). RISC assembly starts with the association of the R2D2/DCR2 protein heterodimer to the double stranded siRNA forming the R1 complex (Pham *et al.*, 2004). The subsequent precursor complex; RISC loading complex (RLC) consists of R1 and several unknown protein factors (Sontheimer, 2005). Recent data suggests that a next intermediate, pre-RISC is formed by recruitment of AGO2 by RLC (Kim *et al.*, 2007).

The mature and active siRISC complex contains one strand of the siRNA duplex, the guide strand, which needs to be separated from the passenger strand during RISC assembly. The separation of the passenger strand of the siRNA duplex is initiated by the 'slicer' function of AGO2 (Matranga *et al.*, 2005; Miyoshi *et al.*, 2005; Rand *et al.*, 2005) which cleaves the passenger strand prior to its dissociation and gives rise to the activated siRISC complex. The activated siRISC complex has been shown to contain additional protein like the vasa intronic gene (VIG) protein, dFXR (an ortholog of human fragile-X mental-retardation protein) and tudor-staphylococcal nuclease (TSN; Pham *et al.*, 2004). The exact roles of these proteins in the siRISC are currently not known. The activated siRISC subsequently uses the guide strand to single out homologous mRNAs (Elbashir *et al.*, 2001) and this endonuclease cleavage is depending on divalent metal ions and the cleaved target is characterised by 3' OH and 5' phosphate termini (Martinez & Tuschl, 2004; Schwarz *et al.*, 2004). After cleavage the guide strand stays associated with siRISC, allowing multiple cleavage cycles (Hutvagner & Zamore, 2002; Martinez *et al.*, 2002).

As mentioned before some organisms encode multiple Dicer enzymes (DCL1, DCL2, DCL3 and DCL4 in *Arabidopsis thaliana* and DCR1 and DCR2 in *Drosophila*) which act differently in siRNA and miRNA mediate pathways of RNA silencing (Lee *et al.*, 2004b; Okamura *et al.*, 2004; Xie *et al.*, 2004). This implies that multiple distinct siRISC and miRISC complexes and precursor complexes could be formed. Currently, it is not known how the single Dicer enzyme in *C. elegans*

and humans controls multiple assembly pathways. The siRNAs guide the assembly of siRISC through the intermediate R1 and RLC complexes, but miRNA molecules can serve as building blocks for the assembly of miRISC. Unlike the siRNA pathway, little is as yet known about the assembly of miRISC.

Argonaute proteins

As discussed in the previous paragraphs, Argonaute proteins play key roles in diverse RNA silencing pathways. Like Dicer, Argonaute proteins contain a PAZ domain and a unique PIWI domain. The PAZ domain consists of approximately 130 amino acids and is composed of two subdomains separated by a cleft. The PAZ domain preferentially binds RNA in a sequence-independent manner and recognises the 3' of ss siRNAs. Since PAZ shows a low affinity for RNA, this suggests that other proteins of the RISC complex or other Argonaute domains contribute to RNA binding (Lingel *et al.*, 2004; Song *et al.*, 2003). Besides the PAZ and PIWI domains an N-terminal and middle domain are present in Argonaute proteins.

Structural analysis of the full length Argonaute from the hyperthermophilic Archaea *Pyrococcus furiosus* showed that the PIWI domain at the C-terminus of Argonaute resembles a RNase H fold (Song *et al.*, 2004). In the catalytic domain of the Argonaute protein is a 'DDH' motif, which shows high similarity to the 'DDE' motif of the catalytic core of RNase H. Mutations in this conserved motif of human AGO2 were shown to inhibit hAGO2 slicer activity (Liu *et al.*, 2004; Rivas *et al.*, 2005). Structural predictions have shown that a large positively charged groove is formed between the PAZ domain and the folded base while a smaller groove between the N-terminal and PIWI domains is present. During slicing the 3' end of the ss siRNA is bound by the PAZ domain and the siRNA-target interacts with the positively charged groove. The 5' region of the target is positioned between the PAZ and N-terminal domain in such a way that the catalytic core of the PIWI domain is able to cleave the phosphodiester bond between position 10 and 11 measured from the 5' end of the siRNA (Rivas *et al.*, 2005), which is recognised by the Argonaute protein (Song *et al.*, 2004).

Of the five *Drosophila* Argonaute proteins (AGO1 to 3, PIWI and Aubergine) AGO1, 2 and PIWI showed slicer activity (Miyoshi *et al.*, 2005; Rand *et al.*, 2004; Saito *et al.*, 2006). Of the eight human Argonaute family members (AGO1 to 4 and PIWI1 to 4), AGO1 to 4 have been tested for slicer activity and only AGO2 showed slicing activity although all show an intact 'DDH' or DDE' motif (Liu *et al.*, 2004; Meister *et al.*, 2004; Qi *et al.*, 2005). For hAGO1 and hAGO4, the absence of slicer activity can be explained by a single amino acid substitution in the 'DDH' motif. Interestingly, hAGO3 shows a 'DDH' motif but is not an active slicer, demonstrating that the presence of this motif is required but not sufficient (Liu *et al.*, 2004; Meister *et al.*, 2004). Of the 24 *C. elegans* Argonaute proteins only ten have an intact 'DDH' motif, which suggests that most are non-slicers. *Arabidopsis* has ten Argonaute family members which all have the required 'DDH' or 'DDE' motif indicating that all ten are potential slicers. So far only AGO1 and AGO4 were tested and showed slicer activity. As mentioned before, AGO1 and AGO4 were shown to be

indispensable for antiviral silencing and TGS, respectively (Baumberger & Baulcombe, 2005; Qi *et al.*, 2006).

Viral suppressors of RNA silencing

In plants, RNA silencing has been demonstrated to be one of the most important antiviral activities. As a response to this highly efficient antiviral RNA silencing pathway, plant viruses have evolved specific suppressor proteins. Many of these proteins were previously described as virulence factors or pathogenicity determinants (Voinnet *et al.*, 1999). Most plant viruses have a (+) ss RNA genome and during viral replication their replication intermediates are proposed to be recognised as dsRNA molecules by the RNA silencing machinery (Ahlquist, 2002). Alternatively, secondary structures in regions of the ss RNA molecules can also be recognised (Szittya *et al.*, 2002). It has been reported that many plant viruses encode suppressor proteins to combat RNA silencing (Li & Ding, 2001; Voinnet *et al.*, 1999). Up to now, no obvious sequence homology was found between the different silencing suppressors and they might act by inhibiting the generation of siRNAs, preventing the incorporation of siRNAs into RISC or by interfering with RISC function. Two of the most studied suppressor proteins, HC-pro and p19 will be discussed in more detail.

HC-Pro of potyviruses

One of the first identified and well studied plant viral suppressors of RNA silencing is the helper component-protease (HC-Pro) of potyviruses. The HC-Pro protein has been reported to be a multifunctional protein involved in different steps of the viral life cycle (Maia *et al.*, 1996). Transgenic plants expressing the P1/HC-Pro sequence developed synergistic disease symptoms when infected with any of a broad range of plant viruses (Pruss *et al.*, 1997). This synergism was lost in transgenic lines with a mutated HC-Pro transgene. First indications that HC-Pro is a suppressor of silencing came with the observation that crossing of a GUS-silenced tobacco plant with a HC-pro expressing tobacco plant restored the expression of the reporter and prevented the degradation of the reporter mRNA (Anandalakshmi *et al.*, 1998). In a viral context, HC-Pro suppressed a silenced reporter transgene in tobacco when infected with *Potato virus X* carrying HC-Pro. Hints on the mode of suppression of RNA silencing resulted from yeast two-hybrid studies. HC-Pro interacted with a plant protein called "*r*egulation of *g*ene *s*ilencing-calmodulin-like" (rgs-Cam). Overexpression of rgs-Cam in plants resulted in phenotypes similar to plants expression HC-Pro, probably by affecting miRNA accumulation (Mallory *et al.*, 2002). Rgs-Cam was found to act as endogenous silencing suppressor and HC-Pro expression was shown to stimulate its expression (Anandalakshmi *et al.*, 2000). Studies on the domains responsible for RNA-binding correlated with the domain responsible for its suppressor action, suggesting that RNA binding could be involved in its suppressor activity (Kasschau & Carrington, 2001; Urcuqui-Inchima *et al.*, 2000; Yang & Ravelonandro, 2002).

P19 of tombusviruses

As was shown for the tombusviral p19 protein, RNA binding activity can indeed be an efficient way to suppress RNA silencing. p19 efficiently binds dsRNA molecules of 21 nt containing 3' 2 nt overhangs; a typical siRNA molecule (Silhavy *et al.*, 2002). The p19 protein is indispensable for an efficient systemic infection. Viral siRNAs were found to accumulate to high levels in wildtype virus infections (Qiu *et al.*, 2002; Silhavy *et al.*, 2002). These results indicate that p19 probably inhibits RNA silencing downstream of Dicer. Transgenic plants expressing p19 and dsRNA specific for a nopallin synthase promoter sequence, showed a change in endogenous miRNA and nopallin synthase specific siRNA patterns. Interestingly, both siRNAs and miRNAs were truncated, suggesting that the p19 binds both classes of molecules and subsequently these are clipped by a nuclease (Papp *et al.*, 2003). Like transgenic plants expressing HC-Pro, also p19 transgenic plants showed defects in leaf and rosette development and reproductive organs (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004).

RNA silencing suppressor proteins of negative strand RNA viruses

At the onset of the research described in this thesis the suppressor proteins of 2 negative (-) strand ss RNA viruses of plants were identified (Bucher *et al.*, 2003; Takeda *et al.*, 2002). By *Agrobacterium tumefaciens* transient assays (ATTA; Johansen & Carrington, 2001) on silenced GFP transgenic plants, the NSs and NS3 proteins were identified as the silencing suppressors of *Tomato spotted wilt virus* (TSWV) and *Rice hoja blanca tenuivirus* (RHBV), respectively (Bucher *et al.*, 2003).

TSWV is the type species of the genus *Tospovirus* in the family *Bunyaviridae*. Like the nucleoprotein (N), the ~52.5 kDa suppressor protein NSs of TSWV is encoded by the ambisense S RNA segment (de Haan *et al.*, 1990). Previously, the accumulation of NSs was shown to coincide with an increase in symptom severity (Kormelink *et al.*, 1991). The tripartite genome further consists of the fully negative stranded L RNA, which encodes the viral RNA dependent RNA polymerase (de Haan *et al*., 1991) and the ambisense M RNA which encodes the envelope glycoproteins Gn and Gc and the viral movement protein NSm (Kormelink *et al.*, 1992; Storms *et al.*, 1995; Fig. 1-3). TSWV is propagatively transmitted by a limited number of thrips species, belonging to the genera *Thrips* and *Frankliniella*. Currently, the Western flower thrips (*Frankliniella occidentalis*) is the most important vector (Falk & Tsai, 1998; Wijkamp *et al.*, 1993). Interestingly, the NSs protein of *La Crosse virus,* an animal-infecting member of the *Bunyaviridae* showed suppression activity against siRNA-induced RNA silencing in human cells (Soldan *et al.*, 2005). This might indicate that in this case, even though a role for antiviral silencing in vertebrate cells was not established at the onset of this thesis, mammalian viruses may encode suppressor proteins. Also for other vertebrate viruses evidence for suppressors of gene silencing was obtained using either heterologous (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004; Li *et al.*, 2004) or homologous test systems (Haasnoot *et al.*, 2007). Previously these proteins have been implicated

in antagonising the interferon pathway through their ability to bind dsRNA (Basler *et al.*, 2000; Wang *et al.*, 2000; Xiang *et al.*, 2002). In general, until now, their property to bind dsRNA makes it difficult to discriminate between an interferon antagonistic function and a possible role in suppressing RNA silencing.

The genomic organisation of RHBV (belonging to the floating genus *Tenuivirus*) much resembles that of TSWV (Fig.1-3). The largest RNA segment (RNA 1) is of complete negative polarity and encodes the putative viral polymerase (L). The other three RNA segments have an ambisense coding strategy encoding two proteins per RNA segment. Only the nucleoprotein encoded by RNA 3 has been functionally characterised (de Miranda *et al.*, 1994; De Miranda *et al.*, 1996; Ramirez *et al.*, 1993; Ramirez *et al.*, 1992). The NS3 protein is encoded on the RNA 3 segment in an ambisense arrangement opposite the N-gene, on an analogues position as the NSs gene of TSWV. Like NSs also the NS3 protein has been shown silencing suppressor activity in the ATTA/GFP silenced plant assay (Bucher *et al.*, 2003). Interestingly, GFP specific siRNAs accumulated in leaves where an *A. tumefaciens* strain, expressing the GFP gene, was co-infiltrated with an *A. tumefaciens* strain carrying the NS3 gene. In leaves co-infiltrated with an *A. tumefaciens* strain, carrying the NSs gene, no GFP and no specific GFP siRNAs could be detected (Bucher *et al.*, 2003). These results hinted towards different suppressor activities of the NSs and NS3 proteins. However, the exact suppressor mode of NSs and NS3 remained unclear. Like TSWV, also RHBV is propagatively transmitted by an insect vector, the leafhopper (*Tagosodes orizicolus*; Falk & Tsai, 1998; Wijkamp *et al.*, 1993).

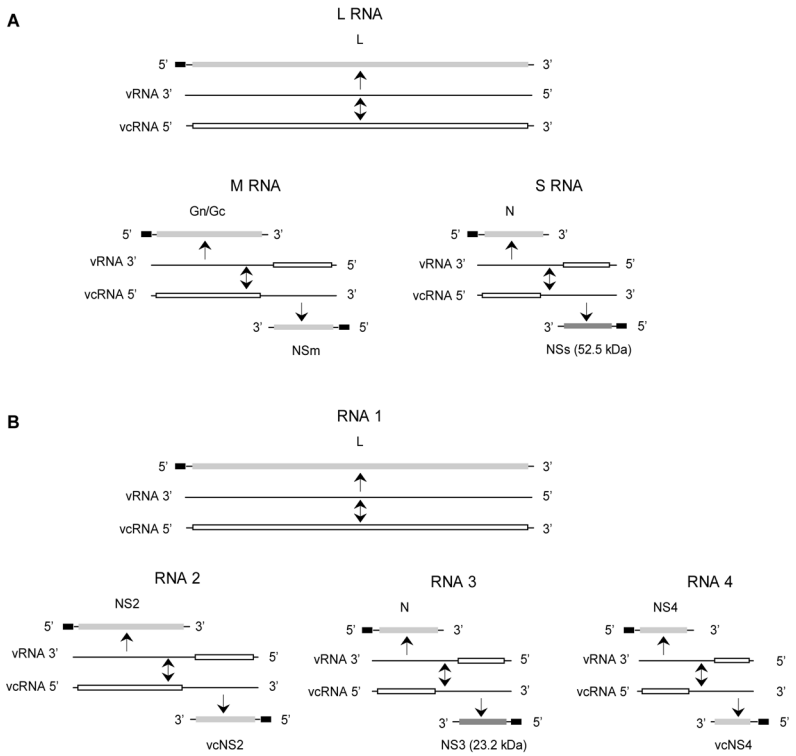

Figure 1-3: Genomic organisation and expression strategies of TSWV (**A**) and RHBV (**B**). vRNA is viral sense RNA, vcRNA is viral complementary RNA. Open reading frames (ORFs) are indicated by light grey boxes, the NSs and NS3 ORFs on the S RNA of TSWV (**A**) and RNA 3 of RHBV (**B**), respectively, are indicated by dark grey boxes.

Outline of the thesis

At the onset of this thesis, a limited number of plant viral silencing suppressor proteins were identified, mainly of positive strand ss RNA viruses and of DNA viruses (Li & Ding, 2001). Positive strand RNA viruses of plants are often transmitted by insects, but they replicate exclusively in their plant hosts. Negative strand RNA viruses of plants are different in this aspect, though, as they replicate also in their insect vector (Falk & Tsai, 1998; Jackson *et al.*, 2005; Wijkamp *et al.*, 1993). For two of such viruses, i.e. *Tomato spotted wilt virus* (TSWV, genus *Tospovirus*) and *Rice hoja blanca tenuivirus* (RHBV, genus *Tenuivirus*) it has been shown that these encode an RNA silencing suppressor protein, NSs and NS3, respectively (Bucher *et al.*, 2003). As antiviral RNA silencing is active in plants (reviewed in Voinnet, 2001) as well as in insects (Galiana-Arnoux *et al.*, 2006; Li *et al.*, 2002; Li *et al.*, 2004; van Rij *et al.*, 2006; Wang *et al.*, 2006) it may be anticipated that the tospoviral and tenuiviral silencing suppressor proteins would be active in both their plant hosts and in their insect vector. Understanding how this would work was the central theme of this research.

In their need to suppress antiviral RNA silencing pathways in both plants and insects, the viral silencing suppressor proteins of tospo- and tenuiviruses will likely block a conserved step in RNA silencing. In eukaryotic RNA silencing pathways, dsRNA molecules are conserved and play a key role, while proteins involved in RNA silencing differ in number and action between organisms. Therefore, the dsRNA molecules are the most likely candidates to be targeted by these suppressors. As during the course of this work the TSWV NSs protein appeared to be difficult to obtain in sufficient quantity most studies were done using RHBV NS3.

Chapter 2 describes the biochemical approaches that were followed using *Drosophila* embryo extracts known to contain all RNA silencing compounds in active form (Tuschl *et al.*, 1999). By using this system the influence of NS3 on the formation of RNA silencing complexes was investigated. The affinity for and structural requirements of dsRNA molecules was investigated for the NS3 protein.

In **chapter 3** a first insight in NS3 protein organisation and in the RNA binding domain within the NS3 protein was investigated by performing an alanine replacement scan of two conserved regions. The RNA binding affinity of the generated mutated NS3 proteins was determined and their suppressor functionality was tested.

The mode of action of the NS3 analogue of TSWV, denoted NSs, was examined in **chapter 4**. For TSWV NSs the affinity for different dsRNA molecules was determined. The RNA binding activity of the proteins of various tospoviruses was tested using virus-infected plant material and the influence on the miRNA pathway verified. Tospoviruses belong to the *Bunyaviridae,* a family which furthermore contains animal-infecting species. Hence, tospoviruses have direct genetic relationship to animal-infecting viruses, not only within the family but also with other families such as the *Orthomyxoviridae*.

For one of these human infecting viruses, *Influenza virus A*, the interferon antagonistic protein NS1 showed cross-species RNA silencing suppressor activity in plant- and insect cell-based assay systems (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004; Li *et al.*, 2004). In **chapter 5** the suppressor activity of NS1 was further investigated using several biochemical approaches. As a clear role in antiviral defence has not been demonstrated for the RNA silencing pathway in mammalian cells, the role of the NS1 protein in RNA silencing was investigated in infectivity assays and linked to the obtained biochemical results.

Finally, in **chapter 6** the results of the experimental chapters are discussed and evaluated in relation to literature data and current insights in the RNAi mechanisms. The RNA binding activity of plant viral suppressors and of innate immunity antagonists of human-infecting viruses are assessed and their mode of action placed in a general perspective. Based on the data described in the experimental chapters 2 to 5 an RNA silencing model is presented with the proposed suppressor action of the NS3, NSs and NS1 proteins.

Chapter 2

The NS3 protein of *Rice hoja blanca tenuivirus* suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs

A modified version of this chapter has been published: **Hemmes H., Lakatos L., Goldbach R., Burgyan J. and Prins M. (2007)**. The NS3 protein of *Rice hoja blanca tenuivirus* suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs. *RNA* **13**, 1079-1089.

RNA silencing plays a key role in antiviral defence as well as in developmental processes in plants and insects. Negative strand RNA viruses such as the plant virus *Rice hoja blanca tenuivirus* replicate in plants and in their insect transmission vector. Like most plant-infecting viruses RHBV encodes an RNA silencing suppressor, the NS3 protein, and here it is demonstrated that this protein is capable of suppressing RNA silencing in both plants and insect cells. Biochemical analyses showed that NS3 efficiently binds siRNA as well as miRNA molecules. Binding of NS3 is greatly influenced by the size of small RNA molecules, as 21 nt siRNA molecules are bound >100 times more efficiently than 26 nt species. Competition assays suggest that the activity of NS3 is based on binding to siRNAs prior to strand separation during the assembly of the RNA induced silencing complex. In addition, NS3 has a high affinity for miRNA/miRNA* duplexes, indicating that its activity might also interfere with miRNA regulated gene expression in both insects and plants.

Introduction

RNA silencing is an evolutionary conserved mechanism in many, if not all, eukaryotes to target and degrade aberrant endogenous or exogenous RNA molecules (Sontheimer, 2005; Tomari & Zamore, 2005; Voinnet, 2005). More recently, related processes were shown to be involved in eukaryotic gene regulation processes through host encoded micro (mi)RNAs (for reviews see Bartel, 2004; Carrington & Ambros, 2003; Herr, 2005).

A common feature of all RNA silencing processes is the endonucleolytic cleavage of longer double stranded (ds)RNA molecules into small interfering (si)RNA or miRNA species. These small RNAs are 21-26 bp in size (Hamilton & Baulcombe, 1999) and contain 2 nt 3'-overhangs and 5'-phosphorylated termini, which are characteristic for their production by RNase-III-type enzymes from the Drosha/Dicer protein family (Bernstein *et al*., 2001; Lee *et al*., 2003). The siRNA guide strand or miRNA strand of the small RNA duplex programmes a ribonucleoprotein complex, RISC, for sequence specific recognition of RNA targets (Khvorova *et al.*, 2003; Lee *et al*., 2004b; Schwarz *et al*., 2003; Tomari & Zamore, 2005). Enzymatic activity of members of the Argonaute (AGO) protein family enables RISC to slice complementary mRNAs or arrest their translation (Fagard *et al*., 2000; Hammond *et al*., 2000).

The core machinery of RNA silencing plays diverse and essential roles in regulation of gene expression by miRNAs, genome defence against transposons and viruses and modification of chromatin structure (Mallory & Vaucheret, 2006). miRNAs originate from long non-coding single stranded RNAs and negatively regulate complementary mRNAs by either guiding RNA slicing activity by RISC or translational repression (Bartel, 2004). In plants, RNA silencing is an important antiviral defence initiated by structured viral RNAs, dsRNA replication intermediates of plant viruses, cytoplasmically replicating viruses or dsRNA production by plant RNA dependent RNA polymerase (RDR) action (RDR1 or RDR6). Subsequent processing of viral specific dsRNAs results in the accumulation of viral siRNAs (Molnar *et al.*, 2005; Silhavy & Burgyan, 2004).

Interestingly, RNA silencing in plants can generate a second class of larger siRNAs (24-26 nt) that seem to be involved in systemic signalling and are proposed to travel to different plant organs in advance of the invading virus (Hamilton *et al*., 2002; Tang *et al*., 2003).

In insects, the miRNA and siRNA mediated RNA silencing processes are initiated by DCR1 and DCR2 respectively (Lee *et al.*, 2004b). DCR1 function requires AGO1, whereas siRNA synthesis and active RISC complex formation is AGO2 dependent (Lee *et al.*, 2004b; Okamura *et al*., 2004). Cultured *Drosophila* cells or animals depleted of or lacking DCR2, R2D2 or AGO2 showed higher accumulation of the insect-infecting *Flock House Virus* (FHV), *Drosophila C virus* (DCV) and *Cricket paralysis virus* (CrPV) suggesting also an antiviral role of RNA silencing in insects (Galiana-Arnoux *et al*., 2006; Li *et al.*, 2002; Li *et al*., 2004; van Rij *et al.*, 2006; Wang *et al*., 2006). RISC complex formation is initiated by the R2D2/DCR2 complex which is a sensor for siRNA strand loading (Pham *et al*., 2004; Tomari *et al*., 2004b). This complex associates with an AGO2-containing protein complex and the passenger strand of the siRNA complex is released after cleavage by the AGO2 slicer function (Matranga *et al*., 2005; Miyoshi *et al*., 2005; Rand *et al*., 2005) to give rise to the activated RISC complex which catalyses sequence specific mRNA degradation (Tuschl *et al*., 1999).

To counteract antiviral RNA silencing, plant viruses were shown to encode specific proteins that were previously mainly associated with the enhancement of viral pathogenicity and accumulation. Over the years many RNA silencing suppressor proteins of plant viruses have been identified (Silhavy & Burgyan, 2004; Voinnet, 2005). Suppression of antiviral silencing can be accomplished by binding to ds siRNAs (Lakatos *et al*., 2006; Merai *et al*., 2006), masking long dsRNA molecules (Merai *et al*., 2005; van Rij *et al*., 2006) or inhibition of active RISC by physical interaction between the suppressor and the slicer component (Zhang *et al*., 2006). Besides suppression of siRNA-mediated antiviral silencing, several plant viral suppressors also induce developmental abnormalities in plants by interfering with the miRNA pathway (Chapman *et al*., 2004; Dunoyer *et al*., 2004).

The identification of RNA silencing suppressors has not remained limited to plant viruses as the B2 protein of the insect-infecting FHV has been identified as viral suppressor in insect cells. Cross-kingdom suppression of RNA silencing was observed for the FHV B2 protein in plants (Li *et al*., 2002). Human-infecting viruses encode proteins that can act as suppressors of RNA silencing as well. The NS1 protein of the *Influenza virus A* is active in insect cells as well as in plants (Bucher *et al*., 2004; Delgadillo *et al*., 2004; Li *et al*., 2004) and the NSs protein of *La Crosse virus* (LACV) shows RNA silencing inhibition in human cells (Soldan *et al*., 2005).

The *Drosophila* embryo extract *in vitro* RNA silencing system was employed for the molecular and biochemical characterisation of the suppressor p19 of tombusvirus (Lakatos *et al*., 2004). Although members belonging to tombusviruses are often transmitted by insects, like many members belonging to positive (+) single strand (ss) RNA viruses, they replicate exclusively in their plant hosts and thus are unlikely to be involved in combating antiviral RNA silencing in

insects. In previous studies we and others reported that two members of negative (-) strand RNA plant viruses also carry a suppressor of RNA silencing (Bucher *et al.*, 2003; Takeda *et al.*, 2002). In contrast to (+) strand RNA plant viruses, the (-) strand RNA plant viruses replicate in both insect vectors and plant hosts (Falk & Tsai, 1998; Wijkamp *et al.*, 1993) and are therefore likely to be a target for antiviral silencing in plant host and insect vector. *Rice hoja blanca tenuivirus* (RHBV) is such a (-) strand RNA virus (Ramirez *et al.*, 1993; Ramirez *et al.*, 1992) which is transmitted by and replicated in a leafhopper, *Tagosodes orizicolus*.

Since RHBV replicates both in insects and plants, we aspired to investigate whether the RHBV NS3 suppressor protein also suppresses RNA silencing in insects. Furthermore, we examined the molecular mechanism of RNA silencing inhibition using the established insect *in vitro* embryo extract system of *Drosophila*. In addition, the ability of the NS3 protein to act on gene regulation through the miRNA pathway was examined by a biochemical approach.

Results

NS3 is a functional RNA silencing suppressor in plants as well as in insects

RHBV infects rice and is transmitted by leafhoppers in which it also replicates (Falk & Tsai, 1998). Therefore, it is likely to be targeted by antiviral RNA silencing in both plants and insects and the NS3 protein of RHBV was shown to be a suppressor of RNA silencing in plants (Bucher *et al*., 2003). In *Drosophila*, DCR2, R2D2 and AGO2 were shown to be involved in the antiviral response (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006). To investigate the RNA silencing suppression activity of NS3 in insects, we expressed a reporter (eGFP) in cultured *Drosophila* S2 cells. Effective RNA silencing of eGFP was achieved by adding long GFP specific dsRNA to the cell culture. After induction, the NS3 protein was detected by Western blotting (Fig. 2-1D) and GFP levels were notably higher compared to non-induced cells (compare Fig. 2-1B and C). These results show that NS3, in addition to being active in plants, is also able to suppress RNA silencing in insect cells.

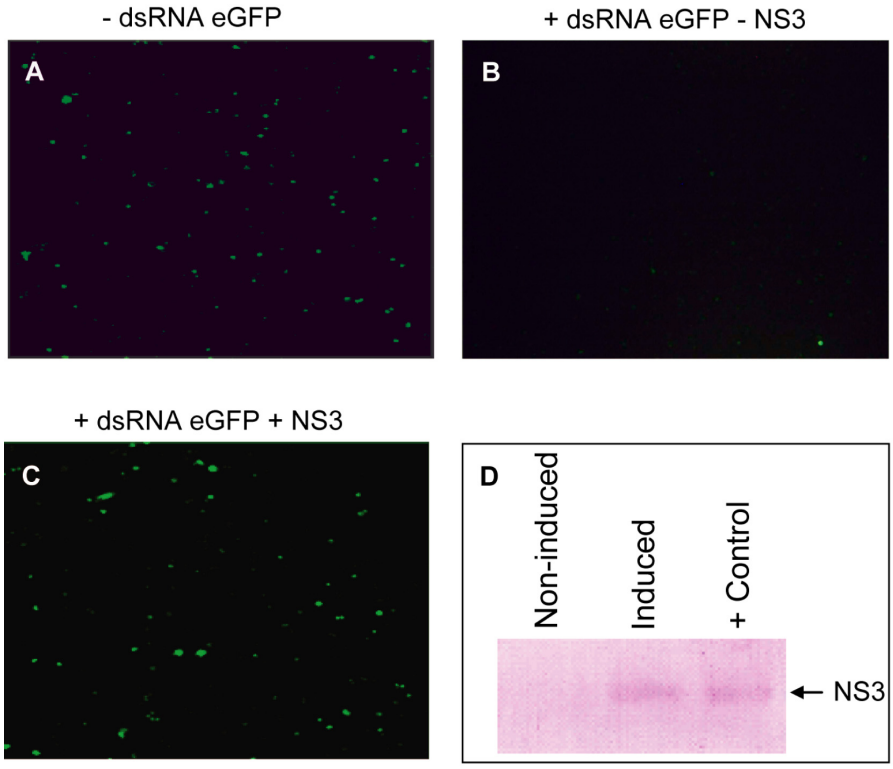

Figure 2-1: Suppression of RNA silencing by NS3 in cultured *Drosophila* cells. Cells were transfected with pAc-eGFP and empty pMK33 (**A**) and treated 1h after transfection with dsRNA specific for eGFP (**B** and **C**). Cells in panel B and C were transfected with the same transfection mixture containing pAc-eGFP and pMK33-NS3. NS3 expression was induced with $CuSO_4$ resulting in an increase of the eGFP signal (**C**) compared to the non-induced cells (**B**). Expression of NS3 was confirmed by Western blot analysis using bacterial expressed HIS-tagged NS3 as positive control (**D**).

The MBP-NS3 fusion protein is an active RNA silencing suppressor

To address the question how NS3 exerts its function as suppressor of RNA silencing a biochemical approach was followed (Lakatos *et al.*, 2006; Lakatos *et al.*, 2004). First the NS3 protein was produced in bacteria as a C terminal fusion to the maltose binding protein for

purification purposes. Suppressor activity of the MBP-NS3 fusion protein was verified in plants using the established *A. tumefaciens* transient expression assay (ATTA; Fig. 2-2) (Bucher *et al*., 2003).

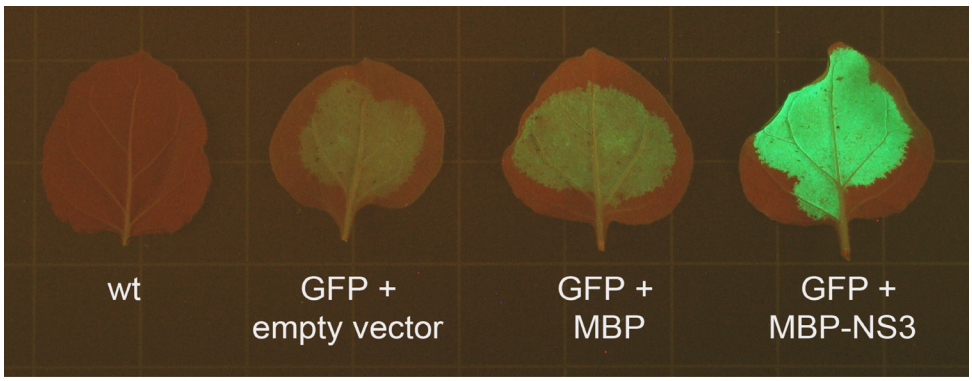

Figure 2-2: GFP silencing suppression of MBP-NS3 in *Agrobacterium* infiltrated *Nicotiana benthamiana* leaves visualised 5 days post-infiltration. From left to right: non-infiltrated wildtype and GFP expression constructs co-infiltrated with an empty binary vector, the MBP construct and MBP-NS3 binary vector, respectively.

NS3 has high affinity for 21 nt, but not for 26 nt siRNAs

Several other strong RNA silencing suppressors have been shown to bind small RNAs with varying affinities. Therefore, it was investigated whether also NS3 is able to bind small RNAs and which size and structural features might influence the affinity of the interaction. The affinity of the NS3 protein to different small dsRNA molecules was determined by electrophoretic mobility shift assays (EMSA). Radiolabelled small RNA molecules were incubated with a dilution serie of the NS3 protein and complexes were resolved by native gel electrophoresis. The dissociation constant (K_d) of NS3 for 21 nt siRNA was calculated to be 2.45±0.26 nM (Fig. 2-3A and D), indicating a high binding affinity in the same order of magnitude as the well-studied suppressor p19 with a K_d of 0.17±0.02 nM for 21 nt siRNA (Vargason *et al*., 2003). Only a slightly lower affinity (K_d 5.7±0.80 nM) was observed for siRNAs lacking the 2 nt overhangs (Fig. 2-3B and D), whereas the K_d increased dramatically (>300 nM) when 26 nt siRNAs were tested (Fig. 2-3C and D). The MBP expression tag by itself was shown not to bind to 21 nt siRNA molecules and had no effect in subsequent control experiments (Fig. 2-3E), thereby excluding a role of the tag in the observed siRNA binding and further biochemical analyses. These experiments showed that NS3 binds short siRNA molecules with high affinity and that the 3' 2 nt overhangs are not essential for NS3 binding.

NS3 binds small RNAs as a dimer

To obtain more insight in the stoichiometry of siRNA binding by NS3, gel filtration experiments were carried out with purified NS3 protein and radiolabelled siRNA molecules. To do this, the EMSA reaction was scaled up and subsequently size-separated. As a control, siRNA without NS3 was loaded at the same concentration onto the column. Gel filtration fractions were tested for the presence of siRNAs by denaturing gel electrophoresis. The results showed that unbound siRNAs were found in the same fractions as control siRNAs.

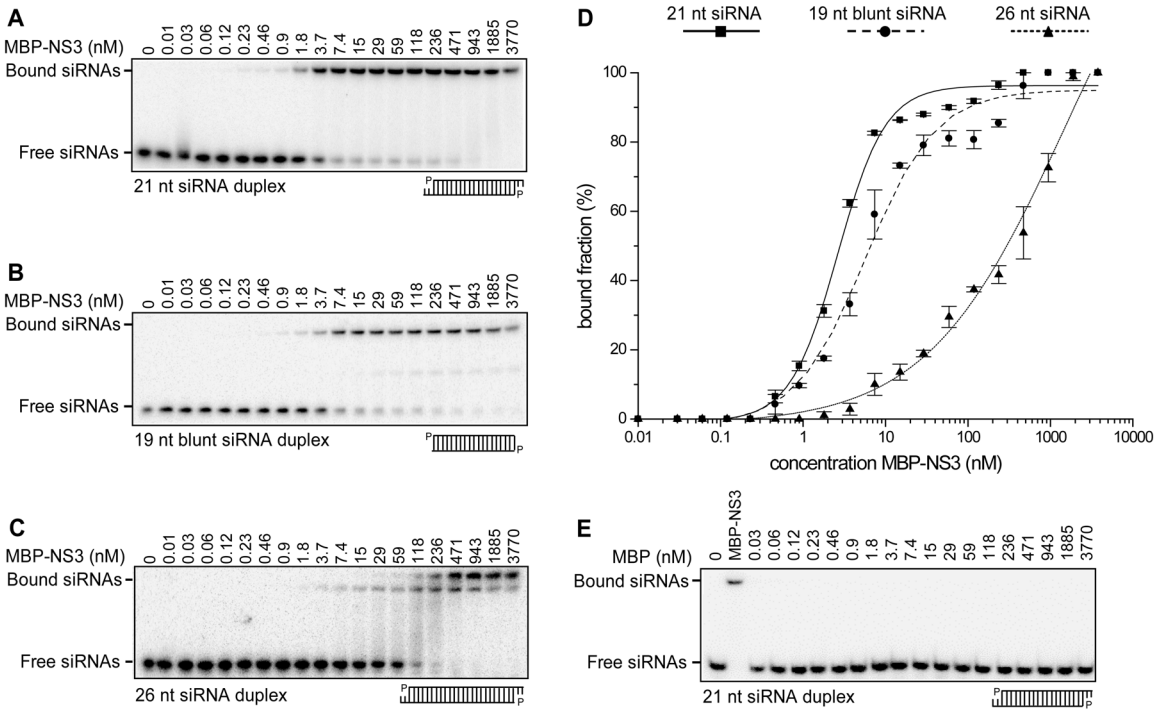

Figure 2-3: Affinity of MBP-NS3 for different RNA duplexes. A dilution serie of MBP-NS3 (0.01-3770 nM) was incubated with 100 pM each of ^{32}P-labelled 21 nt siRNA duplex (**A**), 19 nt blunt ended RNA duplex (**B**) or 26 nt siRNA duplex (**C**) for 20 minutes, then loaded onto a 5% native gel. The K_d was determined of MBP-NS3 for the different small RNA molecules by plotting the bound RNA fraction as function of the MBP-NS3 concentration (**D**). As control, a dilution serie of MBP (0.03-3770 nM) was incubated with 100 pM ^{32}P-labelled 21 nt siRNA duplex (**E**). In panels A, B, C and E the first lane contains only siRNAs.

An additional peak, corresponding to the NS3-siRNA complex, was detected to be migrating at a size similar to 150 kDa (Fig. 2-4). This suggests that a single siRNA pair (~14 kDa) is bound by two molecules of MBP-NS3 (~66 kDa). Binding of RNA silencing suppressors as a dimer has been observed previously for the plant viral suppressor protein p19 (Vargason *et al*., 2003) and the insect viral B2 protein (Chao *et al*., 2005; Lingel *et al*., 2005).

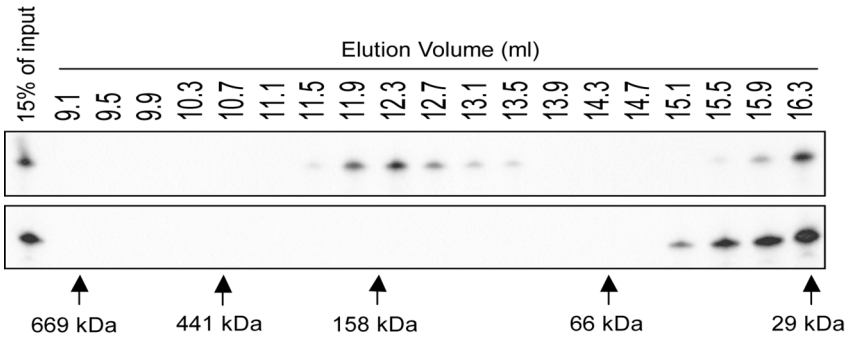

Figure 2-4: Gel-filtration of the MBP-NS3 - siRNA complex. MBP-NS3 was incubated with ^{32}P-labelled siRNAs and size-separated on a Superdex -200 column. Fractions were collected and tested for the presence of ^{32}P-labelled siRNAs (**Top panel**). As control ^{32}P-labelled siRNAs were size-separated in the absence of MBP-NS3 (**Lower panel**). The elution position of protein molecular weight markers is indicated by arrows below the picture: 669 kDa, thyroglobulin (9.1 ml); 441 kDa, ferritin (10.5 ml); 158 kDa, aldolase (12.1 ml); 66 kDa, bovine serum albumin (14.3 ml); 29 kDa, carbonic anhydrase (16.3 ml).

NS3 competes for siRNAs in the RISC assembly process

The biochemical analysis of RISC functionality and assembly is best studied in the *in vitro Drosophila* embryo extract RNA silencing system where mature RISC and intermediate complexes can be visualised (Pham *et al*., 2004; Tomari *et al*., 2004a). RISC complexes are assembled in an organised manner (Pham *et al*., 2004; Tomari *et al*., 2004a). Initial complexes drive the assembly of the mature, active RISC complexes from the R2D2/DCR2 complex (R1) through a distinct intermediary complex, being the RISC loading complex (RLC). The formation of active RISC complexes on siRNAs can be visualised by native gel electrophoresis and the influence on the complex formations studied by adding increasing concentration of the suppressor protein to the *in vitro* reactions. We used the system based on native gel electrophoresis using *Drosophila* embryo lysate (Pham *et al*., 2004) with modifications as described (Lakatos *et al*., 2006).

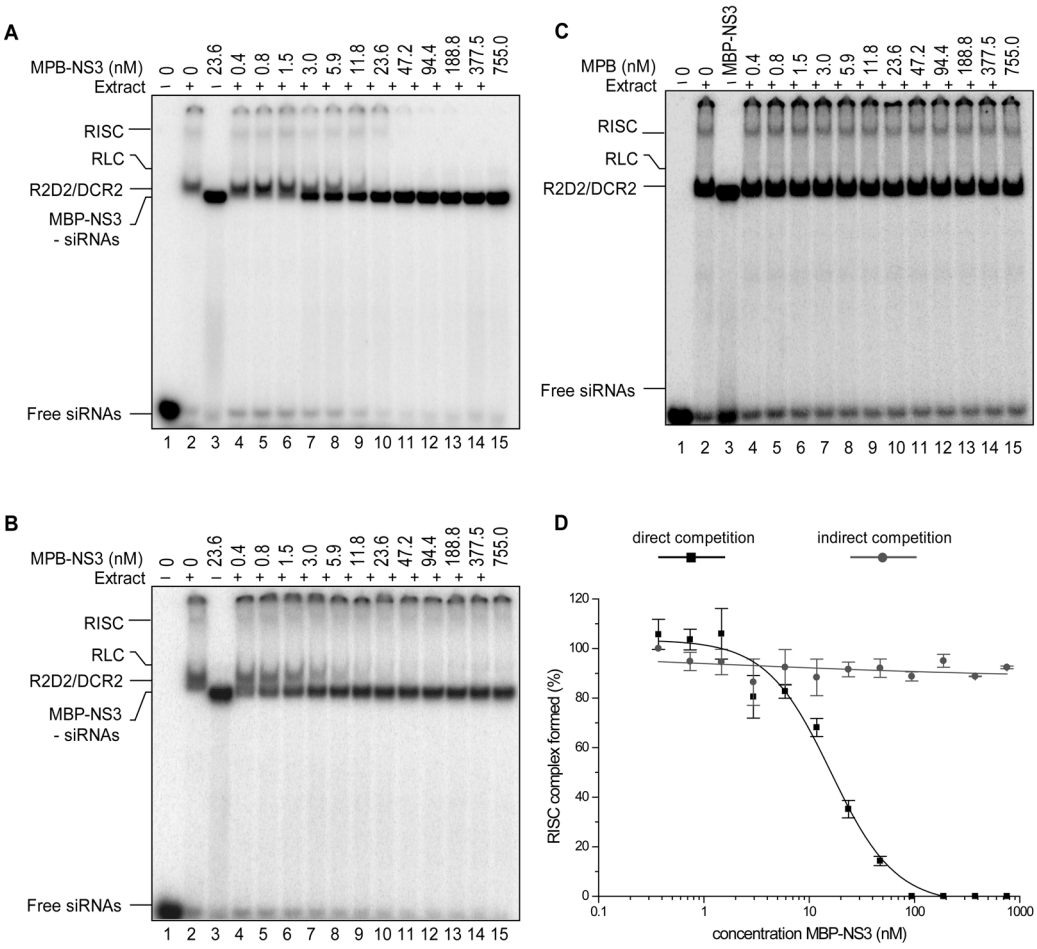

Figure 2-5: Inhibition of RISC assembly by NS3 *in vitro*. (**A**) In direct competition experiments, RISC assembly was monitored by adding ^{32}P-labelled siRNAs and MBP-NS3 (0.4-755.0 nM) to *Drosophila* embryo extract. (**B**) Indirect competition assay where RISC assembly was initiated by adding ^{32}P-labelled siRNAs to embryo extract. MBP-NS3 (0.4-755.0 nM) was added to pre-incubated reactions after 30 minutes. In panel A and B lane 1 contains only free siRNAs, lane 2 ^{32}P-labelled siRNAs and embryo extract, and lane 3 ^{32}P-labelled siRNAs and 23.6 nM MBP-NS3. In lanes 4-15 the competition effect of MBP-NS3 on RISC assembly is shown. (**C**) As control for the direct competition experiment, RISC assembly was monitored by adding ^{32}P-labelled siRNAs and MBP (0.4-755.0 nM) to *Drosophila* embryo extract. (**D**) For direct and indirect competition experiments the formation of RISC complex as a function of MBP-NS3 concentration are plotted relative to the RISC formation in the absence of MBP-NS3 (lane 2).

Labelled siRNAs, *Drosophila* extract and recombinant NS3 protein were incubated simultaneously in direct competition assays and formation of mature RISC decreased gradually with increasing NS3 concentration (Fig. 2-5A and D). Complete inhibition of RISC formation was observed at higher (>90 nM) NS3 concentrations. In a second set-up the effect of NS3 on pre-assembled RISC complexes was tested. RISC was assembled by incubation of *Drosophila* extract with siRNAs and after 30 minutes, different concentrations of NS3 were added to the mixtures. These results showed that in this case increasing NS3 amounts had no effect on the pre-assembled RISC (Fig. 2-5B and D). Though partially masked by the MBP-NS3 protein, both Fig 2-5A and 2-5B suggest a decrease in the accumulation of the R1 complex upon elevation of MBP-NS3 concentrations. This may imply that NS3 is capable of extracting double stranded siRNAs from R1 complexes prior to stand separation or sequestering free siRNAs during passive transient release of siRNAs by the R1 complex in RISC assembly. Control experiments using MBP alone indicated no inhibitory effect of the tag on the formation of silencing complexes (Fig. 2-5C).

RISC-mediated cleavage is inhibited by NS3

RISC mediated cleavage of a target is initiated by siRNAs with sequence complementarity to the target. With the observation that NS3 competes for siRNAs during RISC assembly in *Drosophila* extracts, RISC-mediated cleavage of an RNA target was tested in a way described previously (Lakatos *et al*., 2006). The effect of NS3 on siRNA-mediated target cleavage by RISC was studied in direct and indirect competition assays. In direct competition, *Drosophila* extract, a target RNA (GFP mRNA), synthetic siRNA-inducer molecules with a sequence homologous to the target were mixed with NS3. RISC mediated cleavage was visualised by the 5' cleavage product of the radiolabelled target and cleavage products were quantified at the different NS3 concentrations used. Indirect competition was established by pre-incubation of siRNA inducer and *Drosophila* extract for 30 minutes followed by the addition of target RNA and NS3. At the highest NS3 concentrations used, RISC mediated target cleavage was inhibited in the direct competition assay (Fig. 2-6A and D). As could be expected from the RISC assembly studies, the activity of pre-assembled RISC was not inhibited by NS3, independent of the amount of NS3 added to the reactions (Fig. 2-6B and D). Control experiments using MBP alone indicated no inhibitory effect RISC mediated target cleavage (Fig. 2-6C).

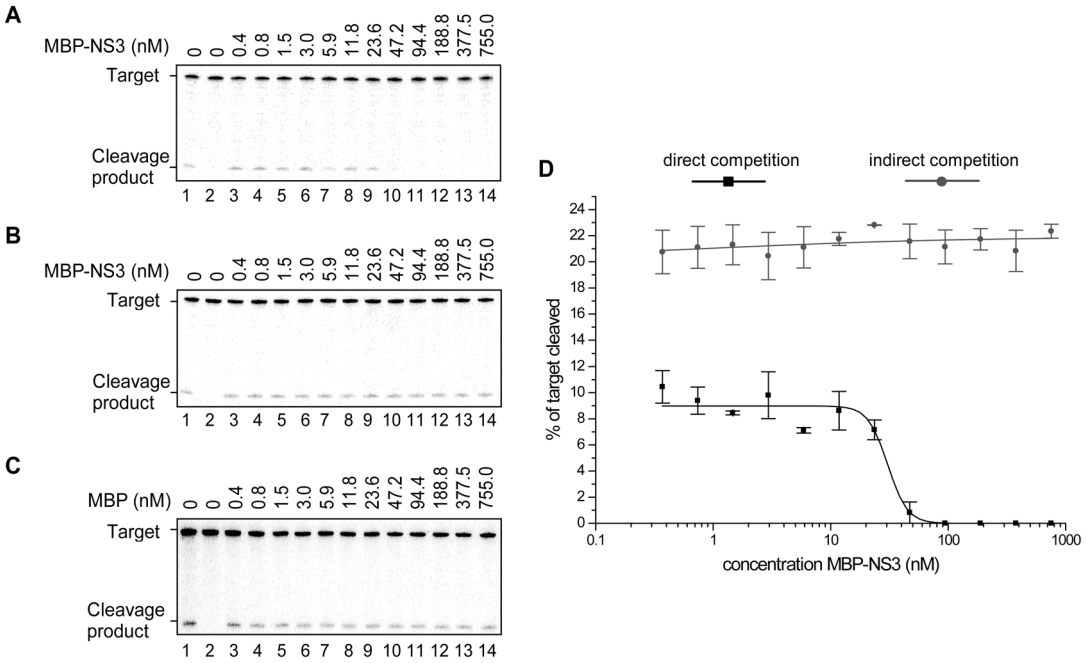

Figure 2-6: NS3 inhibits siRNA-mediated target cleavage in the *Drosophila* embryo extract *in vitro* RNA silencing system. (**A**) In direct competition assays, RISC-mediated target RNA (0.5 nM) cleavage was induced by siRNAs (5 nM) and MBP-NS3 (0.4-755.0 nM), simultaneously added to *Drosophila* embryo extracts. (**B**) In indirect competition, RISC was pre-assembled by adding siRNAs (5 nM) to embryo extract for 30 minutes and target RNA (0.5 nM) and MBP-NS3 (0.4-755.0 nM) subsequently added. (**C**) As control for the direct competition assay, RISC-mediated target RNA (0.5 nM) cleavage was induced by siRNAs (5 nM) and MBP (0.4-755.0 nM) simultaneously added to *Drosophila* embryo extracts. (**D**) For direct and indirect competition experiments the percentage of cleaved target is plotted as a function of the MBP-NS3 concentration relative to the percentage cleaved target in absence of MBP-NS3. In panel A and B lanes 1 include siRNAs and lack MBP-NS3 or MBP, lanes 2 lack inducer siRNA and MBP-NS3 or MBP. We note that *Drosophila* embryo extract was used at the same concentration as we used for RISC assembly experiments (1 µg/µl in the test tube).

NS3 binds miRNAs in vitro

Besides a role in antiviral defence in plants, small RNAs play an essential role in the regulation of gene expression through miRNAs (Mallory & Vaucheret, 2006). It has been reported that several plant viral suppressors also induce developmental abnormalities in plants by interfering with the miRNA pathway (Chapman *et al*., 2004; Dunoyer *et al*., 2004) possibly by interfering with the miRNA/miRNA* duplex unwinding. In order to investigate the base complementarity requirements of NS3 for small RNA complexes including miRNA/miRNA*, three miRNAs belonging to the *Arabidopsis thaliana* miRNA171 family were tested in a similar set-up as was used for siRNAs. Members of the miR171 family show differences in structural features, by containing 2 or 3 wobbles introduced by mismatches between the two strands of the miRNA duplex (Fig. 2-7A-C). The affinity of the NS3 protein to the miRNAs 171a (K_d 6.17±0.80), 171b (K_d 7.19±1.02) and 171c (K_d 6.26±0.78) was compared (Fig. 2-7A-C). Despite their varying degree of base complementarity, all miRNA species were efficiently bound by NS3 (Fig. 2-7D), with an affinity in the same range as observed for the 21 nt and 19 nt blunt siRNA molecules. As indicated for the siRNAs molecules also for the tested miRNAs there was no binding to MBP observed (Fig. 2-7E).

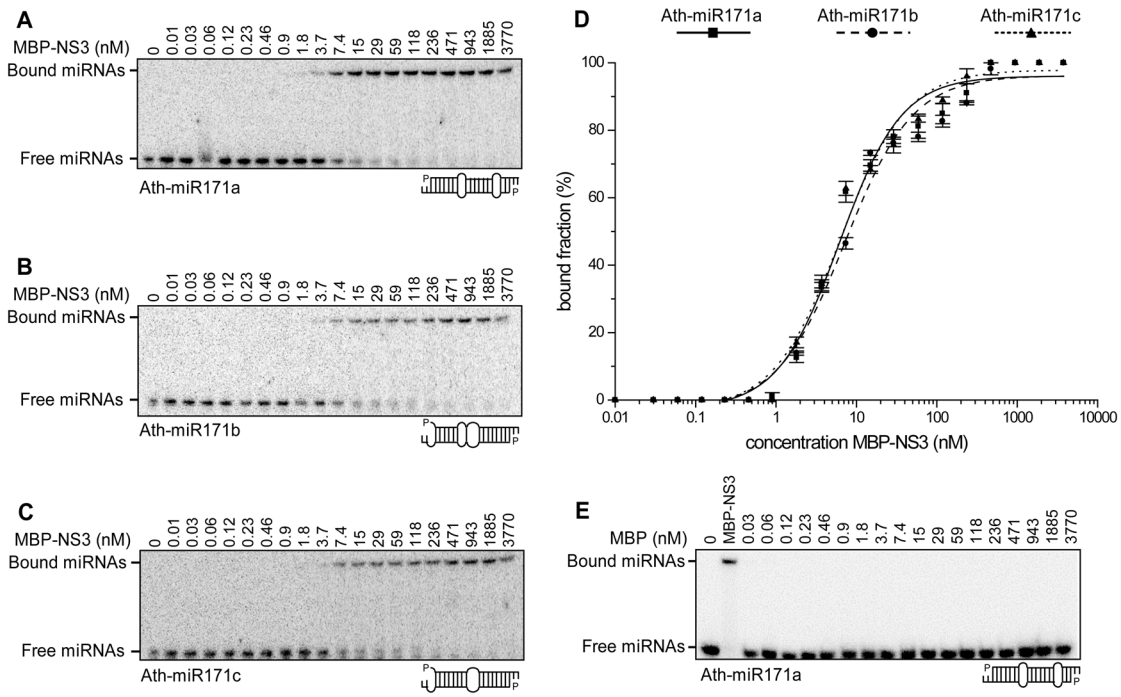

Figure 2-7: Affinity of MBP-NS3 for different miRNA duplexes. A dilution serie of MBP-NS3 (0.01-3770 nM) was incubated with 100 pM each of ^{32}P-labelled Ath-miR171a (**A**), Ath-miR171b (**B**) or Ath-miR171c (**C**) for 20 minutes, then loaded onto a 5% native gel. The K_d was determined of MBP-NS3 for the different small RNA molecules by plotting the bound fraction of as a function of the MBP-NS3 concentration (**D**). As control, a dilution serie of MBP (0.03-3770 nM) was incubated with 100 pM ^{32}P-labelled Ath-miR171a (**E**). In panels A, B, C and E the first lane contains only miRNAs.

This indicates that NS3, besides efficiently binding siRNAs, can interfere with the miRNA regulated RNA silencing pathway by strongly binding miRNAs/miRNA* complexes before these can be incorporated into RISC. Considering that all three members of the miR171 family are bound at high affinity, it can be anticipated that many miRNA/miRNA* complexes can be subjected to NS3 binding. Expression of this protein is therefore likely to influence host gene regulation in infected tissues of plants and insects (Fig. 2-8).

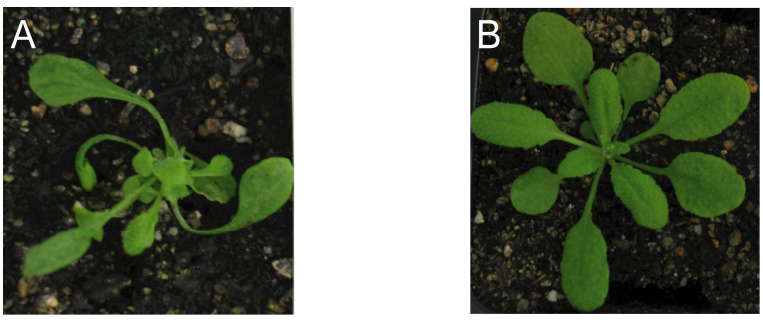

Figure 2-8: Developmental defects induced by the silencing suppressor NS3 (**A**) compared to wildtype plants (**B**). *Arabidopsis thaliana* Col-0 plants were transformed by the floral dip method (Clough & Bent, 1998) by using *A. tumefaciens* LBA4404 carrying a construct to express NS3. Seed from primary transformants was grown under selection for kanamycin resistance in a standard greenhouse.

Discussion

NS3 is an RNA silencing suppressor in cultured insect cells

It has been reported that RNA silencing suppressors of several plant viruses also operate in an insect cell background (Li *et al*., 2002; Li *et al*., 2004; Reavy *et al*., 2004). Additionally, also true insect viruses, FHV and DCV, have been shown to specify such a suppressor, indicating that RNA silencing in insects also acts as antiviral defence mechanism (Li *et al*., 2002; van Rij *et al*., 2006). Here we demonstrate that the NS3 RNA silencing suppressor protein of RHBV, a virus which replicates both in plants and insects, is not only operational in plant cells (Bucher *et al*., 2003) but also in insect cells. The mode of operation of NS3 was hitherto unknown, but based on these observations it must target a conserved part of the RNA silencing pathway.

NS3 suppresses RNA silencing by inhibiting RISC assembly in vitro

NS3 physically interacts with dsRNA molecules with size preference, showing the highest affinity for 21 nt siRNAs. Therefore NS3 is able to recognise the 'standard' siRNA implicated in local silencing, but less efficiently the longer siRNA species which have been implicated in long distance movement in plants (Hamilton *et al*., 2002; Tang *et al*., 2003). Our results showed that the NS3 protein is able to bind siRNAs with a high affinity in the presence or absence of the *Drosophila* extract, suggesting that no additional components are required for efficient siRNA binding.

The siRNAs play an important role by serving as the foundation for the ordered assembly of RISC complexes. In indirect competition experiments, RISC complexes were pre-assembled and it could be shown that NS3 was not able to interfere with sequence specific target cleavage. Active RISC complexes contain ss siRNAs representing the guide strand of the initial ds siRNA complex that give rise to the sequence specificity of RISC for the target. In additional experiments (results not shown) it was shown that NS3 does not efficiently bind to single stranded siRNAs. Consistent with this observation no decrease in RISC complex formation was detected in the presence of increasing concentrations of NS3, because the ss siRNAs present in mature RISC complexes are no substrate for NS3. In contrast, a decrease in the formation of the R1 was observed with increasing concentration of NS3 suggesting that NS3, by having a higher affinity for ds siRNAs, can prevent the formation or maturation of the R1 complex. As the affinity of NS3 for larger dsRNA is low, our results suggest that NS3 action occurs after the cleavage of larger dsRNA complexes by DCR2 and before mature RISC formation.

As a result of RISC formation inhibition, NS3 was shown to inhibit cleavage of a target mRNA in the *Drosophila* embryo *in vitro* RNA silencing system in direct competition experiments, indicating its function as RNA silencing inhibitor in insects where RHBV replicates. *In vitro* mechanistic studies on the *Drosophila* embryo antiviral RNA silencing pathway showed that DCR2 activity is uncoupled from RISC assembly. Long dsRNAs are processed into siRNAs by DCR2, generating

a pool of siRNAs. To initiate RISC assembly, siRNAs are then rebound by R2D2/DCR2 according to the rule of strand preference and facilitate RISC assembly (Preall *et al*., 2006). Thus, the antiviral RNA silencing pathway in insects can be efficiently inhibited either by masking long dsRNA with a dsRNA binding protein (van Rij *et al*., 2006) or sequestering the siRNA pool by a siRNA binding protein (this study).

Inhibition of RISC assembly by sequestering siRNAs was recently also observed for RNA silencing suppressor proteins of plus strand tombusviruses, closteroviruses and potyviruses. However, these plant viruses do not replicate in their insect vectors and are therefore unlikely to encounter antiviral RNA silencing in insect cells. Since all these suppressors bind siRNAs with high affinity, competition for these molecules during RISC assembly may inhibit the assembly of functional RISC complexes in a viral infection, thus inhibiting strand separation of siRNA duplexes. Alternative modes of operation appear to be adopted by the p88 and p27 proteins of *Red clover necrotic mosaic virus* (RCNMV), which seem to recruit Dicer (like) proteins during viral RNA replication (Takeda *et al*., 2005). Though the various viral RNA silencing suppressors have a surprising lack of conservation in their protein sequences, a limited number of modes of action seem to be followed, each targeting a distinct part of the RNA silencing machinery. As the chemical structure of siRNAs is ubiquitous, it seems not surprising for a virus such as RHBV, that has to replicate in both plants and insects, to interfere with a part of the RNA silencing machinery that is identical in both organisms, i.e. siRNA.

NS3 has high affinity for miRNA/miRNA* complexes independent of RNA duplex strand mismatches

RNA silencing not only plays an important role in antiviral defence in plants and insects but is also involved in developmental processes. In the regulation of plant and animal development, host encoded miRNAs are key players in timed targeting of mRNAs for cleavage or translational arrest. The systemic infection of plants by viruses often results in symptoms resembling developmental defects, which can be characterised by loss of leaf polarity, cell division control and reproductive functions (Mallory & Vaucheret, 2006). Occurrence of these phenotypes is regularly associated with viral suppressor proteins or virulence factors. Constitutive expression of NS3 in *Arabidopsis thaliana* causes defects resembling developmental defects, such as loss of leaf polarity similar, but not identical to those reported previously (Chapman *et al*., 2004; Dunoyer *et al*., 2004) (data not shown). These developmental phenotypes are most likely the result of miRNA/miRNA* duplex binding by NS3, independent of mismatches present in the miRNA/miRNA* duplexes, resulting in inhibition of duplex unwinding and preventing miRNA function. Naturally, interference of NS3 with the siRNA part of RNA silencing serves to inhibit this antiviral defence mechanism but it can be speculated that interference of the NS3 protein with plant and insect gene expression regulation may also serve to establish an environment in plants and insects which may be advantageous for the infecting virus.

Binding of NS3 to siRNAs and different miRNAs with comparably high affinities gives rise to the question if physical interactions to these molecules reflect two distinct interference strategies or are variations on a single theme? With the discovery that several sequences of 20–25 nt located within *Arabidopsis* intergenic regions share perfect or near perfect complementarity with a variety of plant virus genomes (Llave, 2004), it is tempting to speculate that plant viruses, besides inducing antiviral RNA silencing and the subsequent production of viral siRNAs, are also targeted by host encoded miRNAs. However, there is no direct evidence that plant host or insect vector of RHBV encode RHBV specific miRNAs able to target RHBV viral sequences during replication. So next to the obvious need to suppress antiviral RNA silencing in both plants and insects, the NS3 protein may perform a similar role as the *Primate foamy virus* type 1 (PFV-1) RNA silencing suppressor protein Tas. The latter was shown to suppress human miRNA-32-mediated translational inhibition and probably functions to inhibit restriction of PFV-1 accumulation by miRNA-32 (Lecellier *et al*., 2005).

Dual roles for NS3 in RNA silencing in plants and insects?

It was shown that a plant virus, which is propagatively transmitted by an insect vector is able to counteract antiviral RNA silencing in both insect and plant hosts. Binding siRNAs offers this possibility as the host organisms generate identical 21 nt siRNAs in antiviral RNA silencing. Targeting such conserved components of RNA silencing, both hosts cannot evade the action of proteins such as NS3. The downside for the virus however is that large quantities of suppressor proteins need to be produced to quench the bulk of the antiviral siRNAs. Alternatively, interfering with specific protein components of the RNA silencing machinery could result in a stronger suppression because a smaller number of targets need to be incapacitated. This approach however, presents the risk that these proteins may alter their primary sequence and become unsuitable as targets or alternatively (partially) redundant gene copies can take over the targeted function. By sequestering siRNAs the NS3 protein prevents this kind of host adaptation strategies. Moreover, by targeting siRNAs the effect of the suppressor may not be perfectly efficient, allowing modulation of virulence.

Materials and Methods

Plasmid constructs

The coding sequence of RHBV NS3 was PCR amplified introducing a unique BamHI site at the 5' end and a unique KpnI site at the 3' end. The PCR product was originally ligated into the bacterial expression vector pQE30 (Qiagen). For recombinant protein expression the NS3 coding sequence was removed from the pQE vector as BamHI-PstI fragment and cloned in frame with the MBP coding sequence into the pMAL-c2x vector (New England Biolabs). For expression of NS3 in *Drosophila* S2 cells (Invitrogen) the NS3 ORF from the pQE30-NS3 was ligated as BamHI-SmaI fragment into the pMK33/pMtHy vector which was digested with SpeI, end-filled with Klenow and subsequently cut with BamHI. pMK33/pMtHy (kindly provided by Lee Fradkin, Leiden University Medical Center, Leiden, The Netherlands; originally constructed by Michael Koelle) was used to clone the NS3 ORF immediately downstream of the $CuSO_4$-inducible metallothionein promoter giving rise to pMK33-NS3.

dsRNA preparation

Double stranded RNA was generated using T7 RNA polymerase (Promega) according to protocol using a gel purified (High Pure PCR purification kit; Roche) PCR template. The primers used introduced T7 RNA polymerase promoters at both ends of the PCR product. Primers used were: T7 ds_eGFP F: 5' GTA ATA CGA CTC ACT ATA GGG GGC GTG CAG TGC TTC AGC CGC 3' and T7 ds_eGFP R: 5' GTA ATA CGA CTC ACT ATA GGG GTG GTT GTC GGG CAG CAG CAC 3' for eGFP specific dsRNA (400 nt). Following transcription the reaction mixture was incubated at 70°C for 10 minutes and cooled down to RT. Template and single stranded RNA molecules were removed by treatment with DNase I and RNase A and dsRNA precipitated using 0.1 volume of 3M NaAc pH5.2 and 1 volume of iso-propanol.

Cell culture, transfection and RNA silencing assay

Drosophila S2 cells (Invitrogen) were cultured at 27°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS). For the RNA silencing assay, 2 ml aliquots of a S2 cell suspension (1 x 10^6 cells/ml) were seeded in a 35 mm tissue culture dish the day before transfection. The cells were then transfected with 1 µg/well pAc-eGFP and 2 µg/well pMK33-NS3 vector. Transfections were performed using Cellfectin (Invitrogen) in serum-free medium as described by the manufacturer. Expression of NS3 protein was induced by adding $CuSO_4$ to 0.5 mM final concentration directly after transfection. RNA silencing was induced by adding 5 µg/well dsRNA 3 h after the transfection. GFP fluorescence was monitored 28-72 h after transfection.

S2 cell expression analysis

Expression of NS3 was analysed by Western blotting. S2 cells were harvested by centrifugation for 5 minutes at 1500 rpm (Heraeus Labofuge I). Cells were resuspended in 1 pellet volume PBS and disrupted by sonification on ice with 30s intervals for 3 times 30 seconds. Proteins were TCA precipitated and separated by SDS-PAGE. Proteins were semi-dry transferred to Immobilon-P (Millipore) and detected using suppressor specific rat primary and goat alkaline phosphatase conjugated secondary antibodies and visualised with NBT-BCIP as substrate (Roche) according to manufacturer's recommendations.

Agrobacterium tumefaciens *infiltration*

A. tumefaciens infiltration was performed according to Bucher and co-workers (Bucher *et al*., 2003). For co-infiltration of *N. benthamiana* leaves, a mixture of a strain carrying the GFP construct (OD_{600}=0.5) and a strain carrying the suppressor construct (OD_{600}=05) was used. The GFP imaging photographs were taken 5 days after infiltration using a yellow 022 Proline B&W filter.

Recombinant protein expression

The RHBV NS3 protein was expressed from BL21 DE3 cells according to manufacturer's recommendations. After induction for 3h at 37°C with 0.3 mM IPTG cells were harvested by centrifugation for 15 minutes at 4000 rpm (Sorvall GSA rotor) at 4°C. Cells were lysed by sonification on ice with 30s intervals for 3 times 30 seconds in buffer A [100 mM NaCl, 20 mM Tris 7.4, 1 mM DTT, 2 mM $MgCl_2$, 10% (vol/vol) glycerol, 0.5% (vol/vol) Triton X-100]. The soluble fraction was recovered by centrifugation at 9000g for 30 minutes at 4°C. Recombinant protein was purified using amylose resin (New England Biolabs) and eluted with 2.5 packed bed volumes (PBV) buffer C (buffer A lacking Triton X-100 and containing 10 mM maltose) after washing with 15 PBV buffer B (buffer A lacking Triton X-100). Protein fractions were flash frozen in aliquots in liquid nitrogen and stored at -80°C until use. Protein concentrations of elution fractions were determined using the Micro BCA protein assay kit (PIERCE) according to manufacturer's recommendations and the purification process analysed by SDS-PAGE and subsequent staining with Coomassie.

Electrophoretic mobility shift assay

Annealing of siRNAs and miRNAs was described previously (Lakatos *et al*., 2004). Bacterial expressed MBP-NS3 was incubated for 20 minutes at RT with 100 pM ^{32}P-labelled siRNAs or miRNAs in 1x binding buffer [100 mM NaCl, 20 mM Tris 7.4, 1mM DTT, 2.5 mM $MgCl_2$, 10% (vol/vol) glycerol] in a 10 µl reaction volume. Separation of NS3 siRNA/miRNA complexes was performed at 4°C at 150V on a 1 mm thick, large format, 5% (38:2 acrylamide:bisacrylamide), 0.5x TBE native gel. After running gels were dried, exposed to a phosphor screen, scanned

(Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences) and bands quantified using Genius Image Analyser software (Syngene).

Gel filtration

A binding reaction of 250 µl was assembled as for the electrophoretic mobility shift assay using 100 pM ^{32}P-labelled siRNAs and 2.45 nM MBP-NS3 and chromatographed at 4°C on a Superdex-200 HR 10/30 column (Pharmacia) at 0.4 ml/min in column buffer containing 100 mM NaCl, 20 mM Tris 7.4, 1mM DTT, 2.5 mM $MgCl_2$, 10% (vol/vol) glycerol. After a void volume of 7.5 ml fifty 200 µl fractions were collected and used for RNA isolation. Per fraction, 80 µl NaCl, 2 µl 10mg/ml glycogen and 3 volumes 96% ethanol were added and RNA precipitated by incubation at -80°C. From 19 out of 50 collected fractions RNA molecules were separated on a 12% polyacrylamide and 8M urea containing sequencing gel.

RISC assembly assays

Drosophila embryo extract preparation, target RNA labelling, and siRNA annealing were described previously (Haley *et al*., 2003). In direct competition assays, embryo extracts were incubated for 30 minutes at 25°C with 5 nM ^{32}P-labelled siRNA duplexes and suppressor protein, diluted with 10 µl of loading buffer [1x lysis buffer, 6% (wt/vol) ficoll 400] and analysed on a 4.1% (40:1 acrylamide:bisacrylamide) native acrylamide gel. In pre-assembled RISC assays, ^{32}P-labelled siRNA duplexes and embryo extracts were pre-incubated for 30 minutes at 25°C to allow RISC assembly prior to addition of suppressor protein. Native gel electrophoresis for separation of silencing complexes was essentially as described previously (Lakatos *et al*., 2006). Gels were dried and exposed to a phosphor screen, and bands were quantified using Genius Image Analyser software (Syngene).

RISC mediated target cleavage assays

Reaction conditions were as described (Lakatos *et al*., 2006). In direct competition assays, reactions were incubated for 1 h at 25°C. In indirect competition assays, siRNA and embryo extracts were pre-incubated for 30 minutes at 25°C to allow RISC assembly prior to addition of target RNA and suppressor protein. Samples were deproteinised and RNA was analysed on an 8% denaturing gel.

Statistical analysis

All *in vitro* target cleavage, RISC formation and RNA binding experiments were performed in triplicate. The percentage of RISC complex formed relative to the control experiment without MBP-NS3 (lanes 2 in Fig. 5A and B) was determined as well as the percentage of cleaved target relative to controls in lanes 1 in Fig. 6A and B. The curves were best fitted to the indicated sets of data with the computer program Microcal Origin 5.00. The average with standard error is shown in all graphs.

Acknowledgments

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Chapter 3

Mutational analysis of the *Rice hoja blanca tenuivirus* RNA silencing suppressor NS3

A slightly modified version of this chapter has been submitted for publication: **Hemmes H., Kaaij L., Lohuis D., Goldbach R., and Prins M. (2007)**. Mutational analysis of the Rice hoja blanca tenuivirus RNA silencing suppressor NS3

The NS3 protein of *Rice hoja blanca tenuivirus* has previously been shown to represent the viral suppressor of RNA silencing that acts by sequestering small interfering RNA molecules as a dimer. To identify functional domains, an alanine scan mutational analysis was performed on selected regions of the protein. Introduction of double and triple alanine substitutions of lysine residues at position 173, 174 and 175 resulted in dysfunctional mutant proteins, which in electrophoretic mobility shift assays exhibited a drastically decreased affinity for 21 nt ds siRNAs. This indicates that siRNA binding, involving lysines 173-175, is essential for the suppressor function of the protein.

Introduction

As a response to the antiviral RNA silencing, plant viruses have developed ways to counteract or evade this host defence mechanism (chapter 1; reviewed in Voinnet, 2005). Among plant viruses, the tenuiviruses take a special position as they also replicate in their leafhopper vectors (Falk & Tsai, 1998). The silencing suppressor protein of tenuiviruses, NS3 (Bucher *et al.*, 2003), is therefore likely to counteract antiviral silencing in both the insect vector and in plants. This view has been substantiated in chapter 2 where it was demonstrated that NS3 of *Rice hoja blanca tenuivirus* (RHBV) is active in inhibiting both plant and insect RNA silencing pathways. A typical suppressor action adopted by plant viruses is the size-specific binding to double-stranded (ds) small RNA molecules (Lakatos *et al.*, 2006; Merai *et al.*, 2006). These 21 nt small interfering (si)RNA molecules play important roles in different RNA silencing mechanisms (reviewed in Brodersen & Voinnet, 2006; Vaucheret, 2006). One strand of the siRNA duplexes guides the sequence specific recognition of complementary targets by the RNA induced silencing complex (RISC), resulting in cleavage (Fagard *et al.*, 2000; Hammond *et al.*, 2000). By sequestering siRNAs, viral suppressors remove these molecules from the RNA silencing pathway, thereby inhibiting RISC assembly (Lakatos *et al.*, 2006). In chapter 2 it has been demonstrated that the tenuiviral NS3 suppressor protein acts by binding 21 nt ds siRNAs, thus explaining it to work in both organisms, as both plant and insect RNA silencing use structurally identical siRNAs. In a search for proteins interacting with the NS3 paralogue p5 of *Rice grassy stunt virus* (RGSV), it was shown that this protein interacts with itself through its N-terminal domain (Chomchan *et al.*, 2003). Also for the *Rice stripe virus* 23.9 kDa protein, another NS3 paralogue, oligomerisation has been observed in both infected insect cells and plant cells (Takahashi *et al.*, 2003). Indeed, also for the NS3 protein dimeric binding to siRNAs was demonstrated (chapter 2). These results indicate that the N-terminal domain of NS3 may be important for homotypic interactions in plants and insects. Hence, for proper functioning NS3 should possess at least two essential domains, one for binding siRNAs and a second for dimerisation. To gain better insight in the functional domains of RHBV NS3, a targeted alanine mutation scan of two conserved sequence stretches within the NS3 protein has been performed and the mutant proteins tested for RNA silencing suppression capability and siRNA binding capacity.

Results

Identification of conserved domains in the NS3 protein

To identify putative domains or amino acid sequence motifs, potentially important for dimerisation or RNA binding, a sequence alignment of the available NS3 protein sequences and their paralogs within the *Tenuivirus* genus was made.

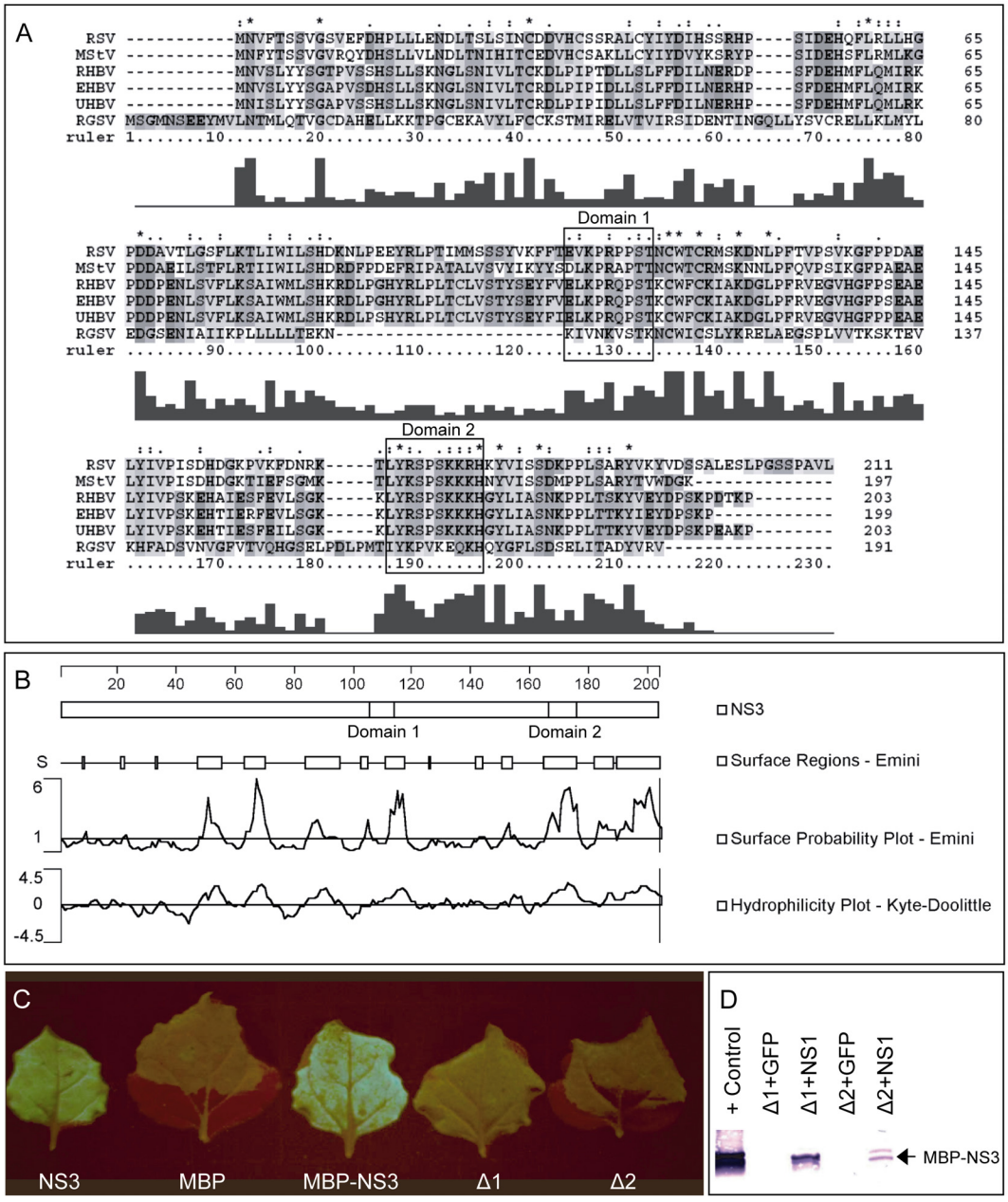

Figure 3-1: Analysis of the tenuiviral NS3-like proteins. Sequence alignment of 6 different NS3-like proteins originating from *Rice stripe virus* (RSV; NS3), *Maize stripe virus* (MStV; p3), *Rice hoja blanca tenuivirus* (RHBV; NS3), *Echinochloa hoja blanca virus* (EHBV; pv3), *Urochloa hoja blanca virus* (UHBV; pv3) and *Rice grassy stunt virus* (RGSV; p5) using the ClustalX computer program (**A**). Surface probability plot and hydrophilicity plot of the RHBV NS3 protein were determined using the Protean (DNASTAR) computer program (**B**). GFP silencing and suppression of MBP-NS3 constructs in *Agrobacterium* infiltrated *Nicotiana benthamiana* leaves visualised 5 days post-infiltration. From left to right: GFP expression constructs co-infiltrated with binary vectors containing NS3, MBP, MBP-NS3, MBP-NS3Δ1 and MBP-NS3Δ2, respectively (**C**). Expression of MBP-NS3Δ1 and MBP-NS3Δ2 was confirmed when co-infiltrated with NS1 by Western blot analysis using bacterial expressed MBP-tagged NS3 as positive control (**D**).

Several conserved areas were found of which two domains stood out based on conservation and surface probability (Fig. 3-1A). One of these domains (amino acid positions 106 - 114; domain 1) is located in the central region of the protein, the other (amino acids 167 - 176; domain 2) near the C-terminus. Using the Protean program both regions were predicted to be surface exposed (Fig. 3-1B). To test the potential functionality of domains 1 and 2 for the protein's suppressor activity, two mutants were constructed lacking either domain 1 or 2 and their suppressor activity was tested (as MBP-fusion, see chapter 2) using the ATTA assay (Bucher *et al.*, 2003). A decrease in GFP fluorescence was observed when MBP-NS3Δ1 and MBP-NS3Δ2 expression constructs were co-infiltrated with GFP, indicating a requirement of both domains for its suppressor function (Fig. 3-1C). The expression of dysfunctional RNA silencing suppressor proteins is susceptible to RNA silencing in the ATTA and therefore no mutant NS3 protein could be observed using Western blot analysis (Fig. 3-1D). To rule out the protein stability as a cause of the observed lack of RNA silencing suppression, the stability of the MBP-NS3Δ1 and MBP-NS3Δ2 proteins was confirmed by Western blotting when these were co-infiltrated with an active suppressor of RNA silencing (Bucher *et al.*, 2004).

Single alanine substitutions in domains 1 and 2 do not eliminate NS3 suppressor activity

As the deletion of entire domains is rather crude and potentially detrimental to the protein structure, more subtle changes were introduced. Based on the surface probability plot, 4 single alanine substitutions were made in domain 1 (E110A, L111A, K112A and P113A) and 3 in domain 2 (K173A, K174A and K175A) and all 7 mutants were tested for their suppressor activity in the aforementioned ATTA assay. Wildtype levels of GFP fluorescence were scored for all mutants in different repetitions, indicating that no single amino acid in region 1 or 2 is critical for suppressor activity *in planta* (Fig. 3-2, top panel).

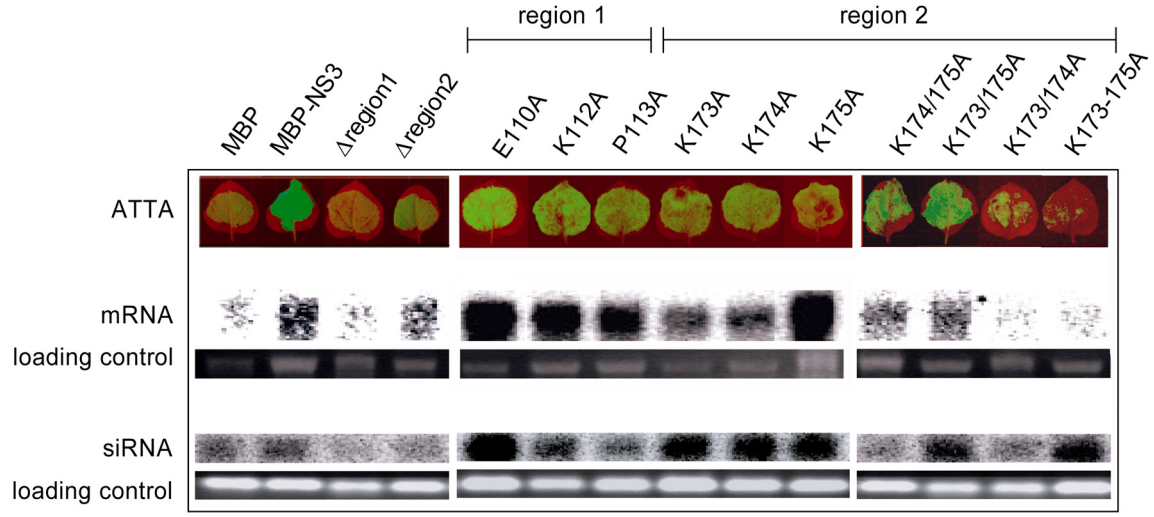

Figure 3-2: Testing alanine scan mutants of RHBV NS3 using the ATTA assay for demonstrating RNA silencing suppressor activity. Single, double and triple alanine replacements in domains 1 or 2 were tested. The upper panel shows GFP silencing and suppression of MBP-NS3 constructs in *Agrobacterium* infiltrated *Nicotiana benthamiana* leaves visualised 5 days post-infiltration. GFP mRNA and siRNAs levels are shown in the middle and lower panel, respectively, with ethidium bromide stained RNA as loading controls.

Also the GFP mRNA levels were similar to those observed in the co-expression with the wildtype NS3, correlating with the observed GFP signals in the ATTA assay (Fig. 3-2 central panel). GFP specific siRNAs were present in all samples (Fig. 3-2, lower panel), confirming our previous conclusion that NS3 operates downstream of Dicer by efficiently sequestering, but not preventing siRNA accumulation (chapter 2; Bucher *et al.*, 2003). Because NS3 exerts its function by binding siRNA molecules, the (unaltered) ability of all mutants to bind siRNA molecules was also tested. As anticipated no significant difference was observed for the estimated siRNA binding coefficient of the mutant NS3 proteins (Fig. 3-3 B-H).

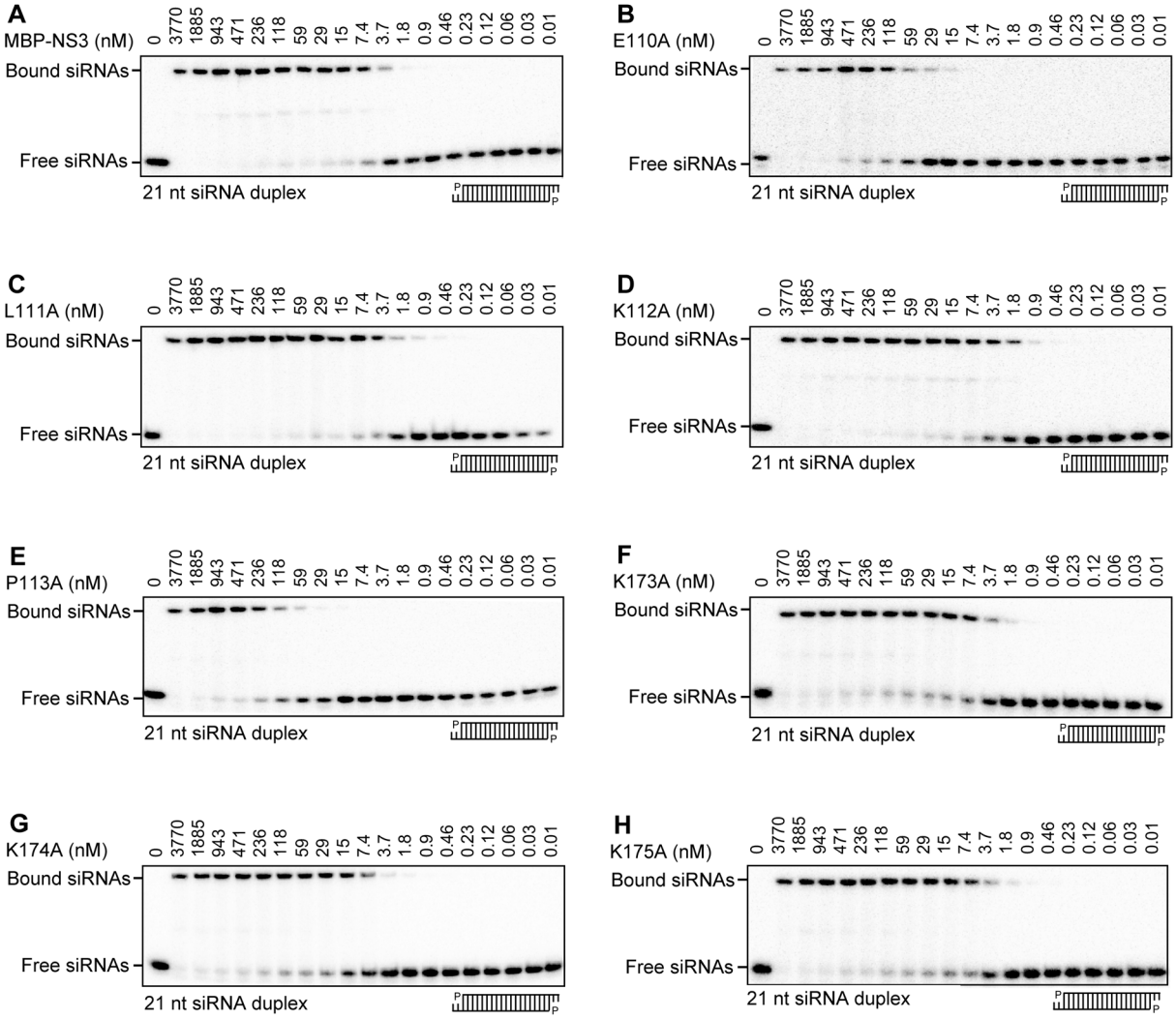

Figure 3-3: Affinity of single alanine mutants of RHBV NS3 for siRNA duplexes. A dilution serie (0.01-3770 nM) of MBP-NS3 mutants E110A (**B**), L111A (**C**), K112A (**D**), P113A (**E**), K173A (**F**), K174A (**G**) or K175A (**H**) was incubated with 100 pM of ^{32}P-labelled 21 nt siRNA duplexes for 20 minutes and then loaded onto a 5% native polyacrylamide gel. In all panels the first lane contains only siRNAs for size reference. As control wildtype MBP-NS3 was used (**A**).

Clustered lysines in domain 2 are important for siRNA binding and suppressor function of NS3

In two previous studies clustered basic amino acids were shown to be important for the suppressor function of the *Influenza virus A* NS1 and tombusvirus p19 proteins (Bucher *et al.*, 2004; Chu *et al.*, 2000; Vargason *et al.*, 2003; Wang *et al.*, 1999). These clusters could be

disrupted by (multiple) mutations. Domain 2 of NS3 encompasses such a cluster (i.e. 3 conserved lysines on positions 173 -175, see Fig. 3-1). To identify the putative RNA binding domain, prime focus was on this KKK cluster and one triple and three double alanine substitutions were introduced to this end (K173/174A, K174/175A, K173/175A and K173-175A) and tested. The double mutant K173/174A and triple mutant K173-175A, but not the two other double mutants, proved to be severely affected in their RNA silencing suppression activity (Fig 3-2 upper panel). In the subsequent siRNA binding studies mutants K174/175A and K173/175A showed a K_d in the order of the wildtype protein (56.9±8.3 nM and 10.8±1.2 nM respectively; Fig. 3-4 A, B and E). Mutants K173/174A and K173-175A on the other hand, effectively exhibited a complete loss of siRNA binding capacity (K_d>>500 nM; Fig. 3-4 C, D and E), explaining their loss of RNA silencing suppression in the ATTA assay. It is therefore concluded that the K173-175 cluster within conserved domain 2 is crucial for siRNA binding and suppressor activity. The presented data further demonstrates that loss of siRNA binding capacity correlates directly with loss of suppressor function.

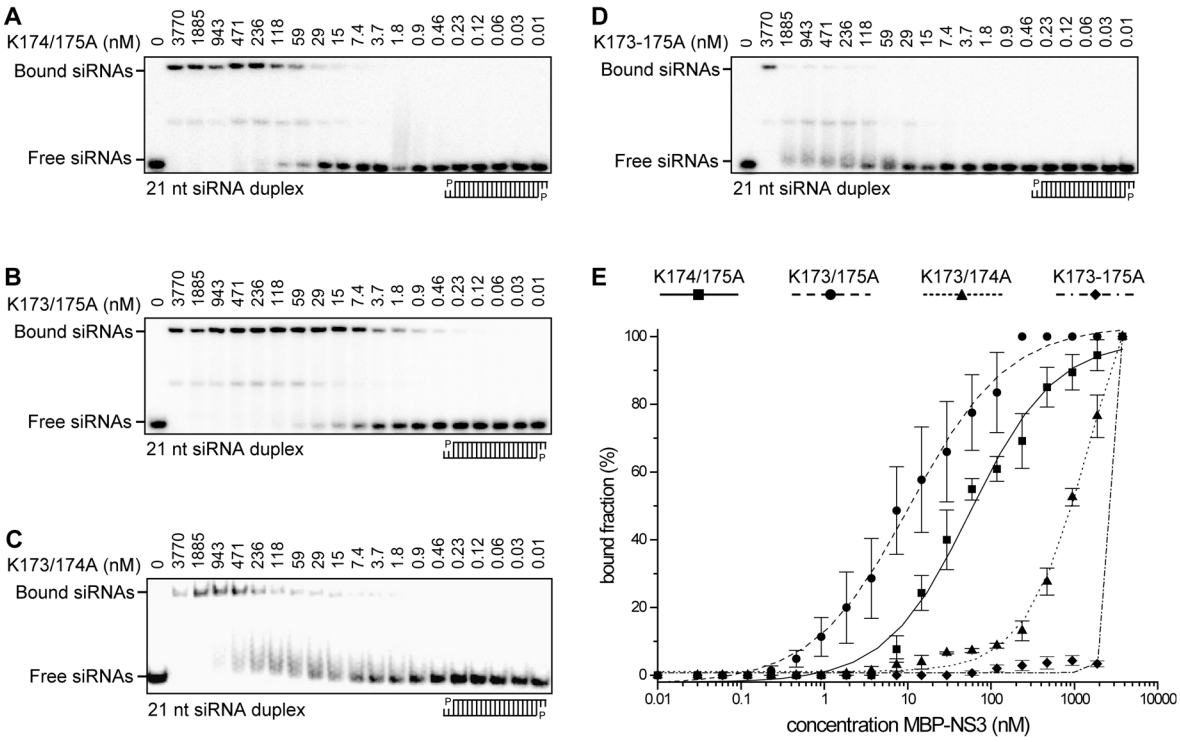

Figure 3-4: Affinity of MBP-NS3 mutated proteins for siRNA duplexes. A dilution serie (0.01-3770 nM) of MBP-NS3 mutants K174/175A (**A**), K173/175A (**B**), K173/174A (**C**) or K173-175A (**D**) was incubated with 100 pM of ^{32}P-labelled 21 nt siRNA duplexes for 20 minutes, then loaded onto a 5% native gel. In panels A, B, C and D the first lane contains only siRNAs. The K_d was determined of MBP-NS3 for the different small RNA molecules by plotting the bound RNA fraction as function of the MBP-NS3 concentration (**E**).

Discussion

The data presented in this chapter demonstrates that one of the investigated domains (domain 2, located near the C-terminus) is involved in siRNA binding, for which a triple lysine cluster is essential. In addition, confirming and underscoring our earlier results (chapter 2), siRNA binding capacity is essential for NS3 suppressor activity. Loss of this capacity leads to loss of RNA silencing suppression. Hence, one of the two crucial functional domains in NS3 has now been identified, the other one i.e. a potential oligomerisation domain still awaits its identification.

Although the dramatic increase in K_d for siRNA binding of mutants K173-175A and K173/174A was measured with a defined amount of purified protein, the loss of RNA silencing suppression activity in ATTA experiments could be explained by a reduction in protein stability. However, this possibility can be regarded as unlikely since the two other double mutants were still able to suppress RNA silencing. Moreover, the MBP-NS3Δ1 and MBP-NS3Δ2 proteins were easily detected on Western blot when co-infiltrated with a functional suppressor of RNA silencing, indicating that even these extensively mutated proteins are intrinsically stable and would be expressed if they would not lack RNA silencing capacity.

Previous studies with dsRNA-binding silencing suppressors of other plant virus genera and human viruses i.e. tombusvirus p19 and *Influenza virus A* NS1 have indicated that clustered positively charged amino acids are important for suppressor function (Bucher *et al.*, 2004; Chu *et al.*, 2000; Wang *et al.*, 1999). Indeed, also the NS3 protein encompasses a stretch of basic amino acid residues that are essential for siRNA binding and RNA silencing suppressor function, again confirming that these two traits are linked. Having mapped an siRNA binding site in NS3 domain 2 we are still left with the question what the biochemical function of conserved domain 1 might be. This domain might be involved in NS3 dimerisation since the p5 prologue RGSV self-interacts through its N-terminal domain (Chomchan *et al.*, 2003), but obviously further mutational analyses need to be performed to confirm the functionality of this domain. From the sequence alignment analysis also other conserved amino acids and domains with high surface probability can be identified and their contribution to dimer formation cannot be excluded.

Single alanine substitutions of several amino acids in both identified domains showed no effect on the suppressor activity in plants. In line with these findings also the RNA binding affinity for siRNA molecules of these mutated NS3 proteins showed no drastic decrease in EMSA experiments. Based on the results of the EMSA experiments, the K_d for siRNAs of NS3 proteins with single alanine substitutions ranged in the region of 5-100 nM. This may indicate that the affinity for siRNAs of pre-RISC (or RISC loading) complexes is lower than 100 nM and can explain the presence of the GFP signal in plants when a mutated NS3 suppressor with relatively high K_d was used in the ATTA experiments. Alternatively, the mutated NS3 protein in the current experimental setup may accumulate to high levels compared to RISC (loading) complexes, making even the proteins with a relatively high K_d sufficiently strong suppressors. 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.

Materials and methods

Plasmid constructs

The coding sequence of RHBV NS3 was PCR amplified introducing a unique BamHI site at the 5' end and a unique KpnI site at the 3' end. The PCR product was originally ligated into the pQE30 vector (Qiagen). For recombinant protein expression the NS3 coding sequence was removed from the pQE vector as BamHI-PstI fragment and cloned in frame with the MBP coding sequence into the pMAL-c2x vector (NEB).

The NS3 protein lacking the first region (Δ1) and alanine substitutions in the first identified region (amino acids 106-114) of the coding sequence of NS3 were introduced by PCR reactions using Phusion high-fidelity DNA polymerase (Bioké, Leiden, the Netherlands) and the region specific primer in combination with one of the primers 1-5 (Table 3-1). For alanine substitutions in the second identified region (amino acids 167-176) or to construct the NS3 protein lacking the second region (Δ2) the region specific primer was used in combination with one of the primers 6-13 (Table 3-1). Alanine substitutions were confirmed by sequencing reactions on all constructs and are indicated in bold in the primer sequences.

Table 3-1: Primers and templates used for the construction of single, double and triple alanine substitution within the NS3 coding sequence

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 c 3'	MBP-NS3 wildtype
2 E110A	5' ccg agg ctt tag t**gc** aac aaa ata ctc 3'	MBP-NS3 wildtype
3 L111A	5' ccg agg ctt t**gc** ttc aac aaa ata ctc 3'	MBP-NS3 wildtype
4 K112A	5' ccg agg c**gc** tag ttc aac aaa ata ctc 3'	MBP-NS3 wildtype
5 P113A	5' ccg ag**c** ctt tag ttc aac aaa ata ctc 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 c**gc** gct agg aga tct gta taa 3'	MBP-NS3 wildtype
8 K174A	5' atg ttt c**gc** ctt gct agg aga tct gta taa 3'	MBP-NS3 wildtype
9 K175A	5' atg t**gc** ctt ctt gct agg aga tct gta taa 3'	MBP-NS3 wildtype
10 K174/175A	5' atg t**gc** c**gc** ctt gct agg aga tct gta taa 3'	MBP-NS3 K175A
11 K173/175A	5' atg t**gc** ctt c**gc** gct agg aga tct gta taa 3'	MBP-NS3 K175A
12 K173/174A	5' atg ttt c**gc** c**gc** gct agg aga tct gta taa 3'	MBP-NS3 K174A
13 K173-175A	5' atg t**gc** c**gc** c**gc** gct agg aga tct gta taa 3'	MBP-NS3 K174A

Entry clones were generated by introducing attB1 and attB2 sites to the MBP-NS3 coding sequences by PCR using the bacterial expression plasmids described above, pDONR207 (Invitrogen) and BP clonase (Invitrogen) according to manufacturer's recommendations. For *A. tumefaciens* infiltration experiments expression clones were generated using the produced entry clones, the pK2GW7 destination vector (Karimi *et al.*, 2002) and LR clonase (Invitrogen) according to manufacturer's recommendations.

Agrobacterium tumefaciens infiltration

A. tumefaciens infiltration experiments were performed as described previously (chapter 2). Detection of MBP-NS3 proteins was performed by Western blotting using equal amount of total protein loaded, primary anti-MBP antibodies (NEB), alkaline phosphatase conjugated secondary antibodies and NBT-BCIP as substrate (Roche) according to manufacturer's recommendations.

Northern blot analysis

RNA was isolated from *A. tumefaciens* infiltrated plant material as described previously (Bucher *et al*., 2004). Enrichment of small RNA was essentially performed as described (Hamilton & Baulcombe, 1999). In total 7 µg small RNA was loaded onto an 16%, 1x TBE denaturing gel, electroblotted onto Hybond N^+ (Pharmacia-Biotech) and hybridised overnight at 50°C in hybridisation buffer [1 mM EDTA, 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 7% (wt/vol) SDS] using a GFP specific probe. For mRNA blots 10 µg total RNA was separated in a denaturing formaldehyde-agarose gel, transferred onto Hybond N^+ (Pharmacia-Biotech) and hybridised overnight at 50°C in hybridisation buffer [1 mM EDTA, 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 7% (wt/vol) SDS] using a GFP specific probe. The used probes were internally labelled by PCR and α-^{32}P dCTP. Following hybridisation, blots were washed briefly with 2xSSC, 0.2% (wt/vol) SDS, 2x 20 minutes with 2xSSC, 0.2% (wt/vol) SDS and 1x 20 minutes with 1xSSC, 0.1% (wt/vol) SDS at 50°C. Blots were exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

Recombinant protein expression

The RHBV NS3 proteins were expressed from BL21 DE3 according to manufacturer's recommendations and purified as described previously (chapter 2).

Electrophoretic mobility shift assay

Bacterial expressed MBP-NS3 and ^{32}P-labelled siRNAs were incubated and separated as described previously (chapter 2).

Acknowledgements

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Chapter 4

The NSs protein of tospoviruses suppresses RNA silencing pathways by binding a broad size range of dsRNA molecules

In response to antiviral RNA silencing, plant viruses encode proteins to suppress this host defence mechanism. Most of the suppressor proteins studied so far operate by specifically binding small interfering RNAs. Here it is shown that the NSs RNA silencing suppressors of tospoviruses also bind longer double stranded RNAs. This enables these viruses not only to sequester siRNAs prior to incorporation into the RNA induced silencing complex, but also to inhibit Dicer cleavage of longer double stranded RNA, thereby inhibiting RNA silencing at an earlier stage. It is also demonstrated that NSs interferes with the generation and function of miRNAs by binding dsRNA precursors of functional micro RNAs. Thus, tospoviral NSs proteins have a dual activity in suppressing siRNA- and miRNA-mediated RNA silencing pathways by inhibiting not only small RNA incorporation into RNA induced silencing complexes, but also by preventing Dicer processing of longer dsRNA.

Introduction

In plants, RNA silencing consists of distinct but overlapping pathways in which small interfering (si)RNAs and micro (mi)RNAs are key players (Mallory & Vaucheret, 2006). siRNAs arise from several endogenous sources and depend on the specific action of RNA dependent RNA polymerase (RDR) and Dicer-like (DCL) proteins (Vaucheret, 2006). Infection of a plant by a virus leads to the accumulation of 21 nt virus-specific siRNAs (Molnar *et al.*, 2005; Silhavy & Burgyan, 2004) and can generate a second class of 24-26 nt siRNAs that are proposed to travel ahead of the invading virus, programming the RNA silencing machinery for destruction of the advancing virus (Hamilton *et al*., 2002; Tang *et al*., 2003). The 21 nt siRNA molecules (Hamilton & Baulcombe, 1999) arise from perfectly complementary double stranded (ds)RNAs that are recognised and cleaved by DCL4, an RNase-III-type nuclease of the Dicer family (Deleris *et al.*, 2006; Dunoyer *et al.*, 2005; Hamilton & Baulcombe, 1999). One strand of the siRNA duplex serves for the structured assembly of the RNA induced silencing complex (RISC) and the subsequent sequence specific degradation of a complementary mRNA. In the active RISC complexes members of the Argonaute (AGO) protein family form the catalytic core for this slicing activity (Baumberger & Baulcombe, 2005).

In contrast to siRNAs, miRNA molecules arise from long host-encoded RNA transcripts that are first processed in the nucleus and subsequently exported to the cytoplasm. Further processing releases a miRNA/miRNA* duplex of which, after unwinding and strand separation, the miRNA strand is loaded into RISC, while the miRNA* is rapidly degraded (Li *et al.*, 2005). miRNAs are mainly involved in directing the repression of genes important for development (Jones-Rhoades & Bartel, 2004; Rhoades *et al.*, 2002) by either guiding RNA slicing activity or translational repression by miRISC complexes (Bartel, 2004).

As a response to antiviral RNA silencing, most plant viruses encode specific silencing suppressor proteins to combat this host response (Voinnet, 2005). Many of the studied suppressor proteins

have been shown to bind siRNA molecules, preferentially those of 21 nt in length (Lakatos *et al*., 2006; Merai *et al*., 2006). Interference with essential protein components of the RNA silencing machinery, such as DCL or AGO, has recently been reported for *Red clover necrotic mosaic virus* (RCNMV, p27 and p88) and *Cucumber mosaic virus* 2b (CMV) and poleroviral p0 proteins (Pazhouhandeh *et al.*, 2006; Takeda *et al*., 2005; Zhang *et al.*, 2006). The expression of viral suppressor proteins in *Arabidopsis thaliana* has been shown to inhibit miRNA activities resulting in developmental defects (Chapman *et al*., 2004; Dunoyer *et al*., 2004).

For *Tomato spotted wilt virus* (TSWV, genus *Tospovirus*) the ~52.5 kDa NSs protein represents the viral RNA silencing suppressor (Bucher *et al.*, 2003; Takeda *et al.*, 2002), earlier reported to be a determinant of symptom severity of tospoviruses (Kormelink *et al*., 1991). So far, the mode of action of NSs has remained unclear. Like the *Rice hoja blanca tenuivirus* (RHBV; see chapters 2 and 3), TSWV and other tospoviruses replicate in both plants and in their insect (thrips) vectors (Wijkamp *et al.*, 1993). Similar to the RHBV RNA silencing suppressor NS3, the suppressor activity of NSs is therefore likely to be operational against antiviral RNA silencing in both the plant and insect part of the viral lifecycle. The ability of the NSs protein to interfere with the RNA silencing pathway has been further investigated in this chapter by determining which size classes of dsRNA this suppressor may bind. Furthermore, its RNA silencing suppressor activity was studied using a Dicer-mediated dsRNA cleavage assay.

Results

Tospoviral NSs binds long dsRNA and siRNA in vitro

The TSWV NSs protein has been shown to represent the viral RNA silencing suppressor using the agro-infiltration assay (Bucher *et al.*, 2003; Takeda *et al.*, 2002), but its mode of action has hitherto remained unknown. To reveal how TSWV NSs exerts its suppressor function, purified bacterially expressed HIS-tagged NSs protein was incubated with radiolabelled dsRNA molecules and separated by native acrylamide gel electrophoresis. Two size classes of dsRNA were tested, i.e. 21 nt ds siRNA ("siRNA") and 114 nt long dsRNA ("long dsRNA").

With increasing NSs concentration, both NSs-long dsRNA (Fig. 4-1A) and NSs-siRNA (Fig. 4-1B) complexes could be visualised in electrophoretic mobility shift assays (EMSA), indicating that TSWV NSs is capable of binding both siRNA and long dsRNA. The dissociation constants (K_d) of NSs for the long dsRNA in EMSA was calculated to be 42.8±1.8 nM, and that for siRNA 200.49±28.5 nM (Fig. 4-1C), indicating a strong affinity to both types of dsRNA.

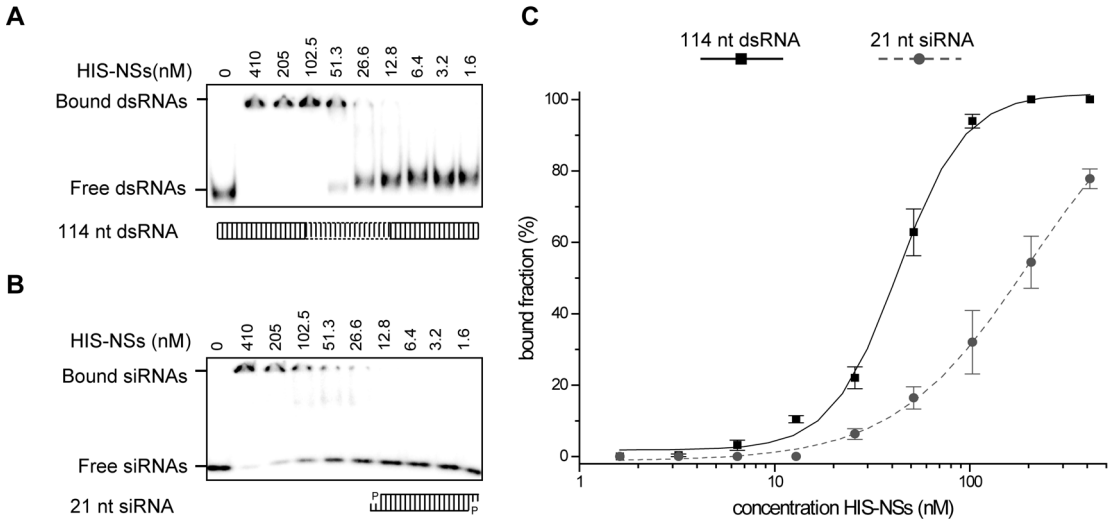

Figure 4-1: Affinity of TSWV NSs for dsRNAs. A dilution serie of HIS-tagged TSWV NSs (410-1.6 nM) was incubated with 100 pM of ^{32}P-labelled 21 nt siRNA (**A**) or (114 nt) long dsRNA (**B**) for 20 minutes, then loaded onto a 5% native gel. The K_d values were determined by plotting the bound RNA fraction as a function of the HIS-NSs concentration (**C**). The first lanes in panel A and B contain only dsRNAs and elution buffer used for protein purification.

Having found the potential inhibitory function of the TSWV RNA silencing suppressor, i.e. binding both siRNAs and long dsRNAs, it was investigated whether this was a general property of the viruses within the genus *Tospovirus*. As it proved extremely difficult to obtain sufficient quantities of soluble bacterial-produced NSs proteins, this was performed by incubating crude extracts of plants infected with a range of tospovirus species (i.e. TSWV, GRSV, INSV, IYSV, TCSV or TYRV; for abbreviations see the materials and methods section) with radiolabelled dsRNA molecules followed by analysis on native gels, similar to earlier reports with other viruses (Merai *et al*., 2006). Infected plant extracts containing the TSWV NSs -studied above in purified form- was included as a positive control. The EMSA results showed that for all viruses tested the

infected leaf extracts were able to bind siRNA (Fig. 4-2B), yet significant binding to long dsRNA was only observed in case of GRSV and INSV and oddly, only to a minor extent for TSWV (Fig. 4-2A).

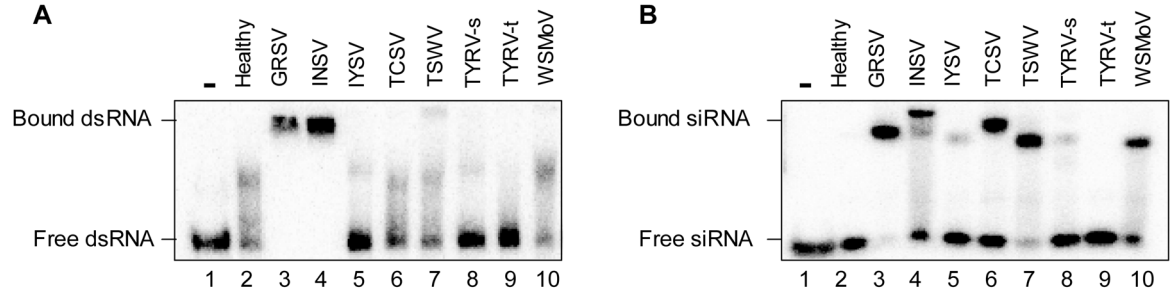

Figure 4-2: Tospoviral activity to bind dsRNA molecules. Systemically infected *N. benthamiana* extracts (GRSV, INSV, IYSV, TCSV, TSWV, TYRV-s, TYRV-t and WSMoV) were incubated with 114 nt dsRNA (**A**) or 21 nt siRNA (**B**) for 20 minutes, then loaded onto a 5% native gel. The first lane in panels A and B contains only dsRNA and extraction buffer, the second lane mock-infected uninfected plant extract and dsRNA.

The differences in intensities of the shifted bands could in part be caused by differences in NSs concentrations within the extracts used. This possibility is substantiated by the observation that purified TSWV NSs binds long dsRNA with high affinity (Fig. 4-1A), whereas TSWV-infected leaf extract shifts dsRNAs only weakly (Fig. 4-2A). The binding preferences of the most obvious long dsRNA binders, INSV and GRSV, was further investigated. With increasing dilution of INSV- and GRSV-infected plant extract, binding to the long dsRNA species was lost at ~0.13 µg total protein in the reaction mixture, whereas binding to the 21 nt siRNA molecules was lost at ~0.03 µg indicating a higher affinity for the shorter dsRNA molecules (Fig. 4-3, panels A-D).

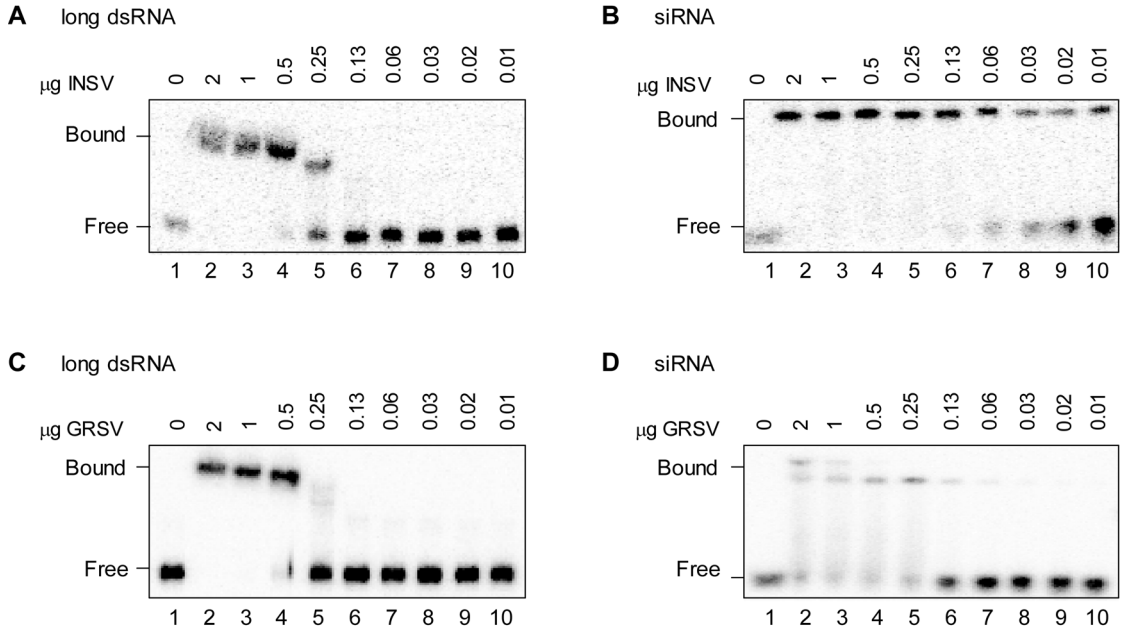

Figure 4-3: NSs suppressors of tospoviruses show binding to long dsRNA and siRNA molecules. Electrophoretic mobility shift assays using a serial dilution series of systemically infected *N. benthamiana* extracts (representing 2 µg - 0.01 µg of protein). Extracts were incubated with RNA for 20 minutes at room temperature, then loaded onto a 5% native gel. INSV (panels **A** and **B**) or GRSV (panels **C** and **D**) infected *N. benthamiana* extracts were incubated with 114 nt dsRNA (panels **A** and **C**) or 21 nt siRNA (panels **B** and **D**). The first lane in all panels contains only dsRNA and extraction buffer, the second lane mock-infected healthy plant extract and dsRNA.

Hence, the lack of long dsRNA binding by the other tospoviruses may indeed be explained by relatively low NSs titres in the infected extracts. This was not further investigated due to lack of specific antisera to most of these proteins. The positive outcome for the tospoviruses TSWV, GRSV and INSV, however, prompt us to assume that binding long dsRNA species likely is a generic property of the NSs proteins of tospoviruses.

NSs can interfere with Dicer-mediated dsRNA processing in vitro

In virus-infected plants, virus-specific siRNA molecules arise from longer dsRNA molecules or double-stranded regions in the viral genome that are being processed by DCL proteins (Molnar *et al*., 2005). If tospoviral NSs is indeed able to sequester long dsRNA species, interference with Dicer-mediated dsRNA processing is likely to occur. This was tested for both GRSV and INSV. For this purpose *Drosophila* embryo extract was used and processing of a 114 nt dsRNA into 21 nt siRNAs was monitored in the presence and absence of extracts from uninfected and INSV or GRSV infected plants. Cleavage of the larger dsRNA into siRNAs was readily detected after addition of embryo extract or in the presence of extracts of uninfected plants. Upon addition of GRSV or INSV infected plant material at the highest extract concentration used (corresponding with 2 µg total plant protein per 10 µl reaction), production of siRNAs decreased significantly (Fig. 4-4, lanes 5 and 7) indicating the ability of the GRSV and INSV silencing suppressors to interfere with Dicer cleavage of dsRNA. Diluting the added infected plant material could reduce this effect (Fig. 4-4, lanes 6 and 8).

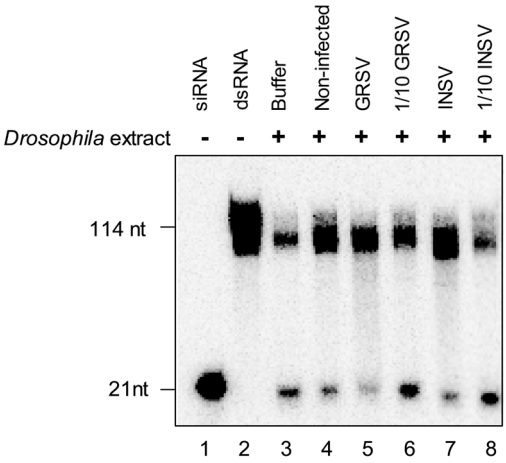

Figure 4-4: Tospovirus-induced inhibition of Dicer-mediated dsRNA cleavage. Extracts of GRSV- or INSV-infected *N. benthamiana* leaves (indicated GRSV and INSV, in 2 concentrations) were mixed with 114 nt dsRNA and *Drosophila* embryo extract, incubated for 60 minutes and RNA loaded onto a 8% denaturing gel. Lanes 1 and 2: untreated 21 nt siRNA and 114 nt dsRNA as size markers, lane 3: positive control reaction with 114 dsRNA and lane 4: negative control using extract of mock-infected *N. benthamiana*.

NSs interferes with miRNA maturation

To investigate whether the tospoviral NSs suppressor proteins bind miRNA duplexes and pre-miRNAs, and could also potentially interfere with miRNA maturation in the plant, RNA was isolated from TSWV-infected plant material and assayed for the presence of miRNA171c or

miRNA171c*. In previous publications miRNA171c was shown to target mRNAs coding for SCARECROW-like transcription factors in *A. thaliana* (Sunkar & Zhu, 2004; Xie *et al.*, 2005a) and is an abundant, well detectable miRNA species in *N. benthamiana* leaves. Interference by the suppressor proteins of potyviruses, tombusviruses and closteroviruses showed to prevent the dissociation of the miRNA/miRNA* duplex and subsequent RISC loading of the miRNA strand and inhibit target cleavage in plants (Chapman *et al*., 2004; Dunoyer *et al*., 2004). In these reports the presence of the miRNA/miRNA* duplexes or even the mere detectability of the miRNA* strand, normally rapidly degraded after strand separation, is regarded indicative for a direct interaction of the viral suppressor with miRNA/miRNA* duplexes. This interaction prevents strand separation, RISC incorporation of the miRNA strand and miRNA* strand degradation. Indeed, both miR171c and miRNA171c* were readily detected using locked nucleic acid (LNA)-based miR171c/c* strand-specific probes (Valoczi *et al.*, 2004) in RNA samples extracted from tospovirus-infected plant material (Fig. 4-5A), whereas in uninfected plants only the miR171c strand could be detected. The interaction of tospoviral NSs with miRNA molecules was further confirmed by EMSA experiments using pre-miRNA (Fig. 4-5B) and miRNA molecules (Fig. 4-5C).

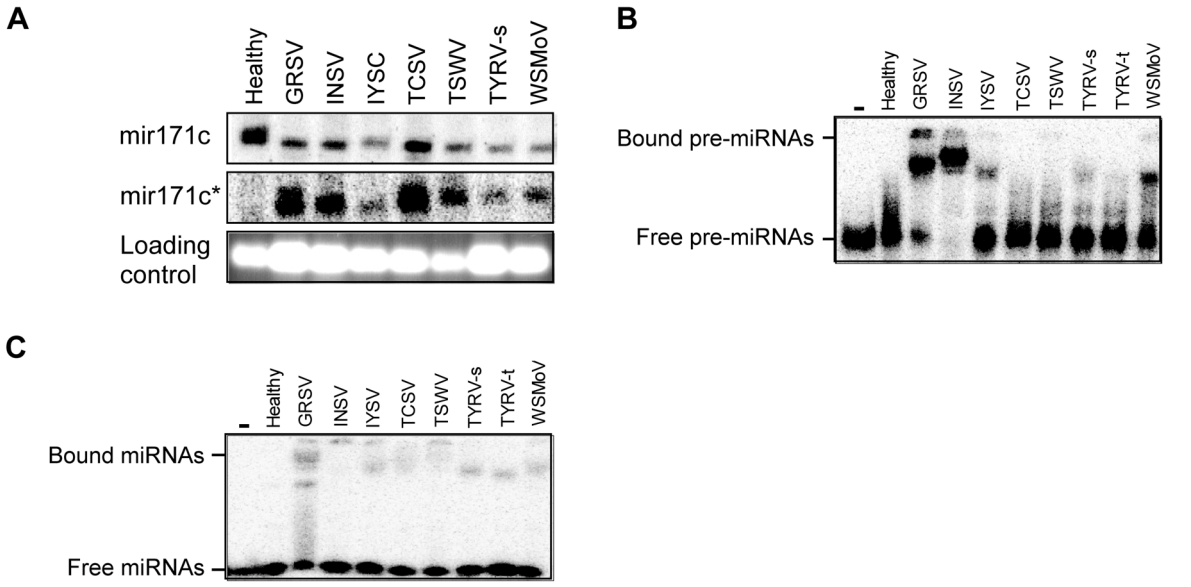

Figure 4-5: The NSs proteins of tospoviruses prevent miRNA duplex unwinding. Northern blot analysis of small RNA isolated from systemically infected *N. benthamiana* leaves using radiolabelled LNA-based probes to detect miRNA171c and miRNA171c* sequences (**A**). Systemically (GRSV, INSV, IYSV, TCSV, TSWV, TYRV-s, TYRV-t and WSMoV) infected *N. benthamiana* extracts were incubated with pre-miRNA (**B**) or 21 nt miRNA (**C**) for 20 minutes and loaded onto a 5% native gel. The first lane in panels B and C contains only dsRNA and extraction buffer, the second lane contains mock-infected healthy plant extract and dsRNA.

Discussion

High affinity binding to siRNA molecules seems to be a common strategy of plant viruses to counteract RNA silencing. Of the approximately 20 plant viral suppressors analysed in more detail so far, 11 have been shown to bind dsRNA (Silhavy & Burgyan, 2004; chapter 1). For most of these suppressors this property was found to be limited to binding siRNAs, but not to longer dsRNAs, as observed for e.g. RHBV NS3, *Tobacco etch virus* (TEV) HC-Pro, *Beet yellows virus* (BYV) p21, tombusviral p19 and *Peanut clump virus* p15 (Lakatos *et al.*, 2006; Merai *et al.*, 2006). Here it is shown that the RNA silencing suppressor protein NSs of tospoviruses is able to bind both siRNA and long dsRNA with high affinity, potentially enabling these viruses to block RNA silencing at two different stages. So far, only within a single other plant viral taxon, i.e. the *Tombusviridae*, such a dual inhibitory activity has been reported; notably for *Turnip crinkle virus* (TCV) p38 and *Aureusvirus* p14 (Merai *et al.*, 2006; Merai *et al.*, 2005).

Although the comparative analyses using extracts of *N. benthamiana* infected with a series of tospoviruses only scored positive for long dsRNA binding in case of GRSV and INSV, the negative score for the other five species tested may be explained as a concentration effect. When bacterial purified HIS-NSs of TSWV was used, longer dsRNA molecules were bound approximately four times more efficiently than siRNAs. This is in contrast with the EMSA experiment where GRSV and INSV plant-infected extracts were used, where the situation was reversed and the affinity for siRNAs was estimated to be four times higher than for longer dsRNAs. Confirming the effective binding of NSs to longer dsRNA molecules, the extracts of INSV- and GRSV-infected leaves showed an inhibitory effect on Dicer mediated processing of dsRNA molecules into siRNAs. Since NSs concentrations used in these Dicer cleavage assays are not precisely known, the effect on dsRNA processing could only be directly compared in dilutions of virus-infected extracts. The variation in experimental outcome of EMSA experiments using the bacterial purified HIS-NSs or plant-infected extracts could be due to differences in assay conditions as have also been described for the BYV p21 protein (Merai *et al.*, 2006; Ye & Patel, 2005). Considering the large quantities of small RNAs in infected plants, it should also be realised that in infected leaf extract a considerable part of the NSs protein is likely to be pre-loaded with viral siRNA, excluding these molecules from binding to radiolabelled dsRNA molecules added in the assay.

The accumulation of miRNA171c/miRNA171c* species in tospovirus-infected *N. benthamiana* material indicates that NSs also binds dsRNA *in planta*. The miRNA171c is predicted to target mRNAs coding for SCARECROW-like transcription factors (Sunkar & Zhu, 2004; Xie *et al.*, 2005a) and therefore a clear role for binding to the miRNA171c sequences by NSs can not be reasoned. However, other miRNA duplexes are likely to be sequestered by NSs as well as they share structural similarities to the miRNA171 duplex feature. Binding to miRNAs might induce viral symptoms and could serve to establish an advantageous environment for virus replication and accumulation. Indeed, the constitutive expression of NSs in transgenic *Arabidopsis* plants led

to grow aberrations which may be attributed to interference with miRNA-guided developmental gene regulation (results not shown).

Like tenuiviruses, tospoviruses are transmitted by insects and are capable to replicate in their insect vector (Wijkamp *et al.*, 1993). Both types of viruses are therefore likely to be a target for antiviral RNA silencing in plants as well as insects. By sequestering long as well as short dsRNA molecules, tospoviruses are able to doubly interfere with the core of the antiviral silencing pathway in both the plant and insect hosts. Size-independent binding to dsRNA enables the virus to interfere with Dicer-mediated processes, assembly of active RISC complexes and the amplification of the silencing signal.

Tospoviruses are the plant-infecting members within the large *Bunyaviridae* family, which is further restricted to animals. Comparing functions related to the suppression of antiviral activity among the members of the *Bunyaviridae* reveal a complicated picture. The phleboviral NSs protein of *Rift valley fever virus* (RVFV), reported to interfere with the basic transcription machinery of the host cell (Billecocq *et al.*, 2004), was tested in the *Agrobacterium tumefaciens* transcient assay for its ability to suppress RNA silencing of a GFP reporter construct in plants. In this system no suppressor action was observed (Bucher, 2006) and also in tick cells, no inhibition of RNA silencing was observed (Garcia *et al.*, 2006b). It is still a matter of debate whether for another animal bunyavirus, i.e. *La crosse virus* (LACV), the NSs protein is a suppressor of RNA silencing (Blakqori *et al.*, 2007; Soldan *et al.*, 2005). At present, an unambiguous antiviral activity of the ubiquitous RNA silencing machinery has not been established in vertebrate systems. Yet a number of putative viral RNA silencing suppressors i.e. animal viral proteins which act as such in plant and insect derived assays, e.g. *Influenza virus A* NS1 and *Vaccinia virus* E3L (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004; Garcia *et al.*, 2006b; Li *et al.*, 2004) have been reported to suppress RNA silencing in mammalian cells (Haasnoot *et al.*, 2007).

Besides being a substrate for Dicer, long dsRNA molecules of cellular or viral origin have been shown to activate the dsRNA-dependent protein kinase, which has been indicated to play a central role in antiviral defence mechanisms in mammals (Garcia *et al.*, 2006a). Sequestering of these longer dsRNA molecules would therefore be advantageous for human viruses because both the putative antiviral role of RNA silencing (RNAi) and the production of interferon can be prevented simultaneously. The ability of the tospoviral NSs to bind dsRNA size-independently is therefore also intriguing in an evolutionary context and may further support the hypothesis that the plant-infecting tospoviruses have evolved from their animal-infecting relatives (Goldbach & Peters, 1996).

Materials and Methods

Bacterial expression and purification of TSWV NSs

The coding sequence of TSWV NSs was PCR amplified introducing a BamHI site at the 5' end and a unique KpnI site at the 3' end. For the expression and purification of N-terminally His-tagged NSs protein, the PCR product was ligated into the bacterial expression vector pQE30 (Qiagen). The NSs protein was expressed from M15[pREP4] cells (Qiagen) according to manufacturer's recommendations. After induction for 3h at 37°C with 1 mM IPTG cells were harvested by centrifugation for 15 minutes at 4000 rpm (Sorvall GSA rotor) at 4°C. Cells were lysed by sonification on ice with 30s intervals for 3 times 30 seconds in lysis buffer [50 mM K_2PO_4, 400 mM NaCl, 100 mM KCl, 10% (vol/vol) glycerol, 0.15% (vol/vol) Triton X-100, 20mM imidazole pH 7.8]. The soluble fraction was recovered by centrifugation at 9000g for 30 minutes at 4°C. Recombinant protein was purified using Ni Sepharose 6 Fast Flow (GE Healthcare) and eluted with 2.5 packed bed volumes (PBV) elution buffer [50mM NaH_2PO_4, 300mM NaCl, 200 mM imidazole, 10% (vol/vol) glycerol] after washing with 15 PBV lysis buffer. Protein aliquots were flash frozen in liquid nitrogen and stored at -80°C until use. Protein concentrations of elution fractions were determined using the standard procedure of the Bio-Rad protein assay according to manufacturer's recommendations and the purification process analysed by SDS-PAGE and subsequent staining with Coomassie brilliant blue.

Preparation of virus infected plant extracts

Groundnut ringspot virus (GRSV), *Impatiens necrotic spot virus* (INSV*), Iris yellow spot virus* (IYSV), *Tomato chlorotic spot virus* (TCSV), TSWV, *Tomato yellow ring virus* soybean and tomato isolates (TYRV-s and TYRV-t) and *Watermelon silver mottle virus* (WSMoV) were mechanically inoculated on *N. benthamiana* plants and extracts prepared from heavily infected systemic leaves, as described previously (Merai *et al*., 2006) with modifications. Virus accumulation was confirmed by ELISA before preparation of virus-infected plant extracts. To prepare extracts, 1 g leaf tissue was ground in liquid nitrogen and 4 ml extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl and 1mM DTT) added. The total protein concentration was determined using the standard procedure of the Bio-Rad protein assay according to manufacturer's recommendations. Crude extracts were centrifuged twice at 15,000 x g for 15 minutes at 4°C. Extracts were flash frozen in liquid nitrogen and stored at -80°C until use.

dsRNA preparation

Double stranded RNA was generated using T7 RNA polymerase (Promega) and α-^{32}P CTP according to protocol using a gel purified (High Pure PCR purification kit; Roche) PCR template. The primers used introduced T7 RNA polymerase promoters at both ends of the eGFP specific PCR product. 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' for 114 nt dsRNA. Pre-miRNA was prepared using T7 RNA polymerase using two annealed primers as template: 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 transcription, reaction mixtures were incubated at 70°C for 10 minutes and cooled down to RT. Template was removed by treatment with DNase I and dsRNA gel purified from an 8% PAGE, 0.5x TBE native gel. Annealing of siRNA and miRNA molecules was performed as described previously (Haley *et al*., 2003).

Electrophoretic mobility shift assay

In a binding reaction, radiolabelled RNA (0.5 ng) was incubated with ~2 µg virus-infected plant extracts per 10 µl reaction and incubated for 20 minutes at RT. As controls RNA was loaded without virus-infected plant extract and RNA with mock-infected healthy plant extracts. Samples were loaded on a 5%, 0.5x TBE native PAGE gel, dried, exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

Dicer cleavage reactions

Drosophila embryo extract preparation was performed as described previously (Haley *et al*., 2003). In Dicer-mediated cleavage reactions embryo extracts were incubated for 60 minutes at 25°C in buffer (Haley *et al*., 2003), while 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 plant extract were incubated. Samples were deproteinised and RNA was analysed on a 8% denaturing polyacrylamide gel. After running, gels were dried, exposed to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

RNA gel blot analysis

RNA extraction of total RNA (Bucher *et al*., 2004) and enrichment of small RNAs (Hamilton & Baulcombe, 1999) was performed as described previously. In total 5-15 µg small RNA was loaded onto an 12%, 1x TBE denaturing gel, electroblotted onto Hybond N^+ (Pharmacia-Biotech) and hybridised overnight at 50°C in hybridisation buffer [1 mM EDTA, 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 7% (wt/vol) SDS] using locked nucleic acid probes (2 µg). Probes specific for miRNA171c or miRNA171c* (kindly provided by Dr. L. Lakatos, Agricultural Biotechnology Center, Gödöllő, Hungary) were labelled using polynucleotide kinase and γ-^{32}P ATP. Following hybridisation, blots were washed briefly with 2xSSC, 0.2% (wt/vol) SDS, 2x 20 minutes with 2xSSC, 0.2% (wt/vol) SDS and 1x 20 minutes with 1xSSC, 0.1% (wt/vol) 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% (wt/vol) SDS for 15 minutes and used for subsequent hybridisation experiments.

Statistical analysis

All *in vitro* Dicer assays and RNA binding experiments were performed in triplicate. The fraction of bound dsRNA as function of the concentration HIS-NSs was determined and the curve was best fitted to the indicated set of data with the computer program Microcal Origin 5.00. The average with standard error is shown.

Acknowledgements

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Chapter 5

Influenza virus A NS1 and RNAi: further evidence for its role in suppressing interferon-independent antiviral response

In eukaryotes RNA silencing serves as important mechanism for the control of developmental process through micro RNA action. In plants and insects, RNA silencing also has been demonstrated to serve as an important antiviral response, which is initiated by virus-specific double stranded RNA molecules. To counteract RNA silencing, plant and insect viruses encode silencing suppressor proteins. Also proteins of human viruses, associated with innate immunity, have recently been shown to act as RNA silencing suppressors in mammalian systems. This suggests that RNA silencing, besides its activity in gene regulation, could also serve as an antiviral response in mammalian cells. Thus far the NS1 protein of *Influenza virus A* was identified as RNA silencing suppressor using heterologous plant- and insect-based assays. To gain more insight in the mode of its suppressor action, the affinity of NS1 for dsRNA of different length and structure was investigated *in vitro*, revealing a Dicer inhibitory function of NS1 by high affinity substrate binding to longer dsRNA. In addition, NS1 was shown to enhance accumulation of both the homologous virus and a Tat-deficient *Human immunodeficiency virus* in infectivity studies. These observations contribute to the suggestion that the multifunctional protein NS1 may be involved in interfering with antiviral RNA silencing in mammalian cells.

Introduction

In plants and insects RNA silencing serves as an important antiviral defence response (Ding & Voinnet, 2007; Galiana-Arnoux *et al.*, 2006; van Rij *et al.*, 2006; Voinnet, 2001; 2005; Wang *et al.*, 2006). This response can be initiated by structured viral genomes, double stranded (ds)RNA viral replication intermediates, cytoplasmically replicating viruses or, in plants, dsRNA produced by RNA dependent RNA polymerase (RDR). By the action of Dicer-like enzymes these dsRNAs are processed into small interfering (si)RNAs, which guide both the assembly of the RNA induced silencing complex (RISC) and homologous target destruction. As a response, plant and insect viruses encode suppressor proteins (Voinnet, 2005). Many plant viral suppressor proteins have been identified and a handful has been studied in more detail. The majority of these suppressors displayed a size-selective siRNA binding (Lakatos *et al.*, 2006; Merai *et al.*, 2006) while only the p38 protein of *Turnip crinkle virus*, p14 protein of *Aureusvirus* and NSs of tospoviruses (chapter 4) showed a preference to binding longer dsRNA (Deleris *et al.*, 2006; Merai *et al.*, 2005). *Cucumber mosaic virus* 2b and *Red clover necrotic mosaic virus* p27 and p88 proteins appear not to bind any dsRNA at all, but interfere with protein components of the RNA silencing machinery (Takeda *et al.*, 2005; Zhang *et al.*, 2006).

In animal cells RNA silencing, also referred to as RNA interference or RNAi (Fire *et al.*, 1998), can be induced by endogenous or exogenous dsRNA molecules. A single Dicer enzyme processes the longer dsRNAs into 21 nt siRNAs (Bernstein *et al.*, 2001) prior to unwinding and loading of one strand of this duplex into RISC to guide sequence specific target recognition (Chendrimada *et al.*, 2005; Liu *et al.*, 2004; Martinez & Tuschl, 2004). For a number of

mammalian viruses, proteins with RNAi suppressor activity have been identified. Among these, the NSs protein of *La Crosse virus* (LACV) has been shown to be able to suppress RNAi induced by transfected siRNAs in mammalian cells (Soldan *et al.*, 2005), providing evidence that also vertebrate viruses can counteract RNAi. There are additional indications for an antiviral activity of the ubiquitous RNAi machinery in vertebrate systems, i.e. by using deficient Tat-minus *Human immunodeficiency virus-1* (HIV-1) mutants (Haasnoot *et al.*, 2007). The *Ebola virus* VP35, the *Vaccinia virus* E3L and *Influenza virus A* NS1 proteins were all shown to functionally complement the Tat silencing suppressor activity (Bennasser *et al.*, 2005) and rescue virus production of the HIV-1 Tat-deletion mutants. Yet, the impossibility to detect viral siRNAs in virus-infected vertebrate cells (Pfeffer *et al.*, 2004) has hitherto hampered unequivocal proof of an antiviral activity of RNAi in mammals.

In most, if not all, eukaryotic organisms RNA silencing also plays a crucial role in the regulation of gene expression and genome integrity. Genome-encoded small RNA molecules, micro (mi)RNAs, play an essential role in these gene regulation processes at the post transcriptional level (reviewed in Bartel, 2004; Carrington & Ambros, 2003; Herr, 2005). miRNAs arise from long primary (pri)-miRNA transcripts that are processed by the nuclear protein Drosha into precursor (pre)-miRNAs of ~70 nucleotides (Lee *et al.*, 2003). Drosha is accompanied by its dsRNA binding partner known as Pasha in *Drosophila* (Denli *et al.*, 2004; Han *et al.*, 2004) and DGCR8 in humans (Han *et al.*, 2004). Following export to the cytoplasm (Lund *et al.*, 2004), Dicer cleavage yields 21-24 nucleotide mature miRNA/miRNA* duplexes of which one strand determines the sequence specific RNA target recognition by miRISC (Bartel, 2004). In addition to their indisputable role in regulation of gene expression, miRNAs also play a role in antiviral defence by restricting virus accumulation (Lecellier *et al.*, 2005).

Demonstrating the existence of animal virus-encoded suppressors of RNAi is complicated by the fact that animal viruses often encode antagonists of the extracellular (Toll-like receptor-mediated) and intracellular (PKR/RIG-I/MDA-5-mediated) defence pathways, which often also bind dsRNA. A well studied example of such an antiviral response antagonist is the NS1 protein of *Influenza virus A*. NS1 is an extensively studied multifunctional protein which is also involved in translational initiation of viral mRNAs (Krug *et al.*, 2003), inhibition of processing and transport of host mRNA (Lu *et al.*, 1995) and preventing induction of type I interferons (IFN-α/β) (Garcia-Sastre *et al.*, 1998; Talon *et al.*, 2000; Wang *et al.*, 2000). Like RNAi, these defence pathways are initiated by dsRNA molecules and modulated by host factors that have been identified as binding partners of human Dicer (Chendrimada *et al.*, 2005; Haase *et al.*, 2005; Kok *et al.*, 2007; Lee *et al.*, 2006), e.g. the Tar binding protein (TRBP) and PACT (Gupta *et al.*, 2003).

The notion that NS1 acts as an RNA silencing suppressor in plants (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004) and insects (Li *et al.*, 2004) triggered us to investigate its proposed suppressor activity in more detail and in homologous animal cell systems. In this chapter the affinity of NS1 for dsRNA in size and structure was determined. Also the potential of NS1 to

inhibit Dicer mediated cleavage of long dsRNAs into siRNAs was investigated. Furthermore, the impact of NS1 was studied in comparative infectivity studies using both the homologous virus encoding a dysfunctional NS1 and a heterologous virus (HIV) deficient in its RNAi suppressor.

Results

NS1 preferentially binds long dsRNA of various origin

Previously, it was shown that the *Influenza virus A* NS1 protein binds dsRNA molecules, which was indicative for its interferon antagonistic function (Wang *et al.*, 2000) and RNA silencing suppressor activity in plants (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004) and insects (Li *et al.*, 2004). However, quantitative data and RNA structural requirements for dsRNA binding by NS1 are lacking (Chien *et al.*, 1997; Hatada & Fukuda, 1992; Liu *et al.*, 1997; Qian *et al.*, 1995). Replacement of the amino acids R35 and R38 was previously demonstrated to result in a NS1 protein dysfunctional for dsRNA binding (Bucher *et al.*, 2004; Wang *et al.*, 1999). This NS1 dsRNA-binding mutant, termed NS1rb was used as control in electrophoretic mobility shift assays (EMSA) to determine NS1 protein-RNA affinities (Fig. 5-1B and D).

Radiolabelled RNA molecules were incubated with a dilution serie of purified NS1 or NS1rb protein and protein-RNA complexes were resolved by native gel electrophoresis. The dissociation constant (K_d) of NS1 for dsRNA of 114 nt was calculated to be 1.1±0.03 nM (Fig. 5-1A and E), indicating a high binding affinity. Also dsRNA species of 400 nt were tested and showed a similar pattern as 114 nt dsRNA (data not shown). A significantly lower affinity was observed for 21 nt siRNA (Fig. 5-1C and E). The NS1rb protein showed no binding to 114 nt or 21 nt dsRNA molecules (Fig. 5-1B and D).

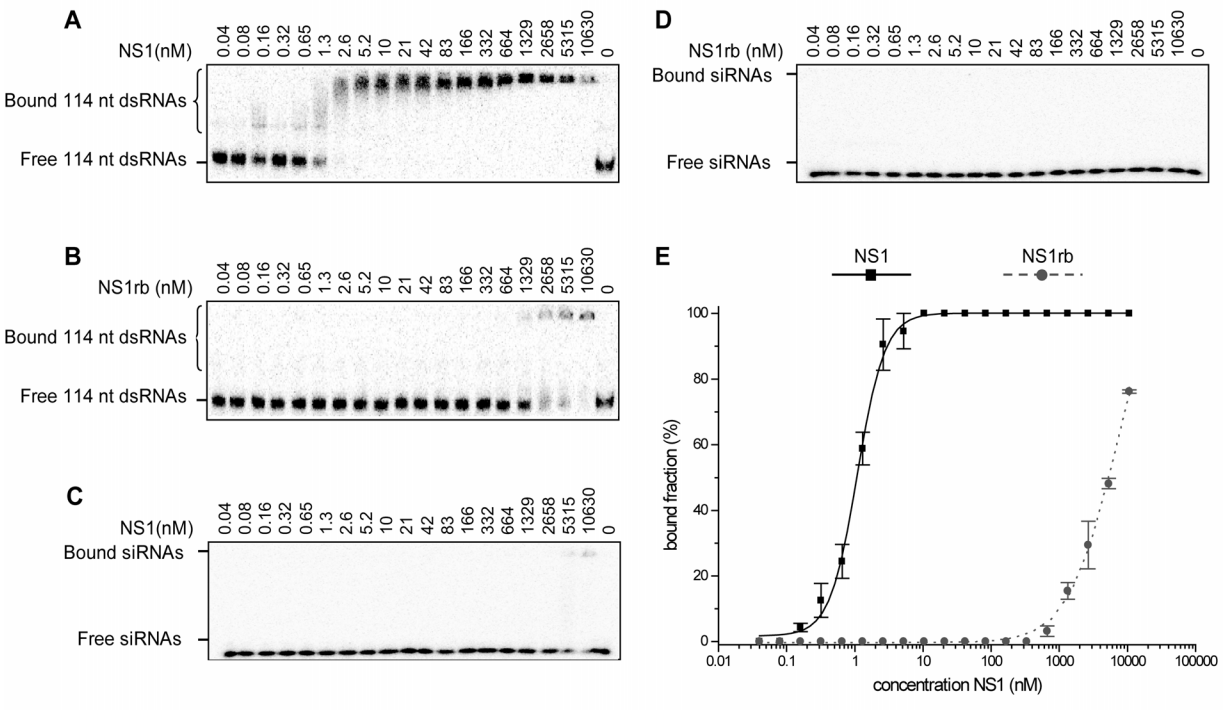

Figure 5-1: Affinity of NS1 and NS1rb for dsRNAs. A dilution serie of HIS-NS1 (**A** and **C**) or HIS-NS1rb (**B** and **D**) was incubated with 100 pM of ^{32}P-labelled 114 nt long dsRNA (**A** and **B**) or 21 nt siRNAs (**C** and **D**) for 20 minutes, then loaded onto a 5% native gel. The K_d values were determined by plotting the bound 114 nt dsRNA fraction as function of the HIS-NS1 concentration (**E**). The last lanes contain only dsRNAs and elution buffer used for protein purification.

The ability to bind to pre-miRNAs (77 nt) and miRNAs (21 nt) that differ structurally to perfect complementary dsRNA and siRNA molecules was tested in a similar set-up. NS1 bound pre-miRNA molecules with comparable affinity (Fig. 5-2A) as 114 nt dsRNA and like NS1rb (Fig. 5-2D) also NS1 showed a drastic decrease in affinity to miRNAs (Fig. 5-2C). These results showed that longer dsRNA molecules of different origin are preferentially bound by the NS1 protein, thereby potentially enabling NS1 to act as suppressor of RNA silencing by blocking Dicer-mediated processing of long (virus-derived) dsRNA into siRNAs or pre-miRNAs into miRNAs.

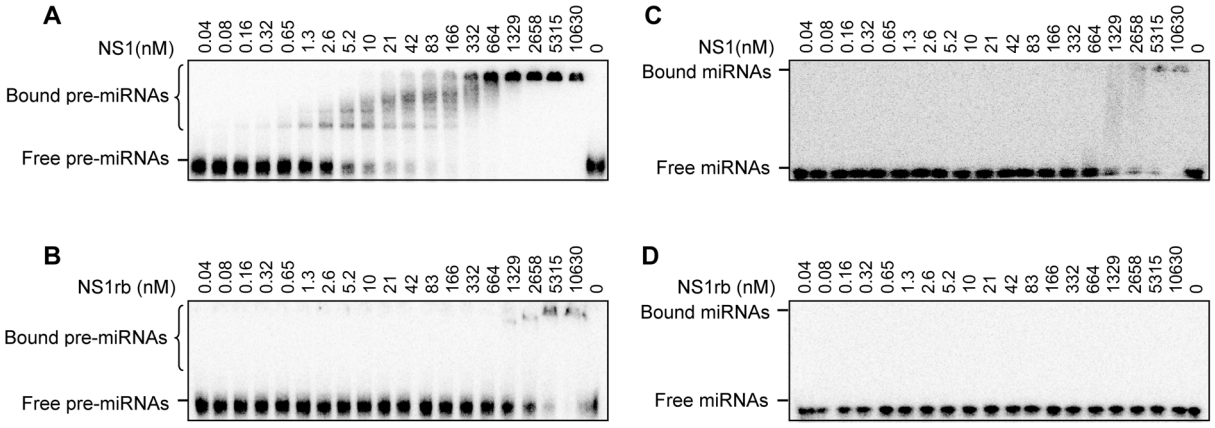

Figure 5-2: Affinity of NS1 and NS1rb for dsRNAs. A dilution serie of HIS-NS1 (**A** and **C**) or HIS-NS1rb (**B** and **D**) was incubated with 100 pM of ^{32}P-labelled pre-miRNA (**A** and **B**) or 21 nt miRNAs (**C** and **D**) for 20 minutes, then loaded onto a 5% native gel. The last lane in each panel contains only dsRNAs and elution buffer used for protein purification.

NS1 inhibits Dicer-mediated dsRNA cleavage in Drosophila extracts

To test whether NS1 indeed has the capacity to inhibit Dicer activity, thereby preventing the formation of siRNAs, the *Drosophila* embryo extract RNA silencing system was used. This system lends itself well for such studies as it allows interference with its endogenous Dicer activity (Bernstein *et al.*, 2001).

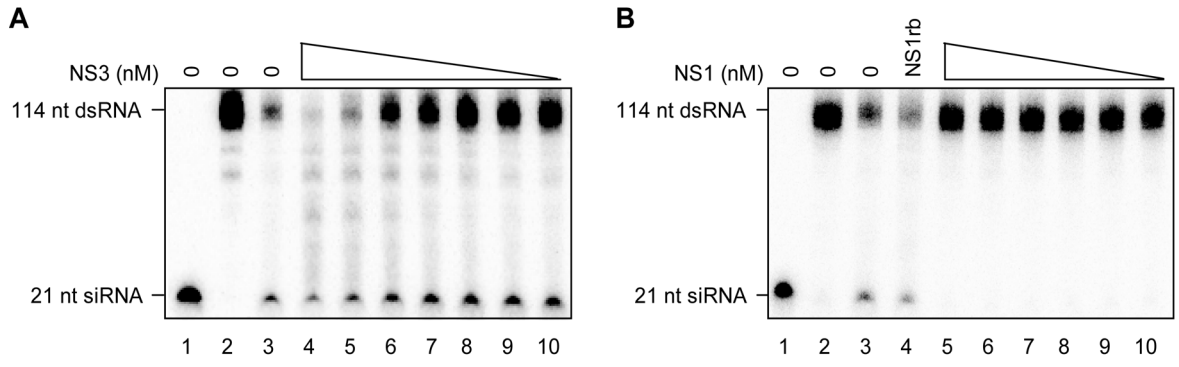

Figure 5-3: NS1 inhibition of Dicer-mediated dsRNA cleavage in Drosophila embryo extracts. As a negative control, purified bacterially expressed RHBV NS3, a plant viral RNA silencing suppressor that only binds short dsRNAs, was mixed with 114 nt dsRNA and Drosophila embryo extract, incubated for 60 minutes at 25°C and RNA loaded onto an 8% denaturing gel (**A**). Bacterial purified NS1 was mixed with 114 nt dsRNA and Drosophila embryo extract, incubated for 60 minutes at 25°C and RNA loaded onto an 8% denaturing gel (**B**). In both panels A and B lanes 1 and 2 contain respectively untreated 21 nt siRNA and 114 nt dsRNA as size markers and lanes 3 positive control reactions of embryo extracts with 114 dsRNA. Lane 4 in panel B: negative control using HIS-NS1rb.

Radiolabelled 114 nt dsRNA, *Drosophila* extract and purified NS1 protein were incubated for 1h at 25ºC and the generation of siRNAs visualised by denaturing gel electrophoresis. The formation of siRNAs was reduced with increasing concentration of NS1 showing that NS1 interferes with Dicer-mediated dsRNA cleavage (Fig. 5-3B). When instead of wildtype NS1, mutant NS1rb protein at the highest concentration was added, siRNAs accumulated to a level (Fig. 5-3B lane 4) comparable to those of the positive control (Fig. 5-3B lane 3), confirming that the RNA binding capacity of NS1, also essential for RNA silencing suppression activity, is responsible for Dicer inhibition. The *Rice hoja blanca tenuivirus* NS3 protein, a plant virus-encoded suppressor exclusively binding 21 nt small RNA molecules but not longer dsRNA (chapter 2), was included as negative control (Fig. 5-3A).

NS1 inhibits human Dicer-mediated dsRNA cleavage

In contrast to *Drosophila* (DCR1 and DCR2) and plants (DCL1-4) human cells encode only a single Dicer enzyme. To test the ability to interfere with human Dicer a similar approach was followed as for the *Drosophila* embryo extract. Again, NS1 was able to prevent Dicer-mediated dsRNA processing into siRNAs (Fig. 5-4A and B) showing its potential to act also as suppressor of RNAi components in a homologous system.

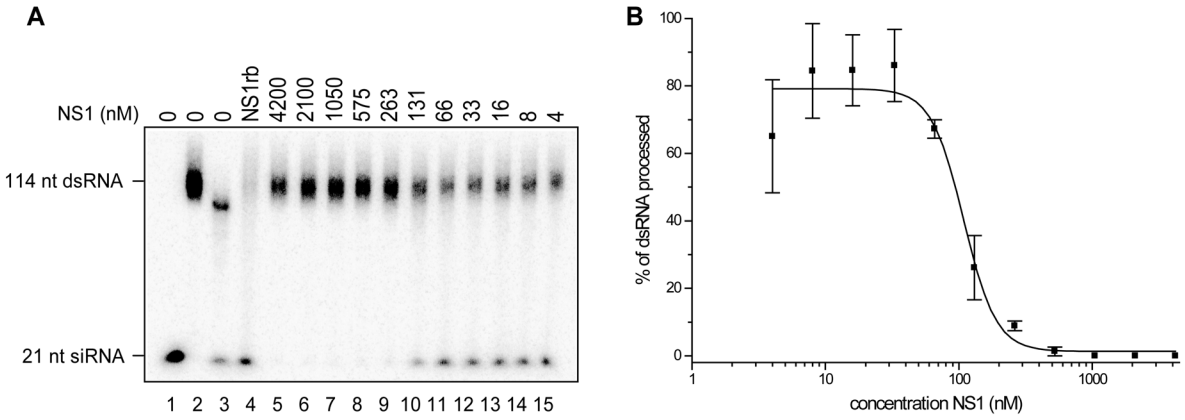

Figure 5-4: NS1 inhibition of human Dicer-mediated dsRNA cleavage. Purified NS1 was mixed with 114 nt dsRNA and *Drosophila* embryo extract, incubated for 60 minutes at 37°C and RNA loaded onto an 8% denaturing gel (**A**). Lanes 1 and 2: untreated 21 nt siRNA and 114 nt dsRNA as size markers, lane 3: positive control reaction with 114 dsRNA and lane 4: negative control using HIS-NS1rb. The percentage of processed dsRNA was plotted against the concentration NS1 (**B**).

The NS1 protein enhances virus accumulation

If RNA silencing indeed would have an effect on human *Influenza virus A* replication by Dicer inhibition, then an *Influenza virus A* expressing a dysfunctional NS1 protein (PR8-NS1rb) is likely to replicate slower and accumulate to lower levels compared to wildtype (PR8-NS1). Of course the additional activity of the multifunctional NS1, i.e. in antagonising the IFN pathway should be carefully ruled out. Therefore, the effect on the IFN pathway was monitored during infection studies. When Madin-Darby Canine Kidney (MDCK) cells and VERO cells were infected with PR8-NS1rb or PR8-NS1 virus stocks at similar MOI, viral titers of the NS1rb mutant virus

developed significantly slower than that of wildtype virus (Fig 5-5). Cells infected with PR8-NS1 or PR8-NS1rb did not show a difference in IFN production (personal communication B.G. van den Hoogen), this in contrast to viruses lacking the complete NS1 protein (Donelan *et al.*, 2003; Garcia-Sastre *et al.*, 1998). This suggests that the decreased replication level of PR8-NS1rb was solely caused by the loss of the Dicer inhibitory function.

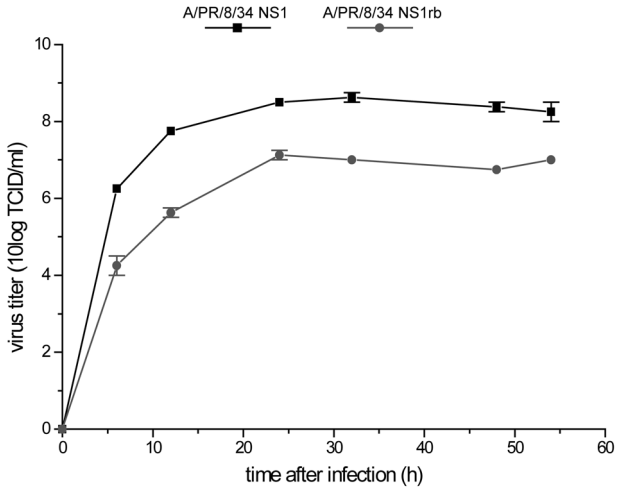

Figure 5-5: Replication kinetics of *Influenza viruses* (A/PR/8/34) expressing NS1 or NS1rb in MDCK cells. Supernatant of cells infected with 0.01 $TCID_{50}$/cell of PR8-NS1 and PR8-NS1rb viruses were harvested at 6, 12, 24, 32, 48 and 54 hours after infection. Geometric mean titers were calculated from two independent experiments.

Indications that small RNAs play a role in mammalian virus infections

Unlike in plants, virus specific siRNAs have thus far not been identified in mammalian cells. A possible explanation is that the processing of viral specific siRNAs by Dicer is inhibited by the long dsRNA binding activity of the suppressor protein encoded by the viruses studied so far. The finding that PR8-NS1rb accumulated to lower levels compared to PR8-NS1 and NS1 activity correlated with its ability to bind dsRNA, made this a good starting point to search for virus specific small RNA molecules in PR8-NS1rb infected cells. Unfortunately, no conclusive results were obtained, as minor amounts of random breakdown products of influenza viral genomic and/or mRNAs seemed to over shade all analyses in search of low amounts of viral siRNAs.

Indirect evidence for an essential role of small RNAs (either siRNAs or miRNAs) came from our recent studies using the NS3 suppressor of RHBV that only binds these small RNAs and not long dsRNA. Previously, HIV-1 was employed as a model system to identify novel RNA silencing suppressors by functional complementation of the HIV-1 silencing suppressor Tat. The *Ebola virus* VP35 protein as well as the previously identified silencing suppressors *Influenza virus A* NS1 and *Vaccinia virus* E3L rescued the production of a Tat minus HIV-1 variant (Haasnoot *et al.*, 2007). When next to NS1 also NS1rb was used to complement Tat function *in trans*, only the NS1 protein showed to complement for HIV-1 Tat function (Fig. 5-6). Interestingly, also the RHBV NS3 protein, but not the dysfunctional NS3 (K173-175A) mutant (see chapter 3), rescued HIV-1 accumulation in HEK-293T cells (Fig. 5-6). This indicates that longer or shorter dsRNA binding is

indispensable for HIV-1 Tat complementation activity and that small dsRNAs e.g. siRNAs or miRNAs have an effect on viral replication and accumulation.

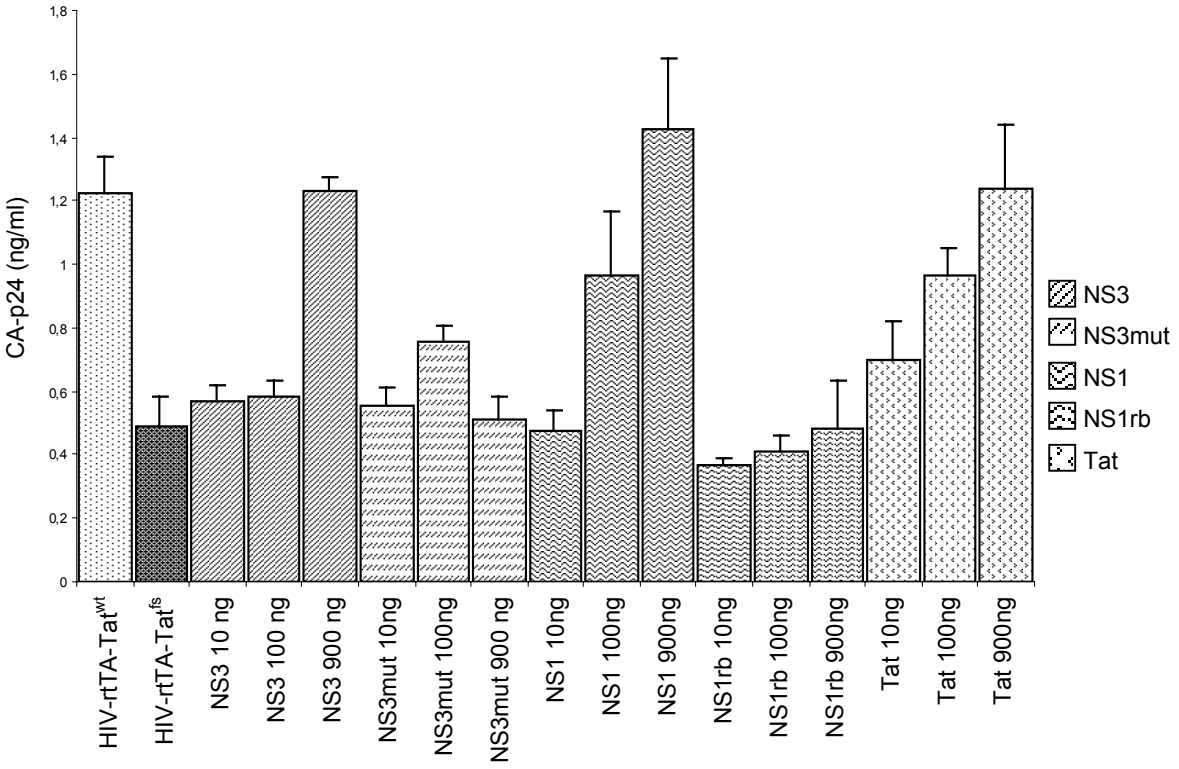

Figure 5-6: Functional complementation of the Tat silencing suppressor function by NS1 and NS3. HEK293T cells were transfected with HIV-rtTA-Tat^{wt}, HIV-rtTA-Tat^{fs}, and expression plasmids for, NS3, NS3mut, NS1, NS1rb and Tat (wt) (10,100 and 900 ng). Virus production was determined 3 days post transfection.

Discussion

While antiviral RNA silencing is a well described mechanism in plants and insects, to date, despite the presence of all necessary components, no clear antiviral role has been ascribed for this mechanism in mammalian cells. If RNA silencing would have antiviral activity in mammalian cells, in analogy to plant and insect systems, it can be anticipated that viral replication would lead to the accumulation of virus specific siRNAs. And, in turn as a response, human viruses would encode suppressors of RNA silencing and interfere by either binding dsRNA or interact with RNA silencing components. Putative suppressor proteins have indeed been identified for a handful of animal viruses that were functional in plant- and insect-derived RNA silencing inhibition assays (Bucher *et al.*, 2004; Garcia *et al.*, 2006b; Li *et al.*, 2004). Yet, virus specific siRNAs have not been detected in infected mammalian cells (Pfeffer *et al.*, 2004), this in contrast to virus-infected plants where viral siRNAs accumulate simultaneously with viral genome replication. The difference may lie in the presence of an amplification step of RNA silencing in plants through RDR action (Mourrain *et al*., 2000), whereas mammalian cells seem to lack this activity. Another difference may be that while (most) plant viral suppressors have adopted a size-selective binding to 21 nt siRNAs (Lakatos *et al.*, 2006; Merai *et al.*, 2006) the animal viruses mostly encode suppressors, exemplified by E3L of *Vaccinia virus*, VP35 of *Ebola virus* and NS1 of *Influenza virus A* (this study), which show a preference for longer dsRNA molecules, thereby interfering at the step upstream of dsRNA processing into siRNAs (Cardenas *et al*., 2006; Xiang *et al*., 2002). Binding longer dsRNA molecules by human-infecting viruses seems a sensible strategy, since dsRNA molecules beside being substrates for Dicer, also induce the IFN response by extracellular immunorecognition of dsRNA by Toll-like receptor (TLR) 3 (reviewed in Galiana-Arnoux & Imler, 2006) or intracellular sensing by the retinoic acid-induced gene I product (RIG-I; Sumpter *et al*., 2005; Yoneyama *et al*., 2004), melanoma-differentiation-associated gene 5 (MDA5; Kato *et al.*, 2006) and PKR (reviewed in Garcia *et al.*, 2006a).

In addition to siRNAs, miRNAs form another pool of small RNA molecules that have indeed been implicated in antiviral RNA silencing in animals. *Primate foamy virus type 1* (PFV-1) accumulation was shown to be restricted by a host-encoded miRNA and this effect was reduced by the PFV-1 Tas suppressor protein (Lecellier *et al*., 2005). The presence of miRNAs has not been restricted to host organisms, but they are also encoded by viruses. For example HIV-1 has been predicted to encode miRNAs that show complementarity to host target genes (e.g. CD28 and CD4) that play crucial roles in HIV-1 pathogenesis (Bennasser *et al*., 2005; Couturier & Root-Bernstein, 2005). An experimentally confirmed miRNA role has been shown for *Simian Virus 40* (SV40). The miRNAs processed from late viral transcripts guide target cleavage of early SV40 transcripts of the large ‘’T’’ and small ‘’t’’ SV40 tumor antigens, resulting in reduced susceptibility to and activation of cytotoxic T lymphocytes (Sullivan *et al*., 2005). To counteract miRNA-mediated RNAi, adenoviruses encode virus-associated (VA) RNAs. Like pre-miRNAs, VA1 RNAs are exported by Exportin-5 from the nucleus to the cytoplasm (Gwizdek *et al*., 2003; Lund *et al*.,

2004). Additionally, VA1 and VA2 are processed into small RNAs by Dicer and subsequently incorporated into RISC (Andersson *et al*., 2005).

Here it is shown that by binding longer dsRNA molecules with high affinity *Influenza virus A* NS1 is able to interfere with Dicer-mediated production of mature siRNAs. It seems reasonable to speculate that by binding to both perfect complementary dsRNAs of viral origin, as well as precursors of possible antiviral miRNAs, the virus is capable to escape several antiviral defence lines, i.e. IFN, RIG-I/MDA5/PKR (Donelan *et al.*, 2003; Lu *et al.*, 1995; Talon *et al.*, 2000; Wang *et al.*, 2000) and siRNA- and miRNA-mediated RNAi. Indeed, the role of the NS1 protein was previously determined to be indispensable for successful viral infection. It was shown here that the dsRNA binding properties of the NS1 protein are not only crucial in inhibiting Dicer action, but likely also for interferon-independent accumulation of *Influenza virus A* in mammalian cells. The *in trans* complementation of the RNAi suppressor role of Tat by NS1 in the HIV-rtTA virus, was recently identified (Haasnoot *et al.*, 2007). Similar results were obtained when the strictly small RNA-binding NS3 suppressor of RHBV was used and its suppressor activity was lost with its ability to bind these RNAs. Current collaborative studies, using the PR8-NS1rb are underway to show *in trans* complementation of RHBV NS3 in *Influenza virus A* infections. This suggests that indeed an active RNA silencing suppressor is needed for successful virus replication and that small RNA molecules of viral (siRNAs) or cellular origin (miRNAs) may both play a role in antiviral RNA silencing. This is in line with the observations that a knockdown of Drosha or Dicer enhances HIV-1 replication, whereas the accumulation of cellular (antiviral) miRNAs is suppressed during infection (Triboulet *et al.*, 2007). Interestingly, also for *Influenza virus A* potential miRNA binding sites have been identified in Polymerase B2 and hemagglutinin genes for mir-507 and mir-136, respectively (Scaria *et al*., 2006). Further investigations will unravel targeting of *Influenza virus A* sequences mediated by siRNA or miRNA molecules.

The fact that NS1 also exhibited IFN antagonistic functions indicates that RNA silencing and IFN responses may act in concert against invading viruses. The dsRNA binding region (amino acids 1-73) of NS1 has been identified as crucial for its IFN inhibitory function. It should be noted that when using *Influenza virus A* lacking the NS1 gene, results hinted in this direction (Garcia-Sastre *et al.*, 1998; Wang *et al.*, 2000). However, when an *Influenza virus A* was modified to express a mutated NS1 protein (R38A/K41A) deficient in RNA binding, a second mutation was adopted (S42G) which restored virus titers and simultaneously repressed IFN action, but was still deficient in dsRNA binding. This suggests that the NS1-dependent inhibition of IFN synthesis is not completely mediated by binding to and sequestering dsRNA generated during virus infection (Donelan *et al.*, 2003). Furthermore, direct interactions to either RIG-1 or PKR by NS1 appear to effect the INF inhibitory mode of action rather than dsRNA binding (Li *et al.*, 2006; Mibayashi *et al.*, 2007). Altogether this implicates that dsRNA binding by NS1 might play a role in antagonising IFN, PKR and RIG-1 pathways and is essential to suppress RNAi.

In the model proposed by Haasnoot and co-workers, dsRNA molecules are the common trigger in these mechanisms and activate IFN and RIG-I/MDA5 responses when exceeding a threshold (Haasnoot *et al.*, 2007). Binding of NS1 to pre-miRNA and dsRNA inhibits Dicer-mediated processing into small RNAs, and results in inhibition of innate immune responses and RNA silencing pathways. The cytoplasmic and nuclear localisation of NS1 (Li *et al.*, 1998; Wolff *et al.*, 1998) can enable NS1 to fulfil its role as inhibitor of innate immune responses as well as suppressor of antiviral RNAi.

Materials and Methods

Plasmid constructs

The coding sequence of *Influenza virus A*, A/Pr/8/34, NS1 was PCR amplified introducing a BamHI site at the 5' end and a unique KpnI site at the 3' end. Construction of the mutant form of the NS1 protein (R35A and R38A), NS1rb, has been described previously (Bucher *et al.*, 2004). For expression and purification of N-terminally His-tagged NS1 and NS1rb proteins, PCR products were ligated into the bacterial expression vector pQE31 (Qiagen).

For the generation of recombinant *Influenza virus A* PR8-NS1rb the same PCR approach was performed by using Phusion high-fidelity DNA polymerase (Bioké, Leiden, the Netherlands) as described above using a plasmid encoding gene segment 8 of *Influenza virus A* (A/PR/8/34) as template. The following primer combination was used for the PR8-NS1rb: 5' ctt gat **gc**g ctt cgc **gca** gat cag aaa tcc c '3 and 5' gaa tgg ggc atc acc tag ttc ttg 3'. Alanine substitutions were confirmed by sequencing reactions on the construct and rescued virus stock and are indicated in bold in the primer sequences.

Recombinant virus production, purification and titration

Recombinant *Influenza virus A* PR8-NS1 and PR8-NS1rb were constructed from eight cDNA fragments as described (de Wit *et al.*, 2004), using the constructed plasmids containing the modified segment 8 described above. Viruses were rescued on MDCK cells, cultured in EMEM (Cambrex) supplemented with 10% (vol/vol) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM HEPES and non-essential amino acids. Supernatant containing virus was harvested after ~48 h.

For large scale infections $3 \cdot 10^6$ MDCK cells were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ and supernatants harvested after ~48 h. Each 50 ml of supernatant was frozen at -80°C, thawed and centrifuged at 2500 g for 5 minutes at 4°C. The virus was further purified from cell debris by adding 0.5 ml 60% (wt/wt) sucrose to 33 ml supernatant and centrifugation for 1.5 h at 4°C at 27000 g. The lower 3 ml of supernatant was harvested and used for subsequent purification. A total of 165 ml of this supernatant was added to 0.5 ml 60% (wt/wt) sucrose topped with 15 ml 30% (wt/wt) sucrose and centrifugation repeated as before. Virus titrations were performed by end-point titration on MDCK cells as described previously (de Wit *et al.*, 2004).

Recombinant protein expression

The NS1 and NS1rb proteins were expressed from BL21 DE3 cells (manufacturer) according to manufacturer's recommendations. After induction for 3 h at 37°C with 0.3 mM IPTG cells were harvested by centrifugation for 15 minutes at 4000 rpm (Sorvall GSA rotor) at 4°C. Cells were lysed by sonification on ice with 30s intervals for 3 times 30 seconds in lysis buffer [50 mM K2PO4, 400 mM NaCl, 100 mM KCl, 10% (vol/vol) glycerol, 0.15% (vol/vol) Triton X-100, 10mM

imidazole pH 7.8]. The soluble fraction was recovered by centrifugation at 9000g for 30 minutes at 4°C. Recombinant protein was purified using TALON CellThru affinity columns (BD Biosiences) and eluted with 2.5 packed bed volumes (PBV) elution buffer [50mM NaH2PO4, 300mM NaCl, 200 mM imidazole, 10% (vol/vol) glycerol] after washing with 15 PBV lysis buffer. Protein fractions were flash frozen in liquid nitrogen and stored at -80°C until use. Protein concentrations of elution fractions were determined using the standard procedure of the Bio-Rad protein assay according to manufacturer's recommendations and the purification process analysed by SDS-PAGE and subsequent staining with Coomassie brilliant blue. Expression and purification of MBP-NS3 was described previously (chapter 2).

dsRNA preparation

Double stranded RNA was generated using T7 RNA polymerase (Promega) and α-^{32}P CTP according to protocol using a gel purified (High Pure PCR purification kit; Roche) PCR template. The primers used introduced T7 RNA polymerase promoters at both ends of the PCR product. Primers used were: T7_dsRNA400 F: 5' GTA ATA CGA CTC ACT ATA GGG GGC GTG CAG TGC TTC AGC CGC 3' and T7_dsRNA400 R: 5' GTA ATA CGA CTC ACT ATA GGG GTG GTT GTC GGG CAG CAG CAC 3' for 400 nt dsRNA and 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' for 114 nt dsRNA. Precursor miRNA 2b was prepared using T7 RNA polymerase using two annealed primers as template: dme-pre2b F: 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 dme-pre-2b R: 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 transcription reaction mixtures were incubated at 70°C for 10 minutes and cooled down to RT. Template was removed by treatment with DNase I and dsRNA was PAGE purified on a 8% native gel.

Electrophoretic mobility shift assay

Annealing of siRNAs and miRNAs was described before (Haley *et al.*, 2003). Bacterial expressed NS1 and NS1rb were incubated for 20 minutes at RT with 100 pM radiolabelled RNA in 1x binding buffer [50mM NaH2PO4, 300mM NaCl, 200 mM imidazole, 10% (vol/vol) glycerol, 0.01% (vol/vol) Triton X-100] in a 10 µl reaction volume. Separation of protein/RNA complexes was performed at 4°C at 150V on a 1 mm thick, large format, 5% (38:2 acrylamide:bisacrylamide), 0.5x TBE native gel. After running gels were dried, exposed to a phosphor screen, scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences) and bands quantified using Genius Image Analyser software (Syngene).

Dicer cleavage reactions

Drosophila embryo extract preparation was described previously (Haley *et al.*, 2003). In Dicer-mediated cleavage reactions embryo extracts were incubated for 60 minutes at 25°C in reaction mixtures as described previously (Haley *et al.*, 2003) where KCl was omitted from the reaction mixture. To 10 µl reactions 125 pmol dsRNA114 and a varying concentration of NS1, NS1rb or MBP-NS3 protein was added. For human Dicer-mediated target cleavage assays 0.25 U of human Dicer (Stratagene) was incubated for 60 minutes at 37°C in reaction buffer (20 mM Tris-HCl 8.0, 150 mM NaCl, 2.5 mM $MgCl_2$), 125 pmol dsRNA114 or pre-2b and a varying concentration of NS1 or NS1rb protein added. Samples were deproteinised and RNA was analysed on an 8% denaturing gel. After running, gels were dried, exposed to a phosphor screen, scanned (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences) and bands quantified using Genius Image Analyser software (Syngene).

Statistical analysis

All *in vitro* Dicer assays and RNA binding experiments were performed in triplicate. The percentage of processed dsRNA into siRNAs relative to the control experiment without HIS-NS1 (lane 3 in Fig. 5-4A) was determined. The curves were best fitted to the indicated sets of data with the computer program Microcal Origin 5.00. The average with standard error is shown in all graphs.

Tat complementation assays

Construction of the expression plasmid was described previously (Haasnoot *et al.*, 2007). The coding sequence of the NS1rb gene was PCR amplified using primers containing attB1 and attB2 sites and PR8-NS1rb as template. The PCR product was subsequently used to generate an entry clone using pDONR207 (Invitrogen) and BP clonase (Invitrogen) according to manufacturer's recommendations. Expression clones were generated using the produced entry clone, the pEF5-V5-DEST destination vector (Invitrogen) and LR clonase (Invitrogen) according to manufacturer's recommendations. Expression plasmids for NS3 and NS3mut were described previously (E. Schnettler *et al*, in press). Transfection of HEK293T cells and complementation studies were performed as described (Haasnoot *et al.*, 2007).

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Chapter 6

General discussion

Since the discovery of RNA silencing in plants about 15 years ago, genetic screens in plants and animals (notably *C. elegans and D. melanogaster*) have revealed that RNA silencing is a complex set of mechanisms implicated in various pathways essential to life. This includes antiviral defence, transposon activity suppression, chromatin structure and regulation of gene expression. Over recent years, a considerable number of proteins and RNAs involved in plant RNA silencing have been identified and their role in the RNA silencing pathways revealed (reviewed in Brodersen & Voinnet, 2006; Vaucheret, 2006). In contrast to elaborate investigations in *Drosophila*, biochemical analyses of RNA silencing pathways in plants have remained limited (Baumberger & Baulcombe, 2005; Qi *et al.*, 2005; Tang *et al.*, 2003; Yu *et al.*, 2005). To date, most advances in this respect have been made in unravelling the interactions between plant-infecting viruses and the host's RNA silencing response. It has become clear that many, if not all, plant viruses combat antiviral RNA silencing by encoding specific suppressor proteins. The identification of these viral suppressors has subsequently contributed to the dissection of the host's antiviral RNA silencing pathways. In this thesis the interaction between the negative strand tenuiviruses and tospoviruses and the antiviral defence in their plant and insect hosts was the central issue. At the onset of this research the suppressor proteins encoded by these viruses had been identified (Bucher *et al.*, 2003), creating an attractive starting point for further research.

Viral proteins NS3 and NSs counteract RNA silencing by binding dsRNA

One of the main questions to be answered in this thesis was how viruses that multiply both in plants and insects, such as tospo- and tenuiviruses, counteract the RNA silencing responses in both types of organisms. In chapter 2 it is shown that the NS3 protein of the tenuivirus RHBV interferes with antiviral RNA silencing by physically interacting with double stranded (ds) siRNA molecules. By binding these 21 nt ds small interfering (si)RNAs with a high affinity, NS3 is able to prevent the incorporation of these ds siRNAs into an active RISC complex and interferes with subsequent antiviral silencing events in insect and plant hosts. The conservation of the structure and the essential role of siRNA molecules between plants and insects makes them perfect candidates to be targeted by NS3 during the replication of RHBV in both systems. Even more so, because the different protein components of the RNA silencing pathways in plants and insects vary not only in their sequence, but also in their number. While for example in *Drosophila* DCR1 and DCR2 show strict separation of functionality in respectively micro (mi)RNA- and siRNA-mediated RNA silencing pathways (Lee *et al.*, 2004b), plants encode four Dicer-like (DCL) proteins, with a partial redundancy in their function (Deleris *et al.*, 2006; Gasciolli *et al.*, 2005).
Several observations described in this thesis provide information where and how the tenuiviral NS3 suppressor interferes with the antiviral RNA silencing. Chapter 2 demonstrates that NS3 is not able to interfere with RISC complexes that have been loaded with an unwound single strand of the siRNA duplex. This is in line with the finding that NS3 showed a >1000 fold lower affinity to ss siRNAs than to ds siRNAs (data not shown). The affinity to longer dsRNA also decreased

dramatically with increasing length, indication that NS3 must act downstream of the Dicer-mediated processing of longer dsRNA into siRNAs. Indeed NS3 could not interfere with Dicer action and the resulting accumulation of 21 nt siRNAs (chapter 5).

The siRNA binding requirements of NS3 were investigated in chapter 3. Comparison of the RHBV NS3 protein to paralogs of other tenuiviruses revealed at least two surface exposed domains present in the NS3 protein. Especially the domain located near its C-terminus was of special interest because mutations in this domain showed a direct correlation between NS3 siRNA binding capacity and NS3 suppressor activity. However, it remains unclear if indeed the sole function of this domain is in siRNA binding. In chapter 2 it was determined that the NS3 protein interacts in dimeric form with a single siRNA molecule, the same stoichiometry as was found for the tombusvirus p19 protein (Vargason *et al*., 2003). It might be possible that in order to sequester siRNAs, NS3 needs to form dimeric structures or NS3 only forms dimers in the presence of siRNAs. The lack of any identity on amino acid level between the RHBV NS3 protein and other identified and studied suppressor proteins, e.g. tospoviral NSs, tombusviral p19 and potyviral HC-Pro, makes comparison even between paralogues suppressor proteins complicated. Therefore, current collaborative crystallography studies with the University of Frankfurt will support any directed search for functional domains in this protein.

In contrast to NS3, TSWV NSs has the capacity to bind dsRNA molecules without obvious size preference (chapter 4). This enables TSWV to suppress RNA silencing at two levels; by interfering with Dicer-mediated dsRNA processing into siRNAs and by the incorporation of these molecules into RISC. In this respect, NSs is distinct from most other plant viral suppressor proteins, since size independent binding has hitherto only been reported for the suppressors of 2 tombusviruses, *Turnip crinkle virus* p38 and *Aureusvirus* p14 suppressor proteins (Merai *et al*., 2006; Merai *et al*., 2005). TSWV and other tospoviruses are of special interest as they are members of the *Bunyaviridae*, a family that mainly encompasses animal-infecting viruses. The need to bind long dsRNA molecules of cellular or viral origin by TSWV NSs is currently unclear, but for the animal viruses within the family it may be assumed that binding both long and short dsRNA molecules is to be preferred over only siRNA binding. This is because in animal cells longer dsRNAs are not only Dicer substrates, but also activate dsRNA-dependent protein kinase (PKR), which plays a central role in interferon-mediated antiviral defence (reviewed in Garcia *et al.*, 2006a). Potentially, a short and long dsRNA-binding suppressor protein could therefore antagonise two main defence systems simultaneously.

As mentioned above, most plant viral suppressors characterised so far were shown to bind size specifically to siRNA molecules (Table 6-1). For some ‘’true’’ plant viruses (i.e. those that exclusively replicate in plants, not in their vector insects) interference with the RNA silencing machinery through protein-protein interactions has been observed. Besides the ability to bind siRNAs, the *Cucumber mosaic virus* (CMV) 2b protein also interacts with AGO1 (Goto *et al*., 2007; Zhang *et al*., 2006).

Table 6-1: Virus-encoded RNA silencing suppressors of plant (white) and animal viruses (grey)

Genome	Virus genus	Virus	Suppressor	Interference				Other functions	References
				siRNA	miRNA	long dsRNA	protein		
(+) RNA	Aureusvirus	Aureusvirus	p14	+		+		Virulence	1
	Carmovirus	Turnip crinkle virus	p38	+		+		Coat protein	2-4
	Cucumovirus	Cucumber mosaic virus	2b	+			AGO1	Movement	5-9
	Closterovirus	Beet yellows virus Citrus tristeza virus	p21 p20, p23, CP	+		-		Replication enhancer, coat protein	2, 10-12
	Comovirus	Cowpea mosaic virus	S protein					Coat protein	13
	Dianthovirus	Red clover necrotic mosaic virus	p27, p88				DCLx	Replication	14
	Hordeivirus	Barley stripe mosaic virus	?b	+		-		Replication enhancer; movement; virulence	2, 15
	Pecluvirus	Peanut clump virus	p15	+		-		Movement	2, 16
	Poleovirus	Beet western yellows virus	p0				RNA silencing component	Pathogenicity determinant	17-18
	Potexvirus	Potato virus X	p25					Movement	19
	Potyvirus	Tobacco etch virus	HC-Pro	+	+	-		Movement; virulence; polyprotein processing; aphid transmission	2, 5, 10, 20-22
	Sobemovirus	Rice yellow mottle virus	p1	+				Movement; virulence	23-25
	Tombusvirus	Carnation Italian ringspot virus	p19	+	+	-		Pathogenicity determinant; movement	25-29
	Tobamovirus	Tobacco mosaic virus Tomato mosaic virus	p126 p130					Replication	25, 30-31
	Tymovirus	Turnip yellow mosaic virus	p69					Movement; virulence	32
(-) RNA	Tospovirus	Tomato spotted wilt virus	NSs	+	+	+		Virulence	33-34
	Tenuivirus	Rice hoja blanca virus	NS3	+	+	-		Unknown	33, 35
DNA	Begomovirus	African cassava mosaic virus Tomato yellow leaf curl virus	AC2 C2					Transcriptional activator	25, 36-37
(+) RNA	Nodavirus	Flock house virus	B2					Plaque formation	38-39
		Nodamura virus	B2	+		+			
	Retrovirus	Human immunodeficiency virus-1	Tat					Transcription; inhibitor of PKR	40-41
		Primate foamy virus-1	Tas						
(-) RNA	Ebolavirus	Ebola virus	VP35			+		Transcription; inhibitor of PKR	42-43
	Orthomyxovirus	Influenza virus A	NS1	-	-	+		Poly A) binding; inhibitor of mRNA export and inhibitor of PKR	44-47
	Orthobunyavirus	La Crosse virus	NSs					Unknown	48
DNA	Adenovirus	Adenovirus	VA RNAs	NA			Dicer, Exportin 5	Translation; inhibitor of PKR	49-51
	Poxvirus	Vacciniavirus	E3L			+		Transcription; inhibitor of PKR	46, 52

1 Merai *et al.*, 2005, 2 Merai *et al.*, 2006, 3 Qu *et al.*, 2003, 4 Thomas *et al.*, 2003, 5 Brigneti *et al.*, 1998, 6 Guo & Ding, 2002, 7 Lucy *et al.*, 2000, 8 Zhang *et al.*, 2006, 9 Goto *et al.*, 2007, 10 Lakatos *et al.*, 2006, 11 Lu *et al.*, 2004, 12 Reed *et al.*, 2003, 13 Liu *et al.*, 2004, 14 Takeda *et al.*, 2005, 15 Yelina *et al.*, 2002, 16 Dunoyer *et al.*, 2002, 17 Pfeffer *et al.*, 2002, 18 Pazhouhandeh *et al.*, 2006, 19 Voinnet *et al.*, 2000, 20 Anandalakshmi *et al.*, 1998, 21 Kasschau & Carrington, 2001, 22 Mallory *et al.*, 2002, 23 Sarmiento *et al.*, 2007, 24 Tamm & Truve, 2000, 25 Voinnet *et al.*, 1999, 26 Qiu *et al.*, 2002, 27 Qu & Morris, 2002, 28 Silhavy *et al.*, 2002, 29 Vargason *et al.*, 2003, 30 Ding *et al.*, 2004, 31 Kubota *et al.*, 2003, 32 Chen *et al.*, 2004, 33 Bucher *et al.*, 2003, 34 Takeda *et al.*, 2002, 35 Hemmes *et al.*, 2007, 36 Dong *et al.*, 2003, 37 van Wezel *et al.*, 2002, 38 Li *et al.*, 2002, 39 Sullivan & Ganem, 2005, 40 Bennasser *et al.*, 2005, 41 Lecellier *et al.*, 2005, 42 Haasnoot *et al.*, 2007, 43 Cardenas *et al.*, 2006, 44 Bucher *et al.*, 2004, 45 Delgadillo *et al.*, 2004, 46 Li *et al.*, 2004, 47 Wang *et al.*, 1999, 48 Soldan *et al.*, 2005, 49 Andersson *et al.*, 2005, 50 Lu & Cullen, 2004, 51 Mathews & Shenk, 1991, 52 Watson *et al.*, 1991.

Another strategy has been adopted by *Red clover necrotic mosaic virus* (RCNMV), which seems to recruit DCL proteins during its replication (Takeda *et al*., 2005), while the p0 protein of *Beet western yellows virus* tags an essential component of the RNA silencing machinery, likely AGO1, for destruction by the plant's own proteasome after guiding it to the E3 ubiquitination ligase machinery (Pazhouhandeh *et al.*, 2006).

Interestingly, despite the identification of a growing number of viral RNA silencing suppressors with overlapping functions, no protein sequence homology between these proteins has been observed. Apparently, high affinity dsRNA binding can be achieved by structurally different proteins. Indeed structural data of e.g. tombusviral p19 and closteroviral p21 show that dsRNA binding can be achieved by rather different protein structures, as dimers or octameric ring structures, respectively (Vargason *et al*., 2003; Ye & Patel, 2005). This is further substantiated by the different interference strategies adopted by the various viral suppressors, even within a single taxon, e.g. the *Tombusviridae*: suppressors of the genera *Aureusvirus* and *Carmovirus* bind both siRNA and long dsRNA, those of the genus *Tombusvirus* bind only siRNA, while the suppressors of the genus *Dianthovirus* bind no RNA at all, but instead recruit DCL proteins during viral replication. This might indicate that viruses that even are closely related have found different ways to combat antiviral RNA silencing that perhaps originally only served as regulatory pathway through miRNA action (Ding & Voinnet, 2007).

As both tospo- and tenuiviruses replicate in plants as well as insects it may not come as a surprise that their RNA silencing suppressors target dsRNAs. This allows these viruses to antagonise RNA silencing in both plant and insect, in which the protein components of the silencing pathways may be less conserved. Most of the ''true'' plant viruses, i.e. those that do not replicate in their insect vector, also specify siRNA-binding suppressors (Table 6-1) while only a few bind protein compounds of the defence system. Possibly, this choice involves different levels of host adaptation, where siRNA-binding suppressors provide a broader host range to the virus. However RCNMV, whose suppressor binds DCL, does not have a narrower host range than plant viruses such as tospoviruses and potyviruses, specifying dsRNA-binding suppressors (Virus Identification Data Exchange; http://image.fs.uidaho.edu/vide/genindex.htm). Hence, a more likely explanation might be that RNA silencing is in fact an ancient mechanism in gene expression regulation, which later adapted into an antiviral defence mechanism. In plants, DCL family members are diversified and functionally specialised during evolution, while animals have only one Dicer. On the other hand, animals have evolved a relatively large family of AGO-related proteins (Carmell *et al.*, 2002), which might provide the components for functional specialisation of RNA silencing pathways. Another explanation could be that dsRNA-binding by many plant viruses is a remnant from evolving from ancestral viruses, which originally replicated in insects (Goldbach, 1987), and in time most of these modern plant viruses had lost their ability to replicate in insects.

Mammalian viruses and RNAi

Having identified the NSs protein of TSWV as suppressor of RNA silencing, also the NSs protein of *Rift valley fever virus* (RVFV), a member of the genus *Phlebovirus* within the *Bunyaviridae* family, has been examined for such activity, using both plant (Bucher, 2006) and mammalian cell-based assays (van der Velden *et al.*, unpublished data). The protein showed no suppressor action in these systems and it became apparent that it is an antagonist of the IFN pathway, acting by suppressing type I interferon (IFN-α/β) induction, not by dsRNA binding but probably via modulation of the basal transcriptional machinery of the host cell (Billecocq *et al.*, 2004).

Notwithstanding this negative outcome, more evidence became available during the course of this PhD research, showing that animal viruses encode dsRNA binding proteins that score positively in established RNA silencing suppressor assays. Our own research and that of others had first indicated that the *Influenza virus A* encoded NS1 protein is able to suppress RNA silencing in plant- and insect cell-based assays by binding dsRNA (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004). Also the E3L protein of *Vaccinia virus* inhibited RNA silencing in an insect-based assay (Li *et al.*, 2004). More recently, also a human cell-based assay has been developed based on *in trans* complementation of the Tat suppressor of a HIV-1 Tat minus virus (Haasnoot *et al.*, 2007). To date suppression of induced RNA silencing was only observed in human cells by the Tat protein of *Human immunodeficiency virus-1* (HIV-1) (Bennasser *et al.*, 2005), the NSs protein of *La crosse virus* (Soldan *et al.*, 2005), VP35 of *Ebola virus* and by the E3L protein of *Vaccinia virus* (Haasnoot *et al*., 2007). Many of these proteins have previously been associated with their antagonistic function of the interferon-induced PKR (Basler *et al*., 2000; Bouloy *et al*., 2001; Brand *et al*., 1997; Chang *et al*., 1992; Garcia-Sastre, 2001; Talon *et al*., 2000; Xiang *et al*., 2002), but also seem to act as RNA silencing suppressors. Additionally, suppressor action of plant and insect viral suppressor proteins in mammalian cells has been shown for tombusviral p19 (Lecellier *et al.*, 2005), *Nodamura virus* B2 (Sullivan & Ganem, 2005) and RHBV NS3 proteins (Schnettler *et al.* in press). Due to the universality of RNA silencing suppression by these dsRNA binding proteins it seems reasonable to anticipate that these suppressors are active in a wide range of organisms. However, also the dsRNA binding RNase III protein of *Escherichia coli*, a prokaryotic organism typically lacking an RNA silencing mechanism, was shown to suppress RNA silencing in a plant-based assay (Lichner *et al.*, 2003). Therefore, to obtain further indications that NS1 is a genuine suppressor of RNA silencing in mammals and how NS1 would exert this function a biochemical approach was followed.

Chapter 5 indicates that NS1 specifically binds longer dsRNA with high affinity and, as a result, prevents the processing of dsRNA into siRNAs by the sole human Dicer protein *in vitro*. Although a clear role for Dicer and other RNA silencing components has not been demonstrated in antiviral silencing, it is essential in miRNA-mediated animal development (Ambros, 2004; Carrington & Ambros, 2003). First indications of the interplay between animal viruses and the RNA silencing machinery of mammalian cells was demonstrated with the identification of pre-miRNA-like

molecules, virus-associated (VA) RNAs, encoded by the adenovirus DNA genome. VA1 RNA was shown to bind to PKR, thereby inhibiting PKR activation by viral dsRNA (Mathews & Shenk, 1991). Like pre-miRNAs, VA1 RNAs are also exported out of the nucleus by Exportin-5, in that way competing for Exportin-5-dependent nuclear export (Lu & Cullen, 2004). In addition, VA1 and VA2 RNAs are cleaved by Dicer into siRNAs which are subsequently incorporated into RISC, thereby saturating the RISC complexes of the RNA silencing machinery with irrelevant decoy RNAs (Andersson *et al*., 2005).

Viral repression by host encoded miRNAs was demonstrated for *Primate foamy virus type 1* (PFV-1). PFV-1 accumulation was restricted by mir-32 and this translational block was reduced by the Tas suppressor protein (Lecellier *et al*., 2005). Interestingly, also for *Influenza virus A* potential binding sites in the Polymerase B2 and hemagglutinin genes have been identified for mir-507 and mir-136, respectively (Scaria *et al*., 2006). This raises the question if mammalian viruses have to cope with multiple ‘’antiviral’’ pathways, i.e. the innate immune response, siRNA-mediated RNAi, and specific antiviral miRNAs. The expression of NS1 enables *Influenza virus A* to interfere with these siRNA- and miRNA-mediated pathways of RNA silencing where the action of a single Dicer enzyme provides the accumulation of these small RNA molecules from longer dsRNA molecules. Although only the RNAi suppression action of NS1 on human Dicer-mediated dsRNA processing was tested (chapter 5), it can be anticipated that in analogy to NSs (chapter 4), NS1 also blocks the processing of pre-miRNA molecules into mature miRNAs with homology to *Influenza virus A* sequences, since these pre-miRNAs were also bound with high affinity by NS1 (chapter 5) and NS1 accumulates both in the nucleus and cytoplasm (Li *et al*., 1998; Wolff *et al*., 1998). The inhibitory action on human Dicer-mediated dsRNA processing by NS1 provides further evidence for the interplay between host defence mechanisms, e.g. the innate immune response, RNA silencing (involving miRNAs, but possibly also viral siRNAs) and the animal virus.

Is there also a role for miRNA molecules in plant antiviral RNA silencing?

Cloning and computational approaches have identified hundreds of animal miRNAs and suggest that a large proportion of animal transcripts are miRNA regulated (Brennecke *et al*., 2005; Doench & Sharp, 2004; Lewis *et al*., 2003). The presence of miRNAs has not been restricted to animals but has also been discovered in their pathogens. Animal viruses have been shown to encode miRNA molecules that can potentially target host sequences. HIV-1 has been predicted to encode miRNAs that show complementarity to host target messenger RNAs, that play crucial roles in HIV-1 pathogenesis, like CD4 and CD28 (Bennasser *et al*., 2005; Couturier & Root-Bernstein, 2005). Herpes simplex-1 (HSV-1) encodes miRNAs which can target host genes of the apoptosis pathway (Cui *et al*., 2006; Gupta *et al*., 2006). Other herpesviruses, like *Epstein Barr virus* (EBV), *Kaposi sarcoma-associated herpesvirus* (KSHV) and *Human cytomegalovirus* (HCMV) encode miRNAs of which only for EBV a host target was identified (Grundhoff *et al*., 2006; Pfeffer *et al*., 2005). A clear role for viral miRNAs in the viral infection cycle has been shown for *Simian Virus 40* (SV40). The miRNAs processed from late viral transcripts guide target

cleavage of early SV40 transcripts of the large ‘’T’’ and small ‘’t’’ SV40 tumor antigens, resulting in reduced susceptibility to and activation of cytotoxic T lymphocytes (Sullivan *et al*., 2005).

In a search for structural requirements of small RNAs binding by NS3, it was also shown that miRNAs were bound with an affinity comparable to siRNAs (chapter 2). This might explain some of the observed phenotypes of NS3 transgenic *Arabidopsis* (chapter 2). Similar findings had been reported by other research groups which showed that expression of a wide range of viral suppressors in *Arabidopsis* resulted in phenotypes resembling those of DCL1 mutant plants (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003). These observations now raise the question whether interference by NS3 with the miRNA pathway in plants is a non-essential side-effect of the siRNA quenching character of the suppressor or whether it happens on purpose: in other words would miRNA binding be required for successful RHBV infection by interfering with the expression of specific host (response) genes?

Evidence for the deliberate interference with the miRNA pathway may come from computational analyses, which showed the presence of several 20-25 nt sequences located within intergenic regions of *Arabidopsis* with perfect or near perfect complementarity to plant viral genomes (Llave, 2004). This observation hints towards the presence of endogenous small RNA molecules able to recognise viral sequences, similar as observed for animal viruses, although this has not been experimentally demonstrated for plant viruses. Also for *Oryza sativa* computational approaches, based on conservation with known miRNAs from *Arabidopsis*, have led to the discovery of rice miRNAs (Llave, 2004; Llave *et al.*, 2002; Park *et al.*, 2002; Reinhart *et al.*, 2002; Sunkar *et al.*, 2005a; Sunkar *et al.*, 2005b; Sunkar & Zhu, 2004). More recently, new miRNA families were identified with no phylogenetic conservation with known *Arabidopsis* miRNAs. Some further investigation indicated that three of the newly identified miRNAs, Os-Mir528, Os-Mir529 and Os-Mir530 could be detected by Northern blot analyses from different tissues and required osDCL1 function. Loss of OsDCL1 function resulted in developmental defects, like root and shoot abnormalities and reduced miRNA levels in rice (Liu *et al.*, 2005). Interestingly, these three novel miRNAs did not show any homology to known rice protein-encoding RNA molecules, which might be explained by the incomplete annotation of the rice genome. However, these three novel miRNAs could also have a function in antiviral defence, as suggested above. The presence of potential target sites for rice-encoded miRNAs (especially Os-Mir528, Os-Mir529 and Os-Mir530) within the RHBV genome was confirmed by using the miRanda program (Enright *et al.*, 2003). Of these, the hit for Os-Mir528 recognising the nucleocapsid (N) gene encoding region was of special interest because of the high degree of complementarity between the 5’ end (seed region) of the miRNA and its target (Fig 6-1). Targeting of the (N) messenger RNA of (-) strand RNA viruses will result in a decrease of available N protein. As a consequence, this would result in a reduction of replication activity of the RNA polymerase of (-) strand RNA viruses, which is depending on the concentration of nucleocapsid proteins (Meyer *et al.*, 2002). Unlike most plant miRNA molecules, the 5’ sequence of Os-Mir528 shows specific binding potential to the 3’ target,

resembling typical binding characteristics of animal miRNAs to their targets (Bartel, 2004). Most plant miRNAs exhibit almost perfect complementarity to their target resulting in the AGO1-catalysed target cleavage (Baumberger & Baulcombe, 2005). Additionally, binding of the 5' region of a miRNA to the 3' end of its target was identified before and mismatch tolerance proposed to be bigger in the central and 3' regions of the miRNA (Mallory *et al.*, 2004). This enables Os-Mir528 likely to interfere with expression of the RHBV N protein by translational repression (Aukerman & Sakai, 2003; Chen, 2004) rather than the more common miRNA-mediated target cleavage observed for plant miRNAs.

```
Os-Mir528      3'  G A G G A G A C - G U A C G G G G A A G G U  5'
                     :  |  |  :  |  |    :  :    |    |  |  |  |  |  |  |  |
RHBV N mRNA    5'  A G U C U U U G A U G A G G C C U U U C C A  3'

Energy: -22.480000 kCal/Mol
```

Figure 6-1: Identification and presentation of a rice-encoded miRNA target within the RHBV genome. The pairing of the heptamer, spanning residues 2-8 (seed region), of the 5' miRNA region to the target is essential in miRNA function.

The presence of an endogenous miRNA will enable the plant to target the invading RHBV directly upon infecting. By encoding the NS3 suppressor protein, RHBV can combat the antiviral defence by recruiting both viral siRNAs and potential endogenous antiviral miRNAs. It is tempting to speculate that binding these miRNAs serves to establish an advantageous viral environment or, even more likely, creates the delicate balance between host and parasite. By allowing targeting of viral RNA by endogenous miRNA molecules, RHBV might eventually accumulate to higher levels by the regulatory action on virus replication by these host miRNAs. The NSs protein of TSWV also interferes with the miRNA pathway of RNA silencing as was shown in chapter 4. However, to date the identification and publication of miRNA sequences of tomato is very limited (Pilcher *et al.*, 2007). Therefore computational identification of potential TSWV targets could not be performed.

Concluding remarks

In this thesis the mode of action of the RNA silencing suppressors of two plant-infecting negative-strand RNA viruses (TSWV, RHBV) has been investigated as well as that of the putative suppressor protein of an animal-infecting negative-strand RNA virus (*Influenza virus A*), and the outcome is schematically presented in Fig. 6-2. Both plant viruses encode suppressors with high affinity for 21 nt siRNA molecules (chapter 2 and 4 respectively) and are therefore able to suppress antiviral silencing in both their botanical hosts and their insect vectors by targeting a conserved part of RNA silencing. Additionally, the TSWV NSs protein showed affinity for longer dsRNA molecules, which might reflect its very close genetic affinity to mammalian viruses. Like TSWV NSs, also the *Influenza virus A* NS1 protein is able to bind long dsRNA but it differs by its inability to bind siRNAs. Therefore, its potential role in suppressing RNAi (besides counteracting the IFN pathway) would be by inhibiting Dicer action, and this possibility has been substantiated

in chapter 5. Given the lack of conserved sequence domains, viral suppressors seem to have evolved independently while key players of the RNA silencing pathway are conserved. This is indicative for the hypothesis that antiviral silencing is a recent spin-off of the ancient miRNA-dependent gene regulation function of RNA silencing. In plants this might be the case since plant viral suppressors seem to have developed independently and show the presence of multiple (specialised) Argonaute and DCL proteins.

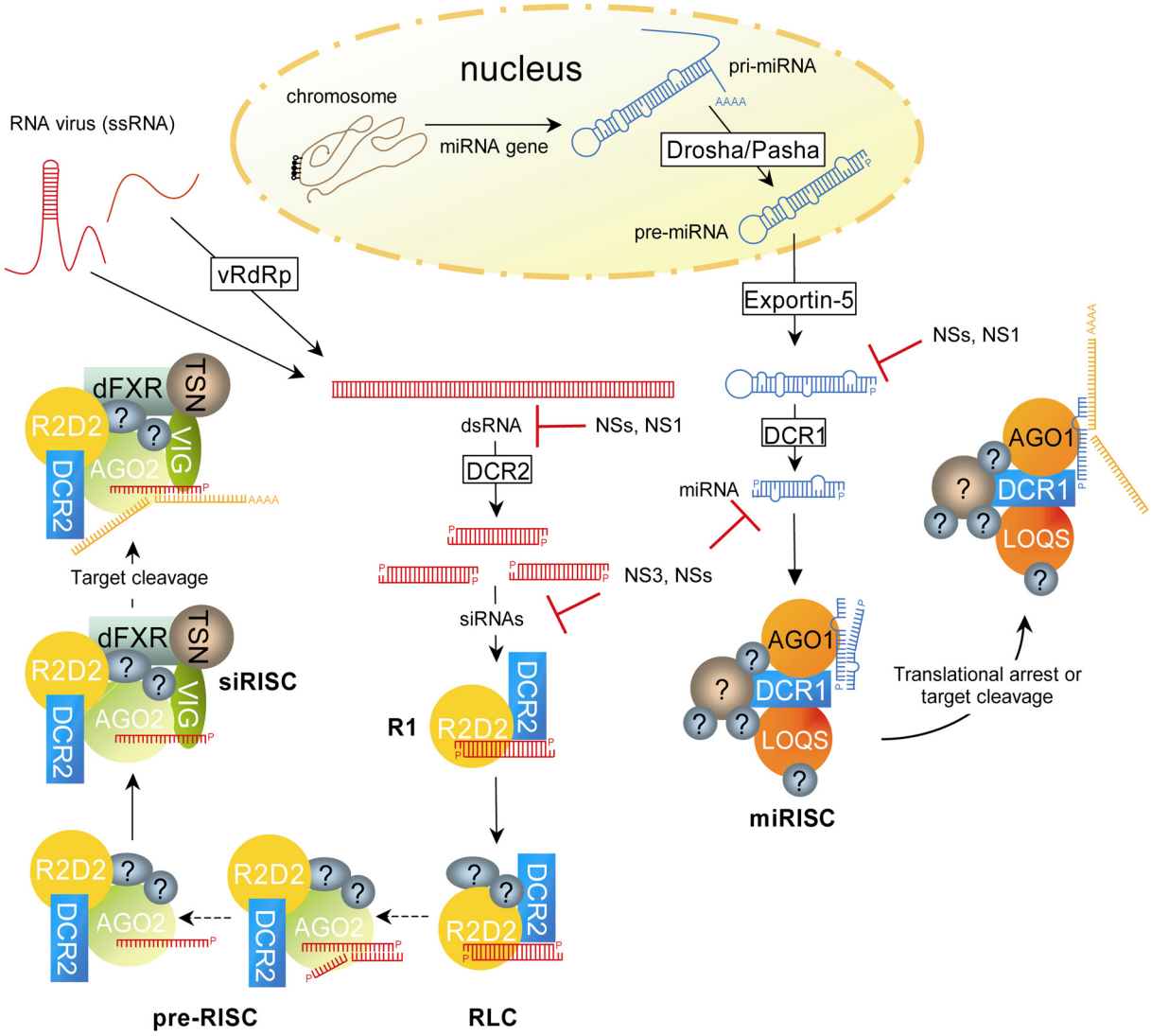

Figure 6-2: A schematic overview of RNA silencing in *Drosophila melanogaster*. The position of the RNA silencing suppressors studied in this PhD research indicate their mode of action. NS3 and NS1 protein show size selective binding to 21 nt dsRNA and long dsRNA, respectively. Whereas NSs shows size independent binding to dsRNA.

Despite providing further evidence in this thesis, a requirement for siRNA-mediated antiviral silencing in mammals still awaits definite proof. This has hitherto been complicated by the inability to demonstrating the presence of virus-specific siRNAs (expected key players in the process) and the existence of another dsRNA-dependent innate antiviral defence response, i.e. the interferon pathway. The need for viral proteins to modulate the miRNA pathway for up- or down regulating

host gene expression and thus their ability to bind miRNA and/or their precursors has become a fact and evidence is compiling that the miRNA-mediated (antiviral) silencing pathway plays a role in viral accumulation. As a response, human viruses may have evolved multifunctional proteins that also act as suppressor in mammalian antiviral RNA silencing pathways.

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Summary

The research described in this thesis focused on the strategies of negative strand RNA viruses to counteract antiviral RNA silencing. In plants and insects, RNA silencing has been shown to act as a sequence specific antiviral defence mechanism that is characterised by the processing of double stranded (ds)RNA 'trigger' molecules into small interfering RNAs (siRNAs) by enzymes of the Dicer family. The siRNA molecules are essential components of the RNA induced silencing complex (RISC), which uses the siRNA sequence to be guided to complementary targets that are subsequently inactivated by the slicing activity of Argonaute proteins, the active component of RISC. To counteract antiviral RNA silencing, plant viruses encode dedicated suppressor proteins. The identified suppressor proteins so far, mostly are encoded by plant positive strand RNA viruses and DNA viruses. This thesis and previous work in our laboratory (Bucher, 2006) centred around the characterisation of the RNA suppressor proteins of negative strand plant RNA viruses. This group of viruses is unique in having a replication cycle in both their botanical host and insect vector, making them likely to encounter antiviral RNA silencing in both types of organisms. At the onset of this thesis research, the suppressor proteins of two negative strand RNA plant viruses, i.e. of *Tomato spotted wilt virus* (TSWV, genus *Tospovirus*) and of *Rice hoja blanca virus* (RHBV, genus *Tenuivirus*), had been identified, but their mode of action remained unknown.

In chapter 2 of this thesis, the RNA silencing suppressor of RHBV, the NS3 protein, was investigated in further detail. Its suppressor action was confirmed in plants and also established in insect cells. Molecular and biochemical analyses of the NS3 protein showed a high affinity for the archetypical 21 nt siRNA molecules, but not for longer dsRNAs. By recruiting these siRNA molecules, NS3 was shown to interfere with the assembly and function of RISC in *Drosophila* embryo extracts. Sequestration of siRNAs, conserved between the RNA silencing pathways of all eukaryotes, enables RHBV to counteract this antiviral response in its insect vector and plant host. RNA silencing also serves a critical role in gene expression regulation and genome integrity. Key players in this part of the RNA silencing are the microRNA (miRNA) molecules. In addition, the binding affinity of NS3 to unwound miRNA duplexes was proven to be comparable to that of siRNAs, which is in agreement with developmental abnormalities observed in transgenic *Arabidopsis* plants after constitutive expression of the NS3 protein.

Knowing the interference strategy of RHBV NS3, the sequence requirements for siRNA binding were examined in chapter 3. By comparing amino acid sequences of the RHBV NS3 protein to its paralogs of other tenuiviruses, two conserved and predicted surfaced-exposed regions were identified. Deletion of either domain resulted in dysfunctional suppressor proteins while deletion of single alanine substitutions in these regions had no effect on their suppressor activity or siRNA binding capacity. However, when three clustered positively charged amino acids (K173-K175), present in one of these domains, were substituted the siRNA binding affinity of this mutated protein was completely abolished, coinciding with complete lack of suppressor activity. This

confirmed the alleged role of siRNA binding as being crucial for the RNA silencing suppression activity of NS3.

The suppressor protein (NSs) of tospoviruses was subject of the studies presented in chapter 4. In contrast to tenuiviral NS3, the tospoviral NSs showed size-independent binding to dsRNA. Its ability to bind also longer dsRNA was shown to result in the inhibition of Dicer-mediated processing of longer substrates into siRNAs. In addition, binding of NSs to miRNA duplexes was confirmed *in planta*. As tospoviruses belong to the large *Bunyaviridae* family, which also hosts many animal viruses, the observed high affinity for longer dsRNA molecules of their NSs proteins may reflect a common ancestry with such animal viruses.

Indeed, for animal infecting viruses the capacity of their host defence antagonistic proteins to bind long dsRNA seems favourable, since these molecules are not only a substrate for Dicer, but are also recognised by alternative innate defence pathways like the interferon response. Although at the time there were few indications for an antiviral activity of the RNA silencing machinery in vertebrate systems, the *Influenza virus A* NS1 protein scored positive as suppressor of RNA silencing in plant- and insect-based assays (Bucher, 2006; Li *et al.*, 2004). Chapter 5 investigates the potential activity of NS1 as RNA silencing suppressor further, now using homologous (human) cell systems. Thus NS1 is shown not to bind siRNAs but exclusively long dsRNA molecules with high affinity and by doing so it is able to inhibit Dicer activity. Two point mutations in its RNA binding domain, previously implicated in both RNA silencing and the interferon response, resulted in the accumulation of siRNAs in Dicer cleavage assays. Recombinant influenza viruses expressing wildtype (PR8-NS1) or the mutant NS1 protein (PR8-NS1rb) were constructed and the effect on virus replication and accumulation was assayed. This demonstrated that viral titers drastically decreased for PR8-NS1rb compared to PR8-NS1 and since interferon production was not induced during PR8-NS1rb infections, this hinted towards an antiviral role for RNA silencing in mammals. A second line of research underscored this interpretation; wildtype NS1 protein, but not the NS1rb mutant protein, was able to complement a Tat-minus *Human immunodeficiency virus-1* (HIV-1) virus. Interestingly, also the NS3 protein of RHBV rescued this HIV mutant, indicating a role of small RNA molecules in vertebrate antiviral silencing.

In conclusion it is shown that negative strand RNA viruses of plants encode suppressor proteins that combat RNA silencing by interacting with dsRNA, thereby ensuring interference of this host response in both plant host and insect vector. Having said this, the suppressors of tenuiviruses and tospoviruses do not act in the same way. While tenuiviral NS3 only interferes with RISC assembly, NSs also inhibits Dicer activity. Both strategies enable the suppression of antiviral silencing in their insect vector and plants. Furthermore, the presented data on the NS1 protein of *Influenza virus A* adds to the recently emerging evidence that also mammalian viruses may encode suppressors to counteract antiviral action of the siRNA or the miRNA pathway.

Samenvatting

Het in dit proefschrift gebundelde onderzoek richtte zich op de strategieën die min-strengs RNA virussen hebben ontwikkeld om tijdens hun infectieproces 'RNA silencing' tegen te gaan. RNA silencing omvat een serie van sequentiespecifieke RNA afbraakprocessen waarvan in planten en insecten is aangetoond dat deze ook dienstdoet als antiviraal verdedigingmechanisme. Hierbij worden langere dubbelstrengs RNA (dsRNA) moleculen tot 'small interfering RNAs' (siRNAs) afgebroken door enzymen van de Dicer familie. De langere dsRNA substraten kunnen daarbij zowel replicatie-intermediairen van het virus zijn als secundaire structuren in niet-replicerend viraal RNA. De gevormde siRNA moleculen zijn essentiële componenten van het 'RNA geïnduceerde silencing complex' (RISC) dat de sequentie van het siRNA molecuul gebruikt om complementaire doelen te vinden die vervolgens worden geïnactiveerd door de knipfunctie van Argonaute eiwitten, de actieve componenten van RISC. Om antivirale RNA silencing tegen te gaan coderen plantenvirussen RNA silencing 'suppressoreiwitten'. Terwijl er bij de aanvang van dit onderzoek vooral veel bekend was met betrekking tot de identiteit van de door plus-strengs RNA virussen gecodeerde suppressoreiwitten was dit niet het geval voor min-strengs RNA virussen van planten. In zoverre is die groep virussen extra interessant omdat zij niet alleen repliceren in hun waardplant maar tevens in hun insectenvector. Derhalve dienen zij zich te beschermen tegen antivirale RNA silencing in zowel plant als insect. In voorafgaand promotieonderzoek (Bucher, 2006), was voor twee groepen van plant-infecterende min-strengs RNA virussen de identiteit van hun suppressoreiwit vastgesteld, te weten het NSs eiwit in het geval van tospovirussen en het NS3 eiwit in geval van tenuivirussen. Hun werkingsmechanismen waren echter nog niet opgehelderd.

In hoofdstuk 2 van dit proefschrift is de RNA silencing suppressie activiteit van het NS3 eiwit van het Rijst 'hoja blanca' virus (RHBV, een tenuivirus) verder onderzocht, en kon ondermeer vastgesteld worden dat deze ook werkzaam is in insectencellen. Verdere karakterisering van het NS3 eiwit liet zien dat dit eiwit een hoge affiniteit heeft voor het archetypische, 21 nucleotiden-lange siRNA molecuul, maar niet voor langere dsRNA moleculen. Door binding van deze siRNA moleculen interfereert NS3 met de opbouw en functie van RISC, zoals aangetoond kon worden in extracten van fruitvlieg-embryo's. Omdat immers siRNAs als onderdeel van het RNA silencing mechanisme sterk geconserveerd zijn onder eukaryoten, stelt deze bindingsactiviteit van het NS3 eiwit RHBV tevens in staat om de antivirale respons te blokkeren in zowel de insectenvector als de plant. RNA silencing speelt ook een cruciale rol in de regulatie van genexpressie en genoomintegriteit. Belangrijke componenten van dit deel van het RNA silencing mechanisme zijn de 'microRNA' (miRNA) moleculen. De bindingsaffiniteit van NS3 voor dubbelstrengs miRNAs bleek vergelijkbaar met die voor siRNAs en verklaart de waargenomen verstoorde ontwikkeling van transgene 'Arabidopsis' planten waarin het NS3 eiwit constitutief tot expressie komt.

Nadat de interferentiestrategie van RVBV NS3 bekend was, werd in hoofdstuk 3 onderzocht welke domeinen binnen het NS3 eiwit betrokken zijn bij siRNA binding. Door onderlinge

vergelijking van de aminozuurvolgorden van enkele tenuivirale NS3 eiwitten werden twee geconserveerde domeinen geïdentificeerd die mogelijk van belang konden zijn voor het functioneren van het eiwit. Deletie van elk van deze twee domeinen resulteerde inderdaad in disfunctionele suppressoreiwitten. Door in één van deze twee domeinen een aanwezige cluster van drie positief geladen aminozuren (lysines 173-175) te vervangen, ging de affiniteit voor siRNAs volledig verloren en dit resulteerde in een defect RNA silencing suppressoreiwit. Dit bevestigde dat siRNA binding cruciaal is voor de RNA silencing suppressieactiviteit van NS3.

De (NSs) suppressoreiwitten van tospovirussen zijn nader onderzocht in hoofdstuk 4. In tegenstelling tot NS3 van tenuivirussen bleek het NSs eiwit van tospovirussen niet alleen korte siRNAs (en miRNAs) te binden, maar tevens langere dubbelstrengs RNA moleculen. Dit gaf het NSs eiwit het vermogen om eveneens het knippen van langere dsRNA moleculen tot siRNAs door Dicer te blokkeren. Tospovirussen behoren tot de Bunyaviridae, een virusfamilie die vooral dierpathogene virussen omvat. Aldus zijn deze plantenvirussen direct genetisch verwant aan diervirussen. Het vermogen van het tospovirale NSs eiwit om ook langere dsRNA moleculen te binden weerspiegelt wellicht deze directe verwantschap, want immers voor diervirussen biedt de capaciteit om ook lang dsRNA te binden een extra voordeel om tevens een antagonist te zijn van een ander afweermechanisme, de interferon respons. Recentelijk zijn reeds enige aanwijzingen verkregen dat mogelijk ook diervirussen coderen voor suppressors van RNA silencing, dat derhalve dus ook antiviraal gericht zou zijn. Zo was van het Influenzavirus A NS1 eiwit vastgesteld dat deze positief scoort als RNA silencing suppressor in zowel een plant- als een insect-gebaseerd testsysteem (Bucher, 2006; Li *et al.*, 2004). In Hoofdstuk 5 is deze potentiële antagonistische functie van NS1 nader onderzocht. Gebruikmakend van homologe (humane) celsystemen kon vastgesteld worden dat NS1 uitsluitend langere dsRNA moleculen bindt, maar geen siRNAs of miRNAs. Dit zou betekenen dat, indien NS1 een suppressor van RNA silencing zou zijn, deze zou werken door remming van Dicer. Dit bleek inderdaad het geval (hoofdstuk 5). Twee puntmutaties in het RNA bindende domein van NS1, dat eerder was aangetoond belangrijk te zijn voor zowel suppressie van RNA silencing als de interferon respons, waren voldoende om deze Dicer remming teniet te doen. De mogelijke betrokkenheid van NS1 bij onderdrukking van RNA silencing kon verder bevestigd worden door dit mutant NS1 eiwit te testen in de context van een volledige virusinfectie in cellijnen. Een recombinant influenzavirus, dat het mutant NS1 eiwit (PR8-NS1rb) tot expressie bracht, bereikte een aanzienlijk lagere virustiter dan virus dat het originele NS1 eiwit (PR8-NS1) tot expressie bracht. Omdat er geen aantoonbare productie van interferon was in geval van infectie met het PR8-NS1rb virus, wees dit resultaat op een antivirale rol voor RNA silencing in zoogdieren. Een tweede test bevestigde deze interpretatie: het NS1 eiwit, maar niet het NS1rb eiwit, was in staat om een Tat-minus Humaan immunodeficiëntie virus-1 (HIV-1) te complementeren. Opmerkelijk was dat ook het NS3 eiwit van RHBV hiertoe in staat was, waarmee tevens aanwijzingen verkregen werden dat kleine dsRNA moleculen een rol spelen in RNA silencing in (zoog)dieren.

Geconcludeerd kan worden dat min-strengs RNA-virussen van planten suppressoreiwitten coderen die RNA silencing blokkeren door dsRNA te binden. De suppressoreiwitten van tenuivirussen en tospovirussen werken daarbij verschillend: terwijl tenuiviraal NS3 alleen interfereert met RISC assemblage, remt NSs bovendien Dicer activiteit. Beide strategieën maken de suppressie van RNA silencing in de plant en insectenvector mogelijk. De behaalde resultaten met het NS1 eiwit van Influenza virus A ondersteunen de hypothese dat ook (zoog)diervirussen dienen te coderen voor een eiwit om siRNA of miRNA geïnduceerde antivirale RNAi te weerstaan.

Dankwoord

Waarschijnlijk worden de volgende pagina's, mijn laatste loodjes, het meest gelezen in dit proefschrift. In dit laatste deel wil ik alle mensen die een kleinere of grotere bijdrage aan dit proefschrift hebben geleverd bedanken.

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Curriculum Vitae

Op 8 januari 1978 ben ik, Johannes Cornelis Hemmes, geboren in Elburg. Na het behalen van mijn VWO-diploma aan ‘de Heemgaard’ in Apeldoorn ben ik begonnen aan de studie Bioprocestechnologie aan Wageningen Universiteit. Tijdens de doctoraalfase heb ik een afstudeervak verricht bij de vakgroep Virologie (dr. Monique van Oers en dr. Stephen Kaba) en een stage van 6 maanden gevolgd bij de Biochemie en Proteomics groep (dr. Wojtek Michalski) aan het Australian Animal Health Laboratory (Geelong, Australië). Een tweede afstudeervak heb ik gevolgd bij de vakgroep Moleculaire Biologie (dr. Joan Wellink) van Wageningen Universiteit in samenwerking met Plant Research International (dr. Daniella Kasteel, Bio-interacties en Plantgezondheid) en het Rijksinstituut voor Volksgezondheid en Milieu (dr. Adam Meijer, Laboratorium voor Infectieziekten en Perinatale Screening). Per 1 december 2002 ben ik begonnen met mijn promotieonderzoek bij de vakgroep Virologie van Wageningen Universiteit onder begeleiding van dr. ir. Marcel Prins en prof. dr. Rob Goldbach. Van het daar uitgevoerde onderzoek, gefinancierd door de Europese Unie en een ‘EMBO short term fellowship’ - ten behoeve van een onderzoeksstage bij de Plant Virology Group van dr. József Burgyán en onder begeleiding van dr. Lóránt Lakatos aan het Agricultural Biotechnology Center, Gödöllõ, Hungary - staan de resultaten beschreven in dit proefschrift. Sinds 1 november 2007 ben ik werkzaam als Postdoc bij het Laboratory of Plant Molecular Biology (prof. dr. Nam-Hai Chua), Rockefeller University (New York, USA).

Account

Kaba, S. A., Hemmes, J. C., van Lent, J. W., Vlak, J. M., Nene, V., Musoke, A. J. & van Oers, M. M. (2003). Baculovirus surface display of Theileria parva p67 antigen preserves the conformation of sporozoite-neutralizing epitopes. Protein engineering 16, 73-78.

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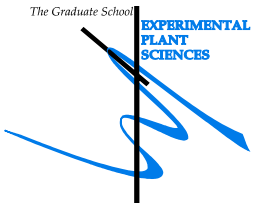
Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Hans Hemmes
Date: 26 November 2007
Group: Laboratory of Virology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
RNA silencing and viral counter defences	Jan 2003
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Radiation course level 5B	2003
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Wageningen University	Sep 13, 2007
► **EPS theme symposia**	
► **NWO Lunteren days and other National Platforms**	
Nederlandse Kring voor Plantenvirologie	Apr 16, 2003
NWO-CW studiegroep nucleinezuren	Dec 01-02, 2003
ALW Experimental Plant Sciences	Apr 07-08, 2004
4th joint meeting of Dutch and German plant virologists	Mar 10-11, 2005
ALW Experimental Plant Sciences	Apr 04-05, 2005
Dutch Annual Virology Symposium (DAVS), Amsterdam, The Netherlands	Mar 26, 2006
NWO-CW studiegroep nucleinezuren	Dec 04-05, 2006
► **Seminars (series), workshops and symposia**	
Symposium on BioNanotechnology, Wageningen, The Netherlands	May 12, 2006
Prof. dr. Rob Martienssen: 'Small RNA, chromosome organization and gene expression'	Oct 23, 2006
► **Seminar plus**	
Prof. dr. Rob Martienssen: 'Small RNA, chromosome organization and gene expression'	Oct 23, 2006
► **International symposia and congresses**	
EU-VIS meeting: Gödöllő, Hungary	2003
Keystone symposium: siRNAs and miRNAs (Keystone USA)	Apr 14-19, 2004
EU-VIS meeting: Heraklion, Crete	2004
EU-VIS meeting: Wageningen	2004
Keystone symposium: Diverse roles of RNA in gene regulation (Breckenridge, USA)	Jan 08-14, 2005
EU-VIS meeting: Dublin, Ireland	2005
Keystone symposium: RNAi and related pathways (Vancouver, Canada)	Jan 26-31, 2006
► **Presentations**	
EU-VIS meeting: Gödöllő, Hungary	2003
Poster presentation Keystone symposium: siRNAs and miRNAs (Keystone USA)	Apr 14-19, 2004
4th joint meeting of Dutch and German plant virologists	Mar 10-11, 2005
EU-VIS meeting: Dublin, Ireland	2005
Plant Virology group, ABC, Plant Biology Institute (Gödöllő, Hungary)	Nov 24, 2005
Poster presentation Keystone symposium: RNAi and related pathways (Vancouver, Canada)	Jan 26-31, 2006
NWO-CW studiegroep nucleinezuren	Dec 04-05, 2006
► **IAB interview**	Jun 03, 2005
► **Excursions**	
De Ruyter Zaden	2004
Subtotal Scientific Exposure	*17.5 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
RNAi course, Gödöllő, Hungary	Jun 01-05, 2004
► **Journal club**	
Literature discussion and work discussions at Virology	Dec 2002-Dec 2006
► **Individual research training**	
EMBO Fellowship at Agricultural Biotechnology Center, Plant Biology Institute, Plant Virology Group (Gödöllő, Hungary)	Sep-Nov, 2005
Subtotal In-Depth Studies	*7.5 credits**

4) Personal development	date
► **Skill training courses**	
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*0.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**28,0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS

** A credit represents a normative study load of 28 hours of study*

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