Antiviral RNA silencing and viral counter defense in plants

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If more of us valued food and cheer and song above hoarded gold,
it would be a merrier world.
J.R.R. Tolkien (1892-1973)

To my beloved Danijela,
my sister and my parents

Contents

CHAPTER 1

General Introduction

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Over the last years RNA silencing in plants and its animal counterpart RNA interference (RNAi) have become intensively studied biological systems. While initially being discovered as a side effect of transgene expression in plants and a process by which transgenic virus resistance could be obtained, it has since been implicated in natural virus resistance and basic biological processes such as development, gene regulation and chromatin condensation. RNA silencing related mechanisms are not only limited to plants, but also play a role in a variety of eukaryotic organisms. Due to the biochemical dissection of components of the silencing pathway in several model organisms, such as *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster*, the general understanding of how RNA silencing works has greatly increased in recent years. The revelation of a striking level of conservation of the RNA silencing pathway between most eukaryotic organisms strengthens its importance. Nowadays, RNA silencing induced by double stranded RNA (dsRNA) molecules such as short hairpins, short interfering RNAs (siRNAs) and long dsRNAs has developed into a standard tool in gene function studies (gene knock-down). It is being applied in large automated genome screens, where a majority of genes of certain organisms (e.g. *C. elegans* and *Homo sapiens*) are knocked-down and analysed using different assays depending on the research interests. In plants RNA silencing is used as a generally applicable antiviral strategy.
In this chapter, I will describe the RNA silencing process with emphasis on the functioning of the mechanisms and its role in natural virus infection in plants. In addition, applications of RNA silencing in plants and implications of RNA silencing for research in other organisms will be discussed.

The discovery of RNA silencing

The first recognized encounter with RNA silencing was when van der Krol, Napoli and their respective co-workers (Napoli, 1990; van der Krol *et al.*, 1990) reported their inability to over-express chalcone synthase (CHS) in transgenic petunia plants. In order to obtain an increase of flower pigmentation, petunia plants were transformed with the CHS gene using different constructs that should have led to over-expression. However, instead of observing an increase of flower pigmentation, the opposite effect was observed: some plants completely lacked pigmentation in the flowers and others showed patchy or reduced pigmentation. It was shown that even though an extra copy of the transgene was present, the CHS mRNA levels were strongly reduced in the white sectors. Since the transgene RNA was suppressing not only its own expression, but also the

endogenous gene this observation was called ‘co-suppression’.

Not much later, another encounter with RNA silencing was made in the field of virus resistance where the concept of pathogen-derived resistance (PDR) was being exploited to produce virus resistant plants. Using different viral systems, three reports demonstrated that in contrast to the original notion, the expression of viral proteins was not required for virus resistance, but untranslatable viral RNA sufficed (Lindbo & Dougherty, 1992; de Haan *et al.*, 1992; van der Vlugt *et al.*, 1992). Since the virus resistance in the recovered plant parts correlated with reduction of transgene mRNA in the cytoplasm, Lindbo and co-workers (Lindbo *et al.*, 1993) proposed this phenomenon to be similar to co-suppression. The observation that a silenced GUS transgene could prevent virus accumulation of Potato virus X (PVX) carrying GUS sequences pointed toward an actual role of, what was then called post-transcriptional gene silencing (PTGS), as a sequence specific antiviral defense mechanism (English *et al.*, 1996). Supporting evidence of the more general nature of this plant response to viral infection was provided by the finding that the recovered parts of virus infected plant would not only be resistant against the initially inoculated virus, but would also cross-protect the plant against other viruses carrying homologous sequences (Ratcliff *et al.*, 1999). In addition, this work showed that viral RNA-mediated cross protection was caused by the same mechanism as transgene induced PTGS. These phenomena are now generally known as virus-induced gene silencing (VIGS). The identification of different *A. thaliana* mutants exhibiting impaired RNA silencing revealed more details about the mechanisms involved in this process (Dalmay *et al.*, 2000; Ratcliff *et al.*, 1999). Certain mutants affected in the silencing pathway showed enhanced susceptibility to virus infection, confirming their involvement in antiviral activity (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). Over recent years, many components of the plant silencing pathways (Fig. 1-1) have now been uncovered and will be further discussed later in this chapter.

Silencing of endogenous and viral genes has now become a commonly used method. Transgene constructs can be arranged as inverted repeats, producing double-stranded RNA (dsRNA), which efficiently trigger silencing of homologous genes (Smith *et al.*, 2000). This can be used to obtain transgenic virus resistance or endogenous gene knock-down. For gene knock-down VIGS is often preferred to the production of transgenic plants, as this fast method can give a first indication on whether a gene knock-down produces the expected phenotype (Lu *et al.*, 2003).

To explain the extreme sequence specificity of the RNA silencing process, small RNA molecules had been envisaged in models throughout the second half of the nineties. However, it was not until 1999 that Hamilton and Baulcombe (Hamilton & Baulcombe, 1999) unequivocally proved

that plants containing a silenced transgene indeed accumulated small (ds)RNA molecules whose sequence was identical to the transgene. They observed the same kind of approximately 25 bp sequence-specific small RNAs in PVX infected plants, suggesting a role of these molecules in a sequence specific antiviral defense mechanism. A further breakthrough pointing to the involvement of RNA silencing in antiviral defense was the discovery of virus specific RNA silencing suppressors (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Voinnet *et al.*, 1999). This will be discussed in detail in subsequent paragraphs.

A next step to an increased general notion of RNA silencing was achieved in animal research. In *C. elegans* sense and anti-sense transcripts were already being used for quite some time to knock-down gene expression. However the real break-through came when Fire and co-workers (Fire *et al.*, 1998) discovered that injection of very low amounts of dsRNA into *C. elegans* could induce what they called RNAi. Like in plants, this method of RNA silencing was much more efficient than just using single-stranded sense or anti-sense RNA. Building blocks of the gene silencing pathway proved to have remarkable similarities in different organisms and hence suggest an ancient role of RNA silencing in development, gene regulation, pathogen resistance, and chromatin structure.

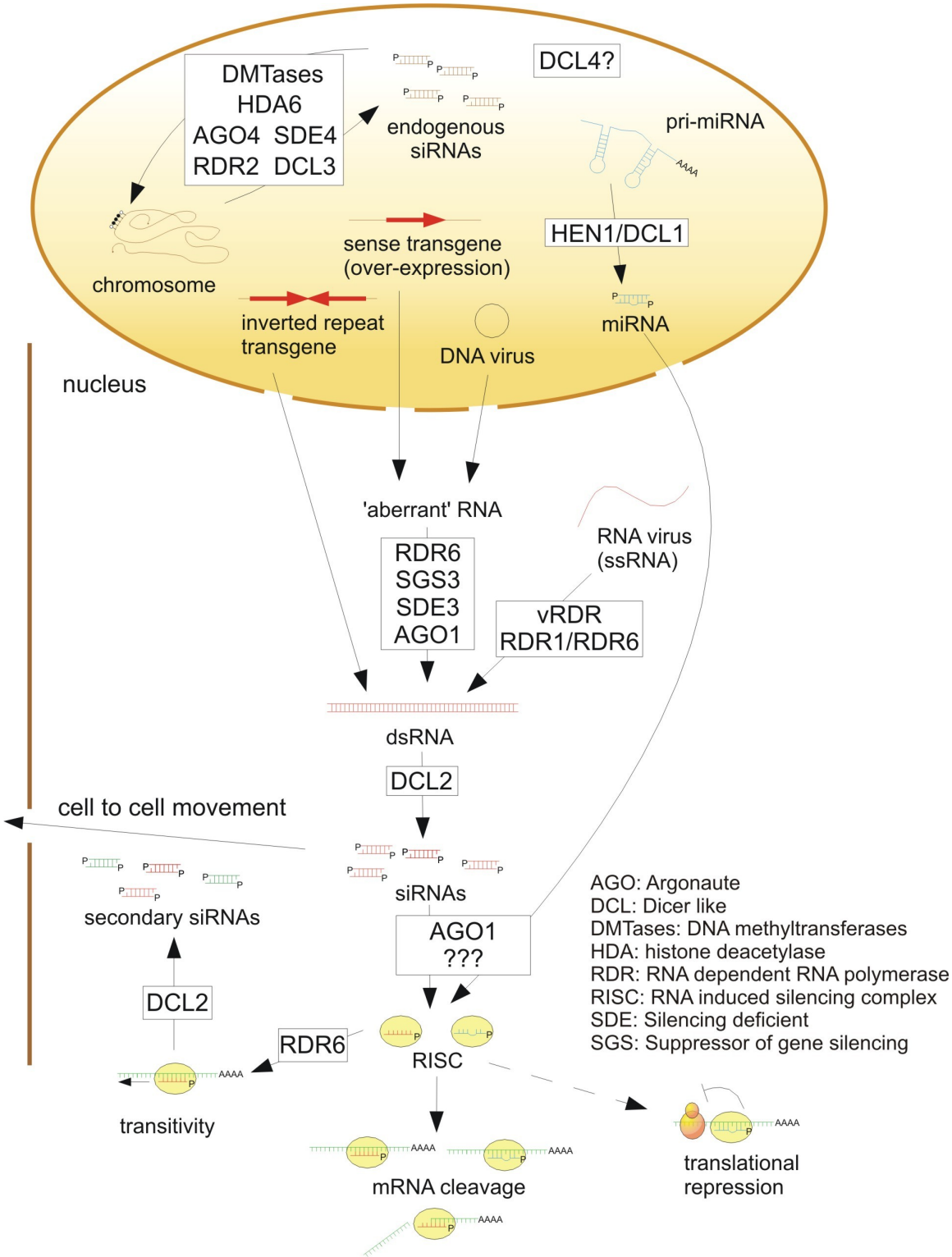

Figure 1-1. A model of the RNA silencing pathways in plants. The squares indicate identified proteins or genes involved in the different silencing processes. ??? indicates the position of proteins associating with DICER such as R2D2 that have been identified in animals but not (yet) in plants.

Mounting the plant antiviral defense

In plants, the control of virus replication is considered as one of the primary roles of RNA silencing. Although expressing viral transgene RNAs can precondition this response, the natural response is adaptive and requires recognition of 'foreign' molecules for initiation. This recognition is subsequently converted into 'effector', 'memory' and 'warning' signals to alert the systemic parts of the plant. DsRNA molecules have been shown to be most potent initiators of RNA silencing (Smith *et al.*, 2000). As most plant viruses are RNA viruses that replicate via double stranded replication intermediates, it is tempting to suggest that these molecules are a trigger for RNA silencing. The situation, however, is more complex. Most, if not all plant RNA viruses may replicate via dsRNA. The chance that these RNAs appear as naked RNA in the cell is very small since replication complexes are protected by viral replication and/or capsid proteins. Viral replication often takes place inside specialized replication structures and dsRNA can immediately be unwound by viral and host RNA helicases (Ahlquist, 2002). Though we do not dismiss the possibility of detection of these structures by RNA silencing, we think viral mRNAs, which might be recognized by the plant as being 'aberrant' (e.g. non-capped or non-polyadenylated mRNAs), are (also) an important target which can be converted into double stranded RNA by plant RNA-dependent RNA polymerases (RdRps). This would explain the generation of virus specific siRNAs in plants infected with geminiviruses (single-stranded DNA viruses) (Vanitharani *et al.*, 2003).

The Arabidopsis genome encodes four Dicer-like enzymes that have the ability to process dsRNA into siRNA molecules (Schauer *et al.*, 2002). In a normal virus infection, plants contain a significant amount of siRNAs originating from the virus (Hamilton & Baulcombe, 1999). These siRNAs can subsequently be used in two ways: either they are unwound and one strand is incorporated into the RNA induced silencing complex (RISC) to target and degrade RNAs homologous to the siRNA, or a plant RdRp uses the siRNA as a primer on homologous mRNAs and synthesizes dsRNA that then is processed by Dicer into secondary siRNAs (a phenomenon called 'transitivity') (Vaistij *et al.*, 2002). This latter step leads to the amplification of the intracellular silencing signal. In plants, RNA silencing generated secondary siRNAs can originate from 5' and 3' parts of the targeted site in the messenger, indicating that the transitivity is bidirectional. This is in contrast to *C. elegans* where secondary siRNAs only originate from the 5' side of the target mRNA in relation to the inducer molecule (Sijen *et al.*, 2001). This may be related to the fact that both siRNA strands seem to be stable in plants (Hamilton & Baulcombe, 1999), while in *C. elegans*, only the antisense strand is maintained. In mammals and insects, transitivity was reported not to be

present. Indeed no endogenous RdRp, which would be required for this activity, has been identified (Schwarz *et al.*, 2002). Next to the predominant 21 nt species of siRNAs observed in all eukaryotes, the plant silencing machinery has the unique ability to produce, a second size class of siRNAs, of around 24 nt (Hamilton *et al.*, 2002). The longer class of siRNAs has been correlated with the long-distance spread of RNA silencing. This ability allows the viral siRNAs produced by the plant silencing machinery to move to adjacent cells advancing the spread of the virus. RISC is thought to be pre-programmed with these siRNAs allowing an immediate recognition and elimination of incoming viruses. The shorter class of siRNAs is thought to operate in local RNA silencing (Hamilton *et al.*, 2002). This size class has also been reported to be able to move from cell to cell, however, spreading no further than up to 15 cells (Himber *et al.*, 2003).

Of great interest and confirming the biological role of RNA silencing in antiviral defense, was the discovery that nearly all plant viruses investigated so far encode RNA silencing suppressors. The interference of plant viruses with the RNA silencing machinery will be discussed in more detail in the next paragraph.

Suppressor proteins: Viral counter measures against RNA silencing

Even though an RNA-based sequence-specific defense against virus infection may be efficient, there are still many viruses that successfully infect plants. The discovery of viral RNA silencing suppressors gave a first hint on how viruses could counteract the plant defense. An indication that these counter measures were developed as an answer to RNA silencing is their great diversity. None of the RNA silencing suppressors discovered so far share any significant sequence homology with those from other viruses. In addition, the RNA silencing antagonists encoded by different plant viruses appear to suppress this virus defense pathway at different points.

It has long been known that certain proteins expressed by viruses played an important role in their virulence (Pruss *et al.*, 1997). It was observed that co-infection of combinations of viruses could cause increased symptom severity compared to each of the viruses alone. These mixed infections indicated that at least one of the viruses possessed a character that could support the replication and spreading of the other virus. Potyviruses were reported early on to increase the virulence levels of another virus. The actual underlying mechanism started to become understood in studies of mixed infections of PVX with different potyviruses. Mutational analysis of the *Tobacco etch virus* (TEV) revealed that the helper component-proteinase (HC-Pro) was required for the

synergistic activity of TEV (Shi *et al.*, 1997). A first indication that HC-Pro could actually block a general plant antiviral pathway was found when transgenic plants constitutively expressing HC-Pro were produced. Heterologous viruses such as TMV and CMV showed enhanced accumulation and pathogenicity in these plants (Pruss *et al.*, 1997). In the case of CMV, virulence could be linked to its 2b protein (Brigneti *et al.*, 1998). These results were later confirmed by studies where the 2b gene of different CMV subgroups were replaced (Shi *et al.*, 2002). Indication that RNA silencing is indeed involved in virus resistance came with the reports that HC-Pro can enhance virulence of heterologous viruses by directly suppressing RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 2001).

Assays used to identify suppressors of RNA silencing

Following the discovery of HC-Pro as a suppressor of RNA silencing many other viruses were shown to express proteins capable of inhibiting this antiviral mechanism (Tab. 1-1). The establishment of relatively simple and reliable functional assays to detect suppressors of RNA silencing greatly accelerated their discovery.

Currently, one of the most commonly used methods for the identification of potential suppressors of RNA silencing is a transient assay using *Agrobacterium tumefaciens* (Johansen & Carrington, 2001; Llave *et al.*, 2000; Voinnet *et al.*, 2000). In this assay, two *A. tumefaciens* strains are used to deliver a reporter gene (often the gene encoding the green fluorescent protein, GFP) and a putative suppressor protein. The *A. tumefaciens* culture mix is infiltrated into a *Nicotiana benthamiana* leaf and reporter gene expression is monitored. Typically, without a suppressor of RNA silencing, the reporter gene becomes silenced after three to five days. However, if an *A. tumefaciens* strain carrying a strong suppressor of RNA silencing between the T-DNA borders is mixed with the ones carrying the reporter gene and co-infiltrated, the reporter gene expression will remain at its high level or even increase during the six days. Using different reporter constructs, such as genes arranged as inverted repeats, one has the possibility to assess at which step of RNA silencing the suppressor protein acts (Takeda *et al.*, 2002).

Another method makes use of GFP or GUS (beta-glucoronidase) silenced transgenic plants. Plants expressing a reporter gene are systemically silenced by the infiltration of *A. tumefaciens* expressing (a fragment of) the RNA of that reporter gene, or plants are genetically silenced (e.g. using inverted repeats). Subsequently, these plants are infected with different viruses or virus constructs and the reporter gene expression is monitored. Restoration of reporter gene expression indicates that the tested virus encodes a suppressor of RNA silencing. PVX encodes a suppressor of

RNA silencing that cannot restore the reporter gene expression in this assay and makes it a good vector to test other viral genes for their silencing suppression capability (Brigneti *et al.*, 1998). Additionally it has been observed that if PVX expresses a heterologous suppressor of RNA silencing it causes more severe symptoms compared to the empty vector (Brigneti *et al.*, 1998; Pruss *et al.*, 1997).

Finally, one can produce transgenic plants that constantly express a suppressor of RNA silencing. A significant drawback with this method is that (high) expression of suppressors of RNA silencing often leads to developmental defects in the plants (Anandalakshmi *et al.*, 1998). Nevertheless, some successes have been reported (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003).

RNA silencing suppressor proteins

Even though many viral suppressors of RNA silencing have been described so far (Tab. 1-1), extensive research was focused on a selection of these proteins.

HC-Pro of potyviruses

The first and best described suppressor of RNA silencing is the potyviral HC-Pro protein (Anandalakshmi *et al.*, 1998). It was shown to suppress RNA silencing in experiments where plants, in which a reporter gene was silenced, were infected with PVX carrying HC-Pro. Upon systemic infection by this chimeric virus, reversal of the silenced state of the reporter gene was observed. Additionally, Anandalakshmi and co-workers (Anandalakshmi *et al.*, 1998) showed that crossing a GUS silenced plant line and a HC-Pro expressing plant line could restore GUS expression. On the molecular level it was shown that HC-Pro prevented the degradation of the reporter gene mRNA (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998). Further analysis revealed that HC-Pro could prevent the degradation of the reporter mRNA into siRNAs (Hamilton *et al.*, 2002). This means that HC-Pro could inhibit, for instance, an RNase III-like enzyme involved in the processing of dsRNA into the siRNAs or a component of the RNA silencing effector complex RISC. Interestingly, HC-Pro did not affect the silencing signal from moving through the plant, even though all siRNAs were eliminated (Mallory *et al.*, 2001). However, HC-Pro was shown to efficiently prevent the plant from responding to the silencing signal in grafting experiments. It is noteworthy that Hamilton and co-workers (Hamilton *et al.*, 2002) reported that HC-Pro could interfere with the silencing signal. These conflicting observations could be a result of different assays being used by the different groups (*A. tumefaciens* infiltration versus grafting). Additionally, there are conflicting reports on

whether or not HC-Pro affects the methylation of a silenced transgene locus in the plant genome (Llave *et al.*, 2000; Mallory *et al.*, 2001).

A first indication on how HC-Pro actually suppresses RNA silencing was shown by protein-protein interaction studies using the yeast two hybrid assay. Anandalakshmi and co-workers (Anandalakshmi *et al.*, 2000) identified a calmodulin related protein rgs-CaM (regulator of gene silencing-calmodulin-like protein) that directly interacts with HC-Pro. In addition, its expression is up-regulated by the suppressor protein. It was found that rgs-CaM could act like an endogenous suppressor of RNA silencing. Transgenic plants over-expressing rgs-CaM showed phenotypic changes very similar to HC-pro transgenic plants such as tumour-like structures at the stem-root junction. From that, it was concluded that HC-Pro suppresses silencing, at least in part, by stimulating the expression of rgs-CaM.

Recently, HC-Pro has been shown to influence microRNA (miRNA)-mediated gene regulation, explaining in part the developmental defects observed in transgenic plants (Kasschau *et al.*, 2003; Mallory *et al.*, 2002). This effect will be discussed further in a later section of this chapter.

A recent report on the structure of the HC-Pro protein confirmed earlier reports that it can form dimers (Plisson *et al.*, 2003). Additionally the structure reveals three domains that correlate with three different functions of that protein. Interestingly, the domain involved in RNA-binding correlates with the domain required for silencing suppression (Kasschau & Carrington, 2001).

Taken together, the data indicates that HC-Pro suppresses RNA silencing downstream of dsRNA and miRNA formation. However, it also acts upstream of the siRNA production and possibly interferes with the systemic silencing signal.

Table 1-1. Suppressors of RNA silencing of different plant viruses that have been identified so far. *Different results have been reported by different groups.

Genome	Genus	Virus	Suppressor protein	Suppressed RNA silencing mechanism	Reference
DNA	Begomoviru	ACMV	AC2	local	Voinnet, et al. 1999; Vanitharani, et al. 2004
			AC4	systemic	Vanitharani, et al. 2004
		TGMV	AL2	-	Wang, et al. 2003
		TYLCV-C	C2	local and systemic	Dong, et al. 2003; Van Wezel. et al. 2003
	Curtovirus	BCTV	L2	-	Wang, et al. 2003
(+) RNA	Carmovirus	TCV	CP (P38)	local	Thomas, et al. 2003; Qu, et al. 2003
	Closteroviru	BYV	p21	local	Reed, et al. 2003
		CTV	p20	local	Lu, et al. 2004
			p23	local and systemic	Lu, et al. 2004
			CP	systemic	Lu, et al. 2004
		BYSV	p22	local	Reed, et al. 2003
	Comovirus	CPMV	S coat protein	local	Voinnet, et al. 1999; Canizares; et al. 2004; Liu, et al. 2004
	Cucumoviru	CMV	2b	local* and systemic	Brigneti, et al. 1998; Lucy et al. 2000
	Furovirus	PCV	P15	local and systemic	Dunoyer, et al. 2002
	Hordeivirus	BSMV	γb	-	Yelina, et al. 2002
	Polerovirus	BWYV	P0	local and not systemic	Pfeffer, et al. 2002
	Potexvirus	PVX	P25	systemic	Voinnet, et al. 2000
	Potyvirus	PVY	HC-Pro	local and systemic*	Brigneti, et al. 1998; Anandalakshmi, et al. 1998
	Sobemovirus	RYMV	P1	-	Voinnet, et al. 1999
	Tobamovirus	TMV	126-kDa protein	-	Voinnet, et al. 1999; Ding, et al. 2004
		ToMV	130-kDa protein	local	Kubota, et al. 2003
	Tombusvirus	TBSV	P19	local and systemic (binds siRNAs)	Voinnet, et al. 1999; Lakatos, et al. 2004
		CymRSV	P19	local and systemic (binds siRNAs)	Silhavy, et al. 2002
	Tymovirus	TYMV	p69	local	Chen, et al. 2004

2b of cucumoviruses

While HC-Pro had a direct and strong effect on the maintenance of RNA silencing, *Cucumber mosaic virus* (CMV) 2b was shown to affect the RNA silencing pathway differently. 2b cannot suppress RNA silencing in tissues where RNA silencing is already established. However, it was shown to be able to prevent the initiation of RNA silencing in newly emerging tissue (Beclin *et al.*, 1998; Brigneti *et al.*, 1998). This suggested that 2b might be involved in inhibiting the systemic spreading of the silencing signal. Further analysis revealed that CMV 2b carries a monopartite nuclear localization signal (NLS) that is required for the 2b silencing suppression activity (Lucy *et al.*, 2000). This was very surprising, since at that time components of RNA silencing were thought to operate in the cytoplasm only. How 2b prevents the silencing signal from spreading throughout the plant remains to be investigated.

Guo and Ding (Guo & Ding, 2002) showed that 2b interferes with the restoration of transgene methylation, giving a first hint on the function of 2b in the nucleus. It was also postulated that 2b was not able to prevent signal-independent RNA silencing initiation of transgene and virus silencing. Additional observations showed that CMV suppresses RNA silencing in mixed infection experiments on transgenic plants expressing dsRNA targeting PVY. The PVY derived dsRNA expressed in these plants renders them immune to PVY infection. However, when PVY was co-inoculated with CMV these plants showed a transient PVY accumulation (Mitter *et al.*, 2003). Additionally, CMV caused a high increase of transgene mRNA levels by preventing its degradation into siRNAs. From these investigations it can be concluded that 2b inhibits the systemic propagation of a silencing signal which would be sent out from the initially infected loci to the rest of the plant and prevent further spreading of the virus.

Finally it is interesting to add that some experiments showed that 2b could also reduce the inhibitory effect of salicylic acid (SA) on virus accumulation (Ji & Ding, 2001). Even though a recent finding reported that a SA inducible RdRp (RDRP1 in Arabidopsis) is involved in TMV resistance, this RdRp had no effect on CMV accumulation (Yu *et al.*, 2003). This indicates that different silencing pathways may be involved in the antiviral defense depending on the infecting virus. Furthermore (Yang *et al.*, 2004) recently showed that the high susceptibility of *N. benthamiana* to viruses in general could at least in part be explained by the fact that its RDRP1 homologue is mutated.

P19 of tombusviruses

One of the most immediate suppressors of RNA silencing is P19 of the tombusviruses, such as

Cymbidium ringspot virus (CymRSV). P19 was found to suppress RNA silencing by binding siRNAs in their double stranded form (Silhavy *et al.*, 2002). P19 only very inefficiently binds single-stranded siRNAs, long dsRNAs, or blunted 21 nucleotide (nt) dsRNAs. However, a 2 nt overhang at the 3' end is sufficient for P19 to bind 21 nt RNA duplexes (Silhavy *et al.*, 2002). The step of the RNA silencing pathway upon which P19 has an effect on was indicated by biochemical experiments performed in Drosophila cell extracts. It was found that P19 activity prevents siRNAs from incorporating into RNA silencing effectors such as RISC (Lakatos *et al.*, 2004). Furthermore, specific binding of siRNAs by P19 efficiently blocks the development of systemic spreading of silencing. This substantiates the previously suggested involvement of siRNAs in the spreading of RNA silencing. Either P19 suppresses systemic silencing by binding the siRNAs, preventing them from moving through the plant, or it inhibits the activity of an siRNA-primed RdRp which is thought to be involved in the formation of the systemic signal (Voinnet, 2001).

The elucidation of the crystal structure of P19 binding a 21 nt siRNA duplex finally provided information on the property of the physical interaction between P19 and siRNAs. The structure of P19 elegantly shows how dimers of this protein are capable of recognizing RNA duplexes with the length of 21 nt and overhanging 3’ nucleotides that are typical for siRNAs (Vargason *et al.*, 2003). The finding that P19 specifically binds siRNAs, the molecule conserved among all silencing-capable organisms, makes it a very potent tool to be used in all kinds of organisms. Indeed P19 has been reported to be active in insect (Lakatos *et al.*, 2004) and mammalian cells (Dunoyer *et al.*, 2004).

Like HC-Pro, P19 was shown to affect the processing and activity of miRNAs, a feature that will be discussed in a later section of this chapter.

RNA silencing suppressors of DNA viruses

In addition to the silencing suppressors of RNA viruses described above, DNA viruses have also been shown to encode suppressors of RNA silencing. This is interesting considering the fact that these viruses replicate in the nucleus and their genomes consist of DNA. Hence geminivirus-derived dsRNA intermediates never occur during replication. It has, however, been reported that geminiviral mRNAs in the plant are targeted by RNA silencing in a plant RdRp (RDR6, previously named SGS2/SDE1) dependent manner (Muangsan *et al.*, 2004). In GFP-silenced plants the bipartite geminivirus *African cassava mosaic virus* (ACMV) was shown to efficiently suppress RNA silencing and AC2 was identified to be its suppressor of RNA silencing (Vanitharani *et al.*, 2004; Voinnet *et al.*, 1999). However, for the *East African cassava mosaic Cameroon virus* (EACMCV)

the unrelated AC4 encodes a suppressor of RNA silencing. Similar to the synergism observed for PVX and PVY, mixed infections of ACMV and EACMCV revealed enhanced virulence. AC2 and AC4 were shown to be involved in this synergism. AC2 of ACMV could enhance EACMCV DNA accumulation and reciprocally AC4 increased the accumulation of ACMV DNA. Although RNA silencing was originally regarded as entirely cytoplasmic, there is evidence that elements of the mechanism also have effects in the nucleus (Fig. 1-1). The fact that AC2 requires a DNA-binding domain and an NLS for its activity as a suppressor of RNA silencing might fit this notion (Dong *et al.*, 2003). Also the AL2 and L2 proteins of the bipartite Tomato golden mosaic virus and the monopartite Beet curly top virus, respectively, were reported to act as suppressors of RNA silencing (Wang *et al.*, 2003). The way these proteins exercise their function is unclear, although they have been shown to increase susceptibility to virus infection by inactivating the SNF1 and ADK kinases (Hao *et al.*, 2003; Wang *et al.*, 2003). Whether and how these inactivated endogenous proteins are involved in RNA silencing is not known.

Considering their range of activities and lack of sequence homology, it appears that RNA silencing suppressors of the geminiviruses evolved independently even within the genus. It remains to be discovered, whether this is a mere reflection of the renowned plasticity of geminivirus genomes, or an indication of a powerful selection pressure (even on DNA viruses) to be able to counteract RNA silencing.

Other functions of RNA silencing

As important as it is, the antiviral activity of RNA silencing is certainly not its only function in plants. By using components of the RNA silencing machinery several other processes are supported. These processes play an important role in plants and perhaps even more so in other multicellular organisms. Among these processes are transposon silencing, transcriptional gene silencing due to sequence specific DNA methylation, chromatin condensation and (developmental) gene regulation by miRNAs. Perhaps more so than the other RNA silencing functions, the latter process, one of the most recent sapling of the RNA silencing tree, has turned out to be of major consequence for molecular biology as it influences gene expression in an unforeseen way and scale.

Transcriptional gene silencing

One of the first indications that RNA is involved in transcriptional gene silencing (TGS) in the

nucleus was done by Wassenegger and co-workers (Wassenegger *et al.*, 1994). Upon viroid infection of plants transformed with T-DNAs containing viroid cDNA sequences, the latter became methylated, while other parts of the T-DNA insertion remained unaffected. They concluded from this that the replicating viroid RNA had lead to specific methylation of homologous sequences in the plant genome. This phenomenon was termed RNA dependent DNA methylation (RdDM). Expression of dsRNA of promoter sequences was shown to be a trigger for sequence-specific RdDM of these promoters and subsequent TGS (Mette *et al.*, 2000). The fact that the promoter-derived dsRNA was processed to siRNAs suggests a role for the siRNAs in the sequence specific targeting of DNA methylation in the nucleus. Endogenous repeat-associated small RNAs possess the ability to trigger de novo methylation of cognate genomic DNA sequences and may thereby contribute to heterochromatin formation (Xie *et al.*, 2004). Recently several components of the RdDM pathway have been identified. While the DNA methyltransferases (DMTase) DRM1 and DRM2 were reported to be involved in the de novo RNA-directed methylation, the DMTase MET1 and the putative histone deacetylase HDA6 maintain or enhance methylation. Recruitment of HDA6 then reinforces CG methylation and finally heterochromatin is formed at the specific targeted loci (reviewed in (Matzke *et al.*, 2004)). Recent reports imply that AGO4 is also involved in long siRNA directed DNA methylation and its maintenance (Zilberman *et al.*, 2003). DCL1, which is required for miRNA processing, was shown not to be required for TGS (Finnegan *et al.*, 2003). The fact that siRNA induced TGS has also been found in human cell lines confirms the importance of RNA silencing in gene regulation through TGS (Morris *et al.*, 2004).

Chromatin modeling

A second role of methylation of perhaps a greater magnitude than TGS was recently discovered in *Schizosaccharomyces pombe*, where RNA silencing was shown to play a role in chromatin structure, centromeric cohesion and cell division. Mutational analyses showed that RNA silencing compounds were required for the pericentromere organisation in *S. pombe* (Volpe *et al.*, 2003). Three genes that encode key enzymes of the RNA silencing machinery, Argonaute (ago1+), Dicer (dcr1+) and an RdRp (rdp1+), were shown to be essential for this process. The RdRp is required for the production of the dsRNA from transcripts originating from the pericentromeric heterochromatin composed of complex repeats. These RNA duplexes are rapidly processed by Dicer and incorporated into what was termed the RNAi-induced transcriptional gene silencing (RITS) complex, a complex with high biochemical similarity to RISC (Verdel *et al.*, 2004). Ago1 of *S. pombe* is a key component of these complexes and binds the siRNA. RITS activity is exerted in the

dividing cell leading to the recruitment of the chromodomain protein Swi6, sequence specific methylation of centromeric regions and ultimately to chromosome condensation (Noma *et al.*, 2004). Though discovered in yeast, these features seem to be conserved among all eukaryotes including vertebrates (reviewed by (White & Allshire, 2004) and (Dawe, 2003)).

Transposon and endogenous repeat associated gene silencing

Like viruses, transposons represent a nucleic acid-based threat to plants. Movement of transposons to new insertion sites can cause major damage to the plant genome. To fight against transposons, plants have evolved a defense system based on RNA silencing. Indeed, it has been shown that plants produce the longer type of siRNAs derived from transposons (Hamilton *et al.*, 2002; Llave *et al.*, 2002a; Xie *et al.*, 2004). As discussed earlier these siRNAs can then lead to sequence specific RdDM and therefore transcriptional silencing of the transposons. Since transposon-derived siRNAs are present in plants, it must be concluded that transposon-derived dsRNA is being produced. Indeed, Arabidopsis mutant studies revealed the involvement of RDR2 and other RNA processing factors to be required for transposon silencing (reviewed in (Bender, 2004)). Similarly, a great body of work in *C. elegans* revealed that several factors involved in RNAi (mut-7 an RNaseD homolog, mut-14 an RNA helicase and mut-16) are required for transposon silencing (Sijen & Plasterk, 2003).

Cloning and sequencing of endogenous naturally occurring siRNAs of *A. thaliana* showed that these originate not only from transposons or retroelements, but also from highly repeated ribosomal DNAs (rDNAs: 5S, 18S and 25S) ((Llave *et al.*, 2002a; Xie *et al.*, 2004). Quite a number of sequenced siRNAs were found to be homologous to expressed and predicted genes. For the majority of these small RNAs it still remains to be investigated whether they act as miRNAs, which will be discussed in the next paragraph, or whether they are implicated in other biological processes yet to be identified.

Development: miRNAs regulating timing and patterning

One of the recent major discoveries in developmental biology was the finding that many higher organisms produce endogenous small RNAs that are essential for the regulation of genes, of which many are involved in development. The most notable of these are the miRNAs. miRNAs are characterized by their phylogenetic conservation across species and their involvement in basic biological processes of development, such as cell death and patterning. Typically, miRNAs are encoded by the genome as more or less imperfect inverted repeats as part of (much) larger processed transcripts, which are actively transported to the cytoplasm (Meister & Tuschl, 2004). Depending on

the degree of homology to the target mRNAs in the cytoplasm, these miRNAs guide the RISC complex for the cleavage or inhibition of translation of mRNAs homologous to the miRNA. Most miRNAs in plants studied so far have a (near) perfect match with their target mRNA in the open reading frame leading to mRNA cleavage (Rhoades *et al.*, 2002). Translational inhibition by miRNA binding but not cleavage was so far only observed in one case (APETALA2, (Aukerman & Sakai, 2003)), while this is the main mode of action for miRNAs in animals (Ambros, 2004).

First hints on the involvement of miRNAs in development were observed in *C. elegans* mutant screens. Worms carrying mutations in the genes producing non-coding small temporal RNAs (stRNAs) lin-4 and let-7 (Lee *et al.*, 1993; Reinhart *et al.*, 2000) were found to modulate developmental timing. The miRNAs encoded by lin-4 or let-7 are incorporated into a miRNA-ribonucleoprotein complex (miRNP) and inhibit the translation of mRNAs containing partial complementarity with the miRNA in the 3' UTR. By this mechanism miRNAs derived from the lin-4 and let-7 transcripts were shown to modulate the translation of their target genes lin-14, lin-28 and lin 41, hbl-1 respectively.

Many miRNAs have been cloned and sequenced in both plants and animals and a great number of genes have in the meantime been identified as being regulated by these miRNAs (an Arabidopsis small RNA database can be found at: http://cgrb.orst.edu/smallRNA/db/). Using computational methods, potential targets of these miRNAs were also indicated in plants (Rhoades *et al.*, 2002). It was found that many predicted miRNA targets are transcription factors involved in development. One group of transcription factors recently found to be regulated by miRNAs in an AGO1 dependent manner are of the Class III HD-Zip gene family. This family directs the polarity establishment in leaves and vasculature (Juarez *et al.*, 2004; Kidner & Martienssen, 2004). Interestingly, these authors propose the miRNAs to be a mobile signal during the establishment of the polarity of developing leaves.

Finally it should be noted that miRNAs are not only involved in development since predicted miRNAs also target genes involved in abiotic stress (Sunkar & Zhu, 2004). The involvement of miRNAs in so many different biological processes underlines its importance in biology (reviewed in (Ambros, 2004) and (Baulcombe, 2004)).

Plant viral RNA silencing suppressors interfere with miRNA action

As mentioned earlier, the expression of viral suppressors of RNA silencing in transgenic plants was shown to lead to strong developmental defects (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003). Further research revealed that these proteins interfere with the action of

miRNAs on the regulation of genes involved in plant development. For instance transgenic plants stably expressing HC-Pro over-accumulate miRNAs and show developmental defects (Kasschau *et al.*, 2003; Mallory *et al.*, 2002). Not only does HC-Pro change the accumulation levels of miRNAs, it also prevents their activity. It has been shown that HC-Pro could prevent the miRNA- guided cleavage of certain mRNAs and therefore cause a higher accumulation of these mRNAs. It appears that HC-Pro might affect the activity and the turnover of the miRNAs by interfering with one of the factors involved in their biogenesis or their cellular localisation.

Also P19 has been shown to interfere with the production of active miRNAs. Since P19 is capable of binding siRNA duplexes it was suggested that P19 could also bind the miRNA/miRNA* duplexes (miRNA* being the partly anti-sense strand of the active miRNA), thereby preventing its incorporation into RISC (Dunoyer *et al.*, 2004).

Whether the inhibition of miRNA function by RNA silencing suppressors, which leads to enhanced virulence, is a genuine role of these proteins in virus infection or a mere side effect of their inhibition of siRNA-mediated RNA silencing remains to be established.

The biochemistry of the RNA silencing machinery

Since the discovery of RNA silencing in animal model systems, the dissection of the RNA silencing machinery has caught up considerable speed. Though the RNA silencing mechanism in plants is the major focus of this chapter, knowledge on the RNA silencing machinery in plants also builds on information gathered from several animal model systems. Parts of the conserved RNA silencing machinery have been studied in many organisms ranging from plants to insects to mammals and back to protozoans. A comprehensive model encompassing the many-shared features is represented in Fig. 1-1.

The key action of RNA silencing involves a sequence-specific cytoplasmic degradation of RNA molecules. It can be induced in a variety of ways. For instance plant viral RNAs can be targeted after the transgenic expression of over-abundant or dsRNA. The key intermediary element in the RNA silencing pathway is dsRNA, which is recognised by a dsRNA-specific nuclease called Dicer, to yield small (21-23 nucleotides long) siRNAs. These siRNAs subsequently serve as guides for cleavage of homologous RNA molecules, mediated by RISC.

Dicer

In plants, several molecular processes can generate small RNAs. Naturally occurring small RNAs can be: (1) miRNAs involved in gene regulation; (2) endogenous siRNAs (also known as repeat associated siRNAs); (3) transposon-derived, and (4) virus-derived siRNAs. DsRNAs can also be produced artificially by the expression of constructs arranged as inverted repeats which will result in the production of siRNAs processed from long dsRNA precursors and destruction of mRNAs with a homologous sequence (Smith *et al.*, 2000). All siRNAs are products of cleavage of dsRNA by members of an RNase III-like enzyme family, first discovered in Drosophila (Bernstein *et al.*, 2001) and termed Dicer in animals or Dicer-like (DCL) in plants. Dicers are multi-domain proteins that typically contain one or more dsRNA binding domain(s), a DExH RNA helicase, a PIWI/ARGONAUTE/ZWILLE (PAZ) domain and two neighbouring RNase III-like domains. It has been reported that human Dicer works as an intra-molecular dimer of its two RNase III domains (Zhang *et al.*, 2004). The products of the endonucleic cleavage by Dicer enzymes are RNA duplexes that have 5' phosphates and 2 nt 3' overhangs, mostly around 21 nt in size. It is interesting to note that while many animals only encode a single Dicer, Drosophila encodes two (Lee *et al.*, 2004), and Arabidopsis has evolved four Dicer homologues (DCL1, DCL2, DCL3 and DCL4) (Schauer *et al.*, 2002). It would appear that the multiple roles Dicer plays in the different branches of the RNA silencing in animals are divided over the different homologues in plants.

In the case of Arabidopsis, the role of DCL4 is yet unknown, while DCL3, in concert with RDR2, plays a role in the production of endogenous siRNAs. As mentioned earlier, these endogenous siRNA are involved in the initiation or maintenance of a heterochromatic state (Matzke *et al.*, 2004), DCL2 was found to be involved in the production of siRNAs derived from viruses (Xie *et al.*, 2004). The fact that viral siRNA accumulation was not completely abolished in DCL2 mutant plants, but just delayed, suggests the existence of another redundant DCL enzyme. In addition to DCL2, the production of virus-derived siRNAs requires two RdRps (RDR1 and RDR6), depending on which kind of virus infects the plant (Muangsan *et al.*, 2004; Xie *et al.*, 2004). DCL1, together with other factors, such as HEN1 and HYL1 (a dsRNA binding protein), was shown to be responsible for the generation of miRNAs (Vazquez *et al.*, 2004; Xie *et al.*, 2004). The processing of the primary miRNA (pri-miRNA) to the miRNA duplex most probably occurs in the nucleus, but is also guided by DCL1. Interestingly, HEN1 is not only involved in miRNA biogenesis but also in transgene silencing and natural virus resistance as was shown by a CMV based sensitivity assay (Boutet *et al.*, 2003).

Compared to plants, processing of miRNA precursors in animals is different. The pri-miRNAs,

synthesised by the RNA polymerase II, are first processed by a nucleus-specific enzyme, Drosha, initially discovered in Drosophila (Filippov *et al.*, 2000), into precursor miRNAs (pre-miRNAs) (Lee *et al.*, 2003). These pre-miRNAs, imperfect hairpins of approximately 70 nt in length, are then exported to the cytoplasm and processed into miRNAs by the cytoplasmic Dicer.

RISC

Regardless of the way different Dicer enzymes produce siRNAs and miRNAs and their final destination, single strands of siRNA or miRNA duplexes are incorporated into RISC, the effector of RNA silencing. RISC provides the different (catalytic) functions such as mRNA cleavage and translational inhibition. RISC is a multi-protein complex of which several components have been identified. Small RNA molecules provide sequence-specificity to RISC. Like these small RNAs, ARGONAUTE (AGO) proteins have been found to be part of RISC in all organisms studied and are essential for its mRNA slicing activity. The term "Argonaute" refers to the squid-like appearance of the leaves of Arabidopsis mutants lacking AGO1 gene function (Bohmert *et al.*, 1998). To date, 10 members of the Argonaute family have been identified in plants. Two of them, AGO1 and AGO4, have been studied extensively. AGO1 mutant plants have been found to develop distinctive developmental defects. miRNAs accumulate normally in these plants, but their target mRNAs are no longer cleaved. Interestingly, the expression of AGO1 itself is regulated by a miRNA (miR168) indicating that the AGO1 protein regulates its own expression in a negative feedback loop (Vaucheret *et al.*, 2004). AGO4 has a role in the production of the 'long' siRNAs of 24 bp. While it is not known yet whether AGO4 mutants are affected in systemic RNA silencing, it was reported that AGO4 is involved in long siRNA mediated chromatin modification (histone methylation and non-CpG DNA methylation) (Zilberman *et al.*, 2003).

In Drosophila, AGO2 is part of RISC and essential for siRNA-directed RNA silencing. AGO2 is not required for the miRNA biogenesis, but a role for AGO1 was indicated (Okamura *et al.*, 2004). R2D2, a Dicer-2 associated protein, was shown to play an important role in binding and strand discrimination of siRNAs and miRNAs for incorporation of the proper RNA strands into RISC (Liu *et al.*, 2003). Though, R2D2 is not involved in the endonucleic cleavage of dsRNA to siRNAs, it stabilizes the association of Dicer-2 to the siRNA.

Generally, it can be concluded that most if not all AGO proteins are involved in different parts of the RNA silencing and possibly define the mode of action of the RISC in which they are incorporated (Baulcombe, 2004).

Thesis outline

Pathogen derived resistance (PDR) has been applied for many years (Goldbach *et al.*, 2003). Both translatable and non-translatable virus sequences were transformed into plants either in sense or anti-sense orientation. However, this technology was limited by the fact that only few transgenic plants obtained actually showed virus resistance. This changed when it was found that RNA silencing, known as RNA interference (RNAi) in animals, was at the base of this mechanism and that double stranded RNA (dsRNA) transcribed from inverted repeat ("hairpin") constructs were an excellent trigger for it (Smith *et al.*, 2000). This system was still limited since it was highly sequence specific and therefore only targeted one virus at once. Chapter 2 describes a improved applied approach allowing a broad multiple virus resistance using minimal sequences from different viruses. This approach is very effective and can still be extended to an even broader spectrum of viruses.

Another aim of this thesis was to better understand host-virus interactions in relation to the RNA silencing based plant antiviral defense and the countermeasures applied by viruses. At the onset of this thesis research, it was already reported that several positive strand RNA viruses encode proteins that can suppress RNA silencing. It was still an open question whether negative strand RNA viruses would also encode such proteins. As negative strand viruses of plants often belong to viral families (Bunyaviridae, Rhabdoviridae) which also encompass animal viruses, the finding of RNA silencing suppressors would meantime give clues whether animal viruses also have to circumvent antiviral RNA silencing (Chapter 3). To examine this intriguing possibility further, in Chapter 4 a true mammalian infecting virus, i.e. Influenza A virus, has been investigated for the possibility that it is encoding an RNA silencing suppressor protein.

Chapter 5 was designed to gain further understanding of the effects that viral silencing suppressors have on a host plant. It describes what kind of transcriptional changes occur in a plant transiently expressing silencing suppressor proteins. It provides insight into what kind of gene families are affected by silencing suppressors and gives some hints into how silencing suppressors work.

Finally, the most interesting and intriguing data obtained during this thesis research are discussed in Chapter 6. In this chapter the experimental data obtained are placed into a general perspective and elucidates some speculative outcomes that can be proposed based on this thesis.

CHAPTER 2

Multiplex RNAi:

High Frequency RNA Silencing of Multiple Targets Using a Single Transgene Construct

Abstract

The use of RNA silencing has become the tool of choice for gene knock-down in many organisms. The discovery that double-stranded RNA (dsRNA), diced into small interfering RNAs (siRNA), is a very potent trigger for RNA silencing is the key element of this technology. By arranging transgenes as inverted repeats (IRs) encoding self-complementary hairpin RNA (hpRNA), which is processed into siRNAs after transcription, it is possible to obtain strong repression of the expression of homologous RNAs. So far, due to the high sequence specificity of RNA silencing, this technology was limited to the targeting of single genes. This chapter demonstrates that efficient simultaneous knock-down of four genes can be achieved in plants by using a single, relatively small transgene. This is shown here based on the production of plants expressing a minimal-sized IR cassette containing the sequences of four related plant-infecting *Tospoviruses*. The transgenic expression of these cassettes rendered up to 82% of the transformed plant lines heritably immune against infection with all four viruses. This was shown to be due to simultaneous RNA silencing of the four targets. This chapter shows that the RNA silencing tool can be further improved for high frequency multiple gene knock-downs by combining a large number of small parts of target genes.

Introduction

Since the discovery of dsRNA as a highly efficient trigger of RNA silencing, the use of this sequence specific tool to engineer gene knock-down in all sorts of organisms has become widely used (Fire *et al.*, 1998; Kennerdell & Carthew, 1998; Ngo *et al.*, 1998; Sanchez Alvarado & Newmark, 1999; Lohmann *et al.*, 1999; Smith *et al.*, 2000). The initial dsRNA transcript is processed into small interfering RNAs (siRNAs), the effector molecules of RNA interference. These hallmark molecules of RNA silencing were first discovered in plants (Hamilton & Baulcombe, 1999). SiRNAs are incorporated into the RNA induced silencing complex (RISC), which specifically recognises and cleaves RNA molecules homologous to the siRNA (Hammond *et al.*, 2000). Currently, siRNAs, short hairpin RNAs (shRNA) (Brummelkamp *et al.*, 2002) or long double stranded RNAs (dsRNA) are delivered into the organism of interest in order to achieve transient RNA silencing. DsRNAs and shRNAs can also be expressed constitutively in a stably transformed cell line or organism.

In plants, RNA silencing has been an important tool in the development of gene knock-downs and virus resistance (Ritzenthaler, 2005). It can be used to render plants resistant against a large range of viruses (reviewed in (Vazquez Rovere *et al.*, 2002) and (Tenllado *et al.*, 2004)). A significant improvement of dsRNA-mediated RNA silencing compared to the originally used (single) sense transgenes is that these dsRNAs render the system very efficient and a much higher frequency of transformed plant lines with such dsRNA constructs will display efficient gene knock-down or virus resistance (Smith *et al.*, 2000; Waterhouse & Helliwell, 2003). It is perceived that this is due to the dsRNAs being directly fed into the silencing pathway at the level of dicer and therefore does not rely on the action of proteins such as AGO1, SGS2/SDE1, SGS3 and SDE3 involved in the production of dsRNAs in (single) sense transgene-induced RNA silencing (Beclin *et al.*, 2002). Before dsRNA technology became available, sense-mediated RNA silencing resulted in a maximum frequency of 20% resistance (Chen *et al.*, 2004; Smith *et al.*, 2000). Lindbo and Dougherty (Lindbo & Dougherty, 1992) discovered already some time ago that transgene mediated virus resistance frequently correlated with very low RNA expression. Further studies showed that this RNA based resistance relied on the specific homology dependent degradation of RNA (reviewed in (Baulcombe, 1996) and (Goldbach *et al.*, 2003)). Interestingly not all virus genes used in these constructs rendered plants resistant. Using the new inverted repeats however, it is now possible to use genes that were not suitable for sense-mediated RNA silencing before (Chen *et al.*, 2004).

In order to take full benefit of these new developments we wanted to achieve high frequency silencing of multiple targets using a single self-complementary hairpin RNA (hpRNA) construct. To test this approach we aspired to design an hpRNA construct capable of targeting four viral genes simultaneously. Using virus resistance assays and detection of specific siRNA accumulation, efficient knock-down of these genes could easily be monitored. Since *Tospoviruses* are among the ten most detrimental plant viruses causing significant economic losses in the cultivation of vegetables and ornamental crops (Prins & Goldbach, 1998), we chose to target four viruses among this genus.

Previous work has shown that from all genes of *Tomato spotted wilt virus* (TSWV, the type species of the genus *Tospovirus*) only the N and NS_M genes used in sense constructs resulted in detectable resistance frequencies. Only the plants carrying the N gene were also resistant at the cellular level (Prins *et al.*, 1997). Additionally work by Pang *et al*. (Pang *et al.*, 1997) and Jan *et al*. (Jan *et al.*, 2000b) showed that sequences of as short as 110 nucleotides of the TSWV N gene were sufficient to efficiently induce RNA silencing and therefore virus resistance, but only when fused to GFP mRNA as a carrier.

In order to produce high frequency broad tospovirus resistance a new approach was taken, which is described here. We aspired to use N gene sequence fragments of the four major tomato-infecting Tospoviruses: TSWV, *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and *Watermelon silverleaf mottle virus* (WSMV) in a single small chimeric hpRNA construct. The viral N gene sequence fragments were fused in a primer overlap PCR reaction and subsequently arranged as an IR in order to obtain multiple virus resistance based on hpRNA (Fig. 2-1). Here we show that it possible to efficiently silence multiple targets using a single construct. These constructs can theoretically be extended to include a large number of gene fragments to produce even broader virus resistance. Finally, this novel technology will enable the knock-down of multiple endogenous genes, such as whole gene families or (parts of) pathways using a single RNA silencing construct, not only in plants, but also in other organisms.

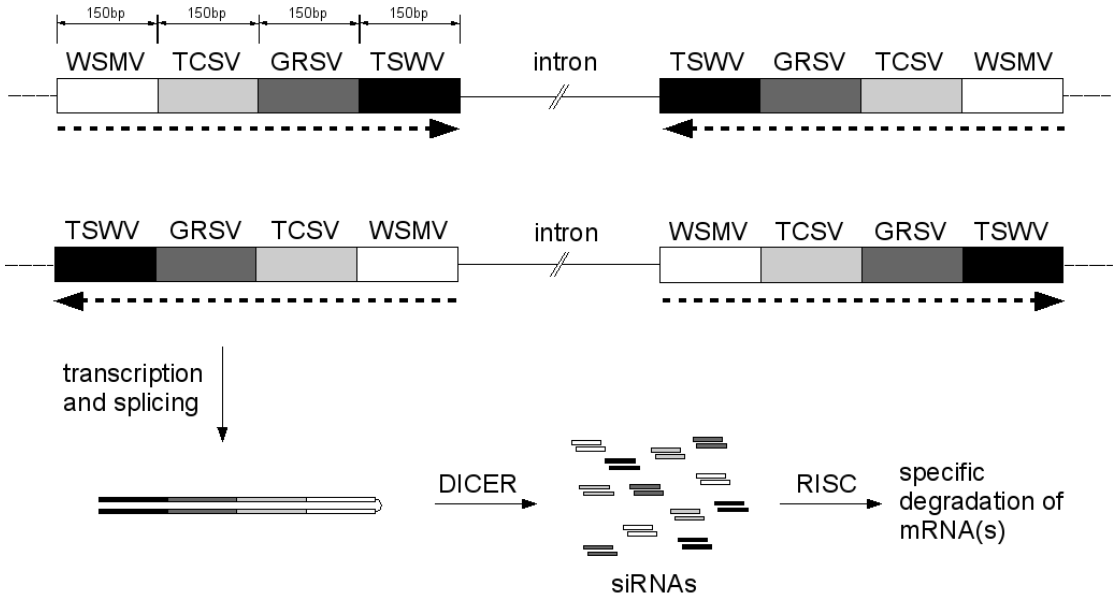

Figure 2-1. Schematic representation of the hpRNA constructs IR-IN and IR-OUT. Four 150 bp consecutive pieces of the N gene of four different tospoviruses were fused to form a chimeric gene and arranged as inverted repeats. The inward facing construct is named IR-IN and the outward construct IR-OUT. Upon transcription and splicing the RNA forms a hairpin structure which is diced into siRNAs. This results in the production of a mixed population of siRNAs originating from the four different N genes.

Results

Cloning of the chimeric tospovirus N gene cassettes

Fusion PCR was used to clone 150 bp of successive parts of the N genes of the four tospoviruses TSWV, GRSV, TCSV and WSMV to result in a chimeric N gene sequence of 600 bp. The homologies of the N genes compared to TSWV are 80%, 78% and 40% for GRSV, TCSV and WSMV respectively. Successive parts of the N genes were chosen to reduce the risk of intramolecular homologous recombination either in bacteria or plants. Due to the lack of a start codon, the construct was rendered untranslatable. The cassettes were subsequently cloned in two orientations as an inverted repeat separated by a 500bp intron sequence of the *Arabidopsis thaliana* actin2 gene resulting in IR-IN and IR-OUT (Fig. 2-1). The intron stabilizes the construct during cloning and enhances its effectiveness to induce hpRNA mediated silencing (Smith *et al.*, 2000). The complete cassette was cloned into a binary vector (pBIN19) between a 35S promoter and a NOS terminator, transformed into *Agrobacterium tumefaciens*, which was further used for *Nicotiana benthamiana* transformation.

The chimeric N gene cassettes confer high frequency of multiple tospovirus resistance

16 IR-OUT and 16 IR-IN independent lines of transgenic *N. benthamiana* were generated and self-fertilised seeds were harvested (S1 progeny). Following selective germination on kanamycin, progeny plants were transferred to the greenhouse and challenged with the four different tospoviruses (TSWV, GRSV, TCSV and WSMV) either separately or co-inoculated. A remarkably high number of 81% of all IR-OUT lines showed high or complete immunity (Tab. 2-1). In almost all cases when an IR-OUT line was resistant to one of the viruses it was also resistant to the other three viruses. Moreover, the plants showed high immunity to highest inoculation pressure using a mixture of the four different tospoviruses at once. Interestingly, the results for the IR-IN lines were less consistent. The resistance incidence for the IR-IN lines, though still high, was lower than for the IR-OUT lines (63% of the lines were highly resistant to the four viruses). This phenomenon of lower silencing induction by inwardly oriented IR constructs has also been observed by others (K. Kalantidis, personal communication). Possibly, ribosome scanning or shunting of the sense RNA prevents the proper folding of the RNA into dsRNA and thereby inhibits effective dicer function.

For both IR-IN and IR-OUT lines, progeny generations of resistant plants (S2 and S3) all remained 100% virus free despite repetitive high inoculation pressure with all four viruses simultaneously (data not shown).

Table 2-1. Resistance analysis of the IR-OUT and IR-IN lines. At least six S1 plants of all the lines were inoculated with each virus or with a mix of all viruses. Resistance was assessed phenotypically and by ELISA three weeks post infection.

Line	Inoculated virus(es)				
	TSWV	GRSV	TCSV	WSMV	Mix
IR-OUT4	100%	100%	100%	100%	83%
IR-OUT5	0%	20%	11%	10%	0%
IR-OUT6	100%	100%	100%	100%	100%
IR-OUT7	94%	100%	100%	100%	100%
IR-OUT8	100%	100%	100%	100%	100%
IR-OUT9	100%	100%	88%	70%	67%
IR-OUT10	94%	90%	89%	80%	67%
IR-OUT11	100%	100%	100%	100%	100%
IR-OUT12	100%	100%	100%	100%	83%
IR-OUT13	7%	0%	44%	10%	33%
IR-OUT14	100%	100%	100%	100%	100%
IR-OUT15	100%	93%	100%	100%	100%
IR-OUT16	100%	100%	100%	100%	100%
IR-OUT17	0%	0%	0%	11%	0%
IR-OUT19	100%	100%	100%	100%	33%
IR-OUT20	100%	100%	100%	83%	83%
IR-IN5	100%	100%	100%	70%	100%
IR-IN6	92%	92%	100%	100%	100%
IR-IN7	8%	0%	10%	8%	0%
IR-IN8	100%	100%	100%	100%	100%
IR-IN9	100%	100%	89%	100%	100%
IR-IN10	100%	100%	100%	92%	100%
IR-IN11	0%	0%	17%	25%	0%
IR-IN12	8%	0%	0%	8%	0%
IR-IN13	9%	0%	0%	0%	0%
IR-IN14	83%	92%	100%	100%	100%
IR-IN15	100%	100%	100%	100%	100%
IR-IN16	0%	0%	0%	10%	0%
IR-IN18	100%	67%	100%	100%	100%
IR-IN19	100%	100%	100%	100%	80%
IR-IN20	100%	100%	100%	100%	80.00%
IR-IN21	17%	25%	20%	75%	33%

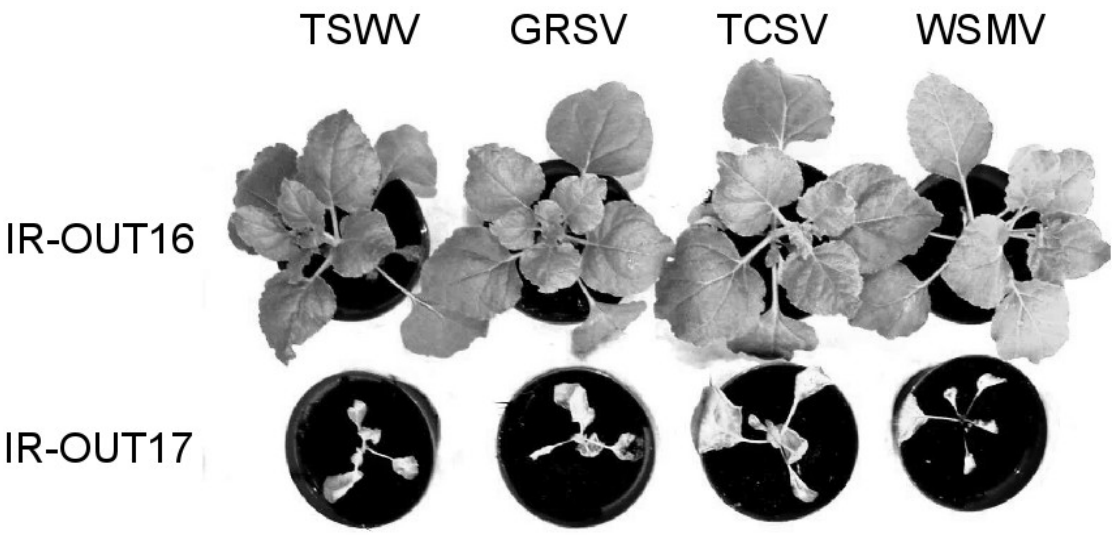

Figure 2-2. Transgenic plants showing multiple virus resistance. The photograph shows two examples of transgenic plant lines inoculated with four different tospoviruses. IR-OUT16 is a line, which is resistant to all viruses and shows no symptoms. IR-OU17 is an example of a totally susceptible line.

Molecular analysis of the expression of the transgenic N gene chimera

The occurrence of siRNAs originating from the transgene in the plant has been shown to be an indicative for a high probability of RNA virus resistance (Kalantidis *et al.*, 2002). We investigated whether siRNAs from the four segments of the cassette could be detected in the resistant and susceptible plant lines. Total nucleic acid was extracted from transgenic and wild-type plants, enriched for siRNAs (Bucher *et al.*, 2004; Chen *et al.*, 2004) and analysed by Northern blot. Using four different probes specific for each segment of the transgene, siRNAs originating from the different parts of the transgene could readily be detected, but exclusively in virus-resistant lines (Fig. 2-3). All susceptible lines, on the other hand, did not show any detectable amounts of siRNAs originating from any part of the transgene even though the transgene was present as was tested by PCR on genomic DNA extracted from these plants (not shown).

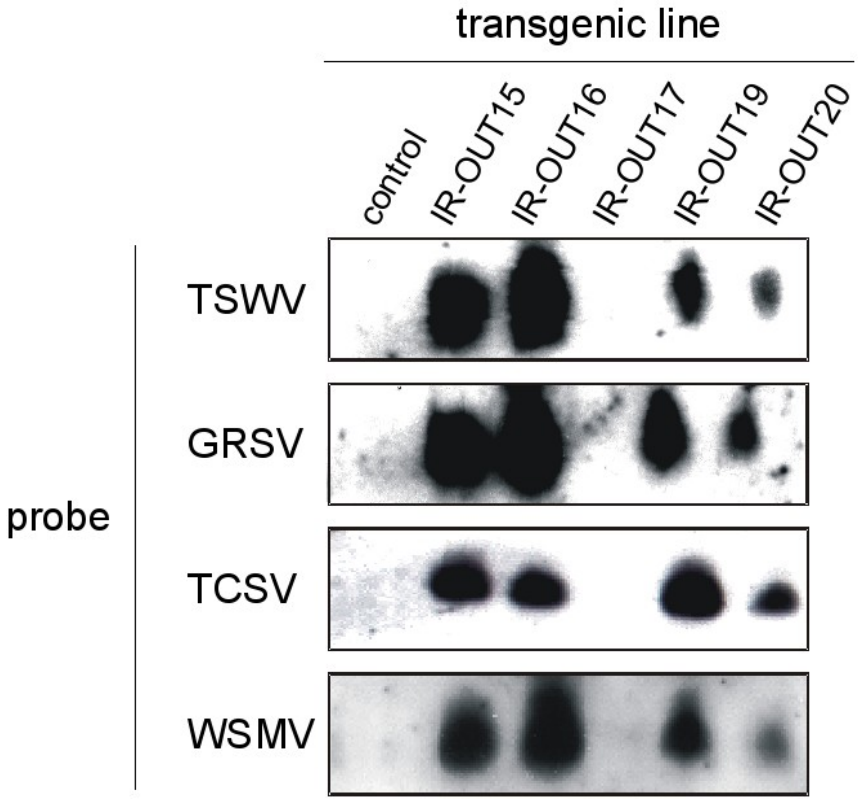

Figure 2-3. SiRNA analysis of several transgenic plant lines. The siRNA blots shown on the right were extracted from transgenic non-inoculated IR-OUT lines. Enriched siRNAs were analysed with four different probes each covering the 150 bp of the piece of the N gene of each tospovirus. The control is total siRNA extracted from a non-transgenic *N. benthamiana* plant.

Discussion

The work presented here demonstrates the possibility to obtain high frequency RNA silencing simultaneously against multiple sequences, which was designated as `multiplex RNAi'. It points towards the possibility to obtain high frequency RNA silencing resulting in multiple tospovirus immunity using a single transgene construct of limited size. Sense mediated multiple virus resistance as reported by Jan *et al.* (Jan *et al.*, 2000a) and Prins *et al.* (Prins *et al.*, 1996) was obtained at a maximum frequency of 20%. This chapter shows that by using multiplex RNAi high frequencies of up to 81% of all transformed plant lines multiple gene knock-down can be achieved. It demonstrates this by showing that the plants become resistant to four different tospoviruses at once and by the detection of siRNA originating from each segment of the cassette. It has been shown before that the detection of specific siRNAs in transgenic plant lines expressing an hpRNA construct usually correlated with virus resistance (Kalantidis *et al.*, 2002). Since our constructs are

entirely hpRNA based they do not rely on the inclusion of silencer sequences such as GFP or *turnip mosaic virus* CP as was used by Jan and co-workers (Jan *et al.*, 2000a). With the new constructs presented here however this problem can be overcome due to the possibility to link as many viral sequences as required, depending on the viruses that can be found in the fields.

The area where genetically modified (GM) crops are being grown world wide has steadily increased in recent years. In 2003 the total area has increased by 15% and just last year Brazil and the Philippines have approved the commercialisation of GM crops (James, 2003). Here is where lies another important advantage of hpRNA mediated silencing can be seen from the perspective of biosafety. No virus-derived mRNAs are accumulating in transgenic plant lines expressing hpRNA constructs (data not shown), thereby reducing the risk of recombination or complementation events. Additionally no viral protein is being produced in these plants circumventing any fears from the population concerning allergic response to novel proteins.

Previously, Crété *et al*. (Crete & Vaucheret, 1999) reported that chimeric sense transgenes containing small fragments originating from the nitrate reducatase (Nii1) and beta-D-glucoronidase (uidA) genes would not lead to the silencing of the endogenous copies of these genes. The new technology presented here, however, shows that it can greatly enhance the chances of knocking-down multiple endogenous genes in a single plant. It would be interesting to investigate whether constructs such as the ones used by Crété *et al*. could work when based on the technology presented in this work.

Based on the recent finding that show that RNA silencing is functional in mammalian cells (Elbashir *et al.*, 2001), several approaches have been made to silence genes in mammals. siRNAs and shRNAs are now being broadly used to inhibit replication of a wide verity of viruses (Ge *et al.*, 2003; Gitlin *et al.*, 2002; Lee *et al.*, 2002). It has recently been shown that long hpRNAs efficiently silence genes in certain cell types (Diallo *et al.*, 2003; Paddison *et al.*, 2002). It can therefore be proposed that multiplex RNAi can also be applied in mammalian cells and therefore providing a new tool for multiple gene knock-down in animals.

Materials and Methods

Vector construction and plant transformation

Primers were designed to amplify the 150bp N gene segments of TSWV, GRSV, TCSV and WSMV and to overlap where the segments of the cassette should be fused (Tab. 2-2). The homologies of the N genes compared to TSWV are 80%, 78% and 40% for GRSV, TCSV and WSMV respectively. After a first round of PCR, the products could be used as primers for another round of PCR resulting in the fusion of the two segments. Using this technique the four segments were fused resulting in the chimeric N gene cassette. The cassettes, flanked by *Bam*HI and *Not*I sites were then cloned into an inverted repeat as previously described (Chen *et al.* 2004). The primers MH17 to MH20 were used to amplify the *A. thaliana* actin2 gene intron sequence with compatible cloning sites to the cassette. The constructs were cloned into a modified pBIN19 binary vector containing the restriction sites *Asc*I and *Pac*I between the right and left border. Finally the binary vector was transformed into the *A. tumefaciens* strain LBA 4404. The plasmid DNA was rescued from A. tumefaciens and sequenced. These A. tumefaciens were then used to inoculate small leaf squares of *N. benthamiana* essentially as described by Horsch *et al.* (Horsch *et al.*, 1985a; Horsch *et al.*, 1985b). Each line originates from a single independent callus.

Table 2-2. Primers used in the fusion-PCR in order to clone the chimeric N-gene cassette. Restriction sites are underlined and a "^" indicates the gene fusion sites.

Primer	Description	sequence
FP1	*Not*I-WSMV	CCCGCGGCCGCTCTAACGTTAAGCAGCTCACAG
FP2	WSMV-TCSV	GAGATAACTTTCACAAACTGC^TTGACTTTCCTGAAAAATCGCC
FP3	TCSV-GRSV	GATAGCATGATAAGAGTTAAGC^TCATAGAAGAGACTGCAAACAATG
FP4	GRSV-TSWV	GAGGTAGTATCCCTCTCATTGC^TTCAGTTGATAGCTTTGAGATG
FP5	TSWV-GRSV	CATCTCAAAGCTATCAACTGAA^GCAATGAGAGGGATACTACCTC
FP6	GRSV-TCSV	CATTGTTTGCAGTCTCTTCTATGA^GCTTAACTCTTATCATGCTATC
FP7	TCSV-WSMV	GGCGATTTTTCAGGAAAGTCAA^GCAGTTTGTGAAAGTTATCTC
FP25	*Bam*HI-TSWV	CCCGGATCCTGATCTTCATTCATTTCAAATG
MH17	*Not*I-Intron sense	CCCGCGGCCGCCAAGGTAATAGGAACTTTC
MH18	*Not*I-Intron antisense	CCCGCGGCCGCGAGCTGCAAACACACAAAAG
MH19	*Bam*HI-Intron sense	CCCGGATCCAAGGTAATAGGAACTTTC
MH20	*Bam*HI-Intron antisense	CCCGGATCCGAGCTGCAAACACACAAAAAG

siRNA analysis of the transgenic plants

Total nucleic acids of transgenic plants was extracted and siRNAs enriched as described (Bucher *et al.*, 2004; Hamilton & Baulcombe, 1999). 20 µg of total siRNAs per sample were separated on a 15% PAGE gel and analysed by Northern blot using specific digoxigenin (Roche) labelled TSWV, GRSV, TCSV and WSMV probes covering the 150 bp of each fragment of the cassette.

Inoculation of transgenic plants

The tospovirus strains TSWV, GRSV, TCSV (De Avila *et al.*, 1992) and WSMV (Heinze *et al.*, 1995) were propagated in *N. benthamiana* plants. Inoculum to infect the transgenic plants was obtained by grinding systemically infected plant leaves in a 0.5% Na_2SO_3 solution. The inoculum was applied by softly rubbing the leaves using carborundum powder. In mixed inoculation experiments equal amounts of diluted sap of each systemically infected plant was mixed. For each inoculum used in all experiments non-transformed control plants were inoculated after the transgenic lines were inoculated. To maximize inoculation pressure in the experiments shown in

table 1 and 2, all plants were inoculated twice using an interval of one week between the two inoculations. The plants were then monitored for 30 days for symptom development and plants were analysed by ELISA to exclude symptomless infections.

CHAPTER 3

Negative-Strand Tospoviruses and Tenuiviruses Carry a Gene for a Suppressor of Gene Silencing at Analogous Genomic Positions*

Abstract

Posttranscriptional silencing (PTGS) of a green fluorescent protein (GFP) transgene in *Nicotiana benthamiana* plants was suppressed when these plants were infected with *Tomato spotted wilt virus* (TSWV), a plant-infecting member of the *Bunyaviridae*. Infection with TSWV resulted in complete reactivation of GFP expression similar to *Potato virus Y* (PVY), but distinct from *Cucumber mosaic virus* (CMV), two viruses known to encode silencing suppressor proteins. *A. tumefaciens*-based leaf-injections with individual TSWV genes identified the NS_S gene to be responsible for the RNA silencing suppressing activity displayed by this virus. The absence of siRNAs in NS_S expressing leaf sectors suggest that the tospoviral NS_S protein interferes with the intrinsic RNA silencing present in plants. Suppression of RNA silencing was also observed when the NS3 protein of the *Rice hoja blanca tenuivirus* (RHBV), a non-enveloped negative strand virus, was expressed. These results indicate that plant-infecting negative stranded RNA viruses encode a suppressor of RNA silencing.

*Bucher E. , Sijen T. , De Haan P. , Goldbach R. , Prins M. (2003). Negative-strand Tospoviruses and Tenuiviruses Carry a Gene for a Suppressor of Gene Silencing at Analogous Genomic Positions. *J Viro*l 77: 1329-36.

Introduction

RNA silencing involves a sequence-specific degradation which is induced by over-abundant and by double stranded RNA (dsRNA) molecules and can target transgenes as well as homologous endogenous genes. RNA silencing was first described in plants (Napoli, 1990; van der Krol *et al.*, 1990) and over recent years, has been described in other organisms, where it is also referred to as co-suppression, post-transcriptional gene silencing (English *et al.*, 1996) or RNA-mediated virus resistance (Baulcombe, 1999; Lindbo *et al.*, 1993; de Haan *et al.*, 1992) in plants, quelling in fungi (Cogoni & Macino, 1997) or RNAi in animals (Fire *et al.*, 1998). Building blocks of the gene silencing pathway proved to have remarkable similarities in the different organisms and hence suggest an ancient role of gene silencing in pathogen resistance or development (Cogoni & Macino, 2000; Ketting *et al.*, 2001; Voinnet, 2001). One of the key intermediary elements in the RNA silencing pathway is double stranded RNA, which is recognized by a dsRNA-specific nuclease (Bernstein *et al.*, 2001) to yield small (21-23 nucleotides long) short interfering RNAs (siRNAs) (Hamilton & Baulcombe, 1999). These siRNAs subsequently serve as guides for cleavage of homologous RNA molecules. In plants, versions of transgenes that produce double stranded RNA molecules have been shown to be very potent activators of RNA silencing (Smith *et al.*, 2000). As all RNA viruses replicate through formation of double stranded RNA intermediates, these are potential targets of the RNA silencing mechanism. Indeed, antiviral RNA silencing has been shown to occur in nature and has been proposed as a natural defense mechanism protecting plants against viruses, resulting in resistance (Al-Kaff *et al.*, 1998; Ratcliff *et al.*, 1997).

To counteract the RNA silencing mechanism of their host, plant viruses have developed ways to evade or neutralize this response. Over recent years, RNA silencing inhibiting proteins have been identified in several plant viruses. Among the best studied examples are the helper component-proteinase (HC-Pro) of the potyvirus *Potato virus Y* (PVY) and the 2b protein of *Cucumber mosaic virus* (CMV) (Brigneti *et al.*, 1998; Pruss *et al.*, 1997). Also other plus strand RNA (and some DNA) viruses have been found to suppress gene silencing and for some of them the viral protein involved was identified (Dunoyer *et al.*, 2002; Pfeffer *et al.*, 2002; Silhavy *et al.*, 2002; Voinnet *et al.*, 2000). The viral suppressor proteins of PVY and CMV act differently by targeting different steps in the RNA silencing pathway. HC-Pro was shown to prevent degradation of dsRNA into small interfering RNAs (siRNAs), which are considered a hallmark of RNA silencing (Hamilton & Baulcombe, 1999), however it did not prevent the silencing signal from going systemic (Brigneti *et al.*, 1998; Mallory *et al.*, 2001). The 2b protein of CMV, a function required for long distance

movement, is targeted to the nucleus (Lucy *et al.*, 2000) and prevents silencing in newly emerging tissues, but unlike HC-Pro is not able to reverse RNA silencing once established (Beclin *et al.*, 1998). Interestingly, the required nuclear accumulation of 2b for efficient suppression of RNA silencing indicated a possible blocking of silencing from the nucleus while RNA degradation takes place in the cytoplasm. A third step of the silencing pathway is targeted by the *Potato virus X* (PVX) p25 protein, namely systemic signaling of silencing (Voinnet *et al.*, 2000). Recently, the P19 protein of tombusviruses was implicating in inhibiting RNA silencing by physically interacting with siRNAs, thus providing another mechanism to interfere with RNA silencing (Silhavy *et al.*, 2002).

An increasing number of positive strand RNA viruses of plants have been shown to counter-act the RNA silencing defense system. Though often transmitted by insects or fungi, plant-infecting plus strand RNA viruses exclusively replicate in plant hosts. In contrast, plant-infecting negative strand RNA viruses, i.e. tospoviruses, tenuiviruses and rhabdoviruses, have life cycles in both plants and their arthropod vectors (Falk & Tsai, 1998; Jackson *et al.*, 1999; Wijkamp *et al.*, 1993). Recently, RNA silencing has also been demonstrated in insects (Li *et al.*, 2002; Pal-Bhadra *et al.*, 1997). Negative strand viruses therefore have to cope with the RNA silencing defense systems of both the plant and insect hosts. For this reason it is of interest to investigate whether negative strand RNA viruses infecting plants also encode RNA silencing suppressors, which enable them to overcome the plant and insect intracellular defense response. To test this, two representatives of the genera *Tospovirus* and *Tenuivirus,* i.e. TSWV and RHBV, respectively, have been investigated for this feature.

Like all bunyaviruses, the genome of TSWV is tripartite, of which the fully negative stranded L RNA encodes the viral RdRP (Fig. 3-1). Both M and S RNAs have two genes, in an ambisense arrangement (Kormelink *et al.*, 1992; de Haan *et al.*, 1990; de Haan *et al.*, 1991). The M RNA codes for the precursor to the membrane glycoproteins G_1 and G_2 and the viral movement protein NS_M (Storms *et al.*, 1995). The S RNA codes for the nucleoprotein (N) and a non-structural protein (NS_S). No clear function could be assigned to the NS_S protein, although, as accumulation of this protein coincides with increase of symptom severity, it has been implicated in viral virulence (Kitajima *et al.*, 1992; Kormelink *et al.*, 1991). Like for TSWV, the largest RNA segment of RHBV (RNA 1) is of complete negative polarity and encodes the putative viral polymerase. The other 3 RNA segments have an ambisense coding strategy, thus encoding a further 6 proteins of which only the nucleoprotein encoded on RNA 3 has been functionally indicated (Ramirez *et al.*, 1992; Ramirez *et al.*, 1993; de Miranda *et al.*, 1994; de Miranda *et al.*, 1996). The genome arrangements of TSWV and RHBV are indicated in figure 3-1.

Using a testing system previously applied to identify silencing suppressors of positive strand RNA viruses, i.e. a combination of GFP-silenced reporter plants and *A. tumefaciens*-based transient transformation (Voinnet *et al.*, 1999), the occurrence of a possible silencing suppression gene of TSWV and RHBV was investigated.

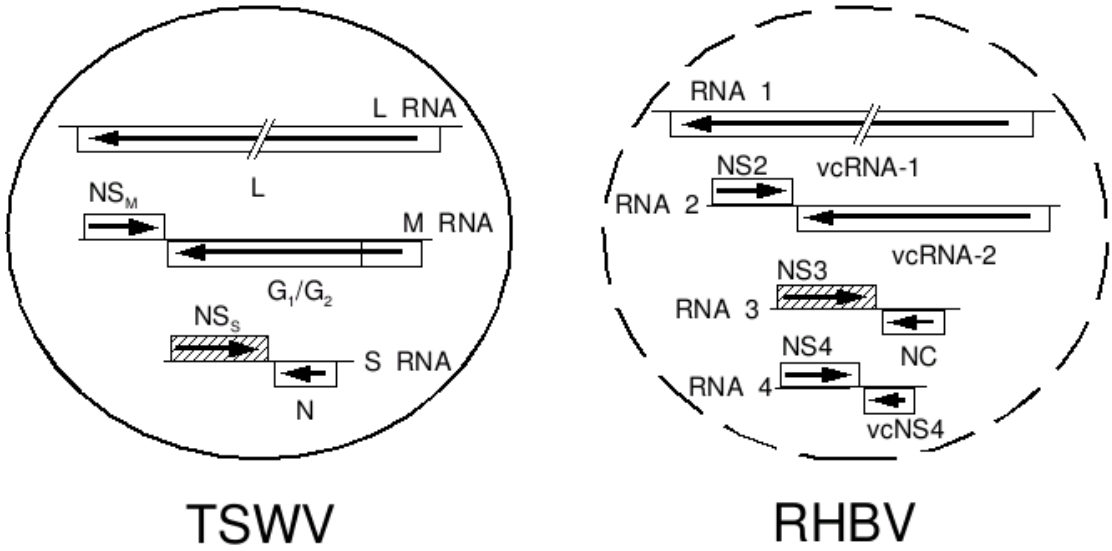

Figure 3-1. Schematic representation of the coding strategies of the TSWV and RHBV genomes. The dashed line surrounding the RHBV Tenuivirus indicates that no membranous particles have been found for these viruses, in contrast to Tospoviruses and other members of the Bunyaviridae. Viruses of both genera have a fully negative stranded large RNA (L RNA and RNA 1, resp.), encoding the viral RdRP. The nucleoprotein is encoded on the vc RNA strand of the third largest segment (S RNA or RNA 3) in which the NS_S and NS3 proteins are encoded on the viral RNA strand. The remaining RNA segments of both viruses are all ambisense.

Results

TSWV-infection counteracts RNA silencing

TSWV is capable of infecting many host plants, in many cases causing severe disease symptoms (Goldbach & Peters, 1994; Prins & Goldbach, 1998; Van Regenmortel *et al.*, 2000). We therefore aspired to investigate whether the virulence of TSWV may be enhanced by its ability to suppress gene silencing. For this purpose a series of virus inoculation experiments was performed on *N. benthamiana* plants in which a GFP transgene was silenced. Transgenic *N. benthamiana* were inoculated with TSWV and GFP fluorescence was monitored. Two positive stranded viruses known to effectively suppress gene silencing (PVY and CMV) were used as controls in these experiments.

In the transgenic plant line used in these experiments, the silencing of GFP fluorescence occurs only in leaf parenchyma, whereas stems and major veins still express GFP (Fig. 3-2A). After further aging of the plant (several weeks) stems and veins also become silenced resulting in totally silenced plants showing no fluorescence.

Starting around six days post inoculation, and concomitant with the occurrence of virus symptoms, all TSWV-infected plants (26 replicates) showed a complete reversal of GFP silencing and became highly GFP fluorescent in infected tissue (Fig. 3-2A). GFP expression in mock-inoculated control plants remained silenced, indicating that the observed fluorescence is GFP specific and not related to fluorescence of necrotised tissue. Inoculation of the (GFP transgenic) test plants with PVY and CMV also resulted in a reversion of the GFP silenced phenotype, although the effect of CMV was markedly less than that of PVY or TSWV. A clear relation between viral symptoms and GFP expression was visible by distinct sectoring of GFP reactivation. For both PVY and TSWV this phenomenon was observed at an early stage of infection (Fig. 3-2B). At a later stage (3 weeks post inoculation) not only newly developed systemically infected leaves were completely suppressed in GFP silencing but also older leaves (Fig. 3-2C). Similar inoculation experiments performed with two additional tospovirus species, *Groundnut ringspot virus* and *Impatiens necrotic spot virus,* also revealed a reversal of gene silencing, indicating that silencing suppression is a general feature of tospoviruses (data not shown).

Inoculation of GFP silenced plants with two different CMV isolates did not result in immediate suppression of GFP silencing. Strikingly, after 3 weeks only older leaves infected with CMV subgroup I started to show some suppression of GFP silencing coinciding with viral symptoms. However this was restricted to the older leaves and not observed in young leaves. Only after one month, newly emerging leaves of CMV subgroup II infected plants started to show similar RNA silencing suppression patterns as observed by Brigneti and co-workers (Brigneti *et al.*, 1998).

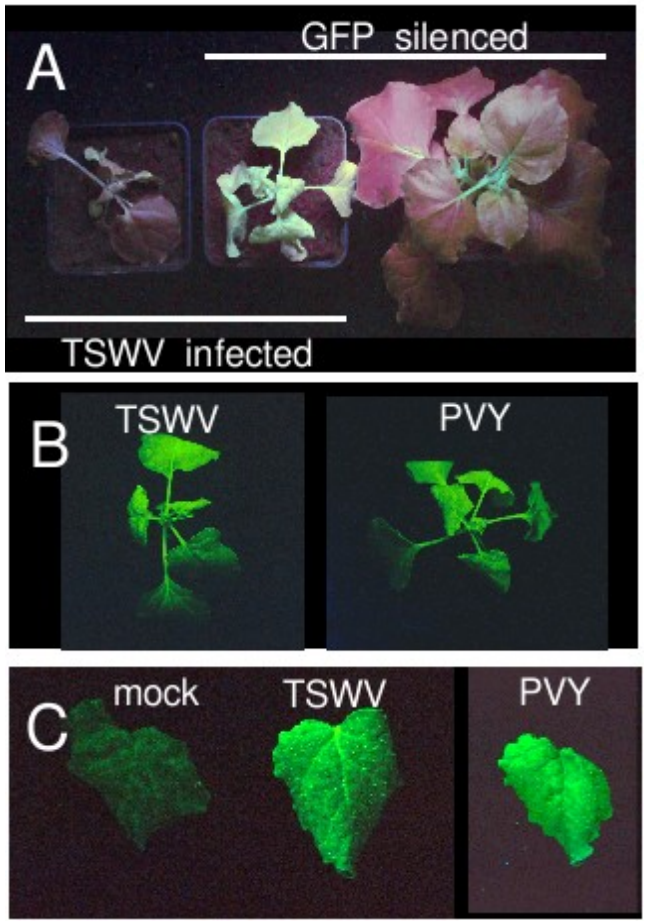

Figure 3-2. Virus inoculation of transgenic N. benthamiana plants containing a silenced GFP gene. (A) Suppression of RNA silencing by TSWV. The plant on the left is a non-transgenic plant infected with TSWV to show that the infection does not cause auto-fluorescence. The plant on the right is a non-infected, GFP silenced, control plant. The photograph was taken without filter. (B) Recovery of GFP expression in GFP silenced plants by infection with TSWV or PVY 10 days post inoculation. (C) Suppression of GFP expression in older leaves. Photographs were taken 3 weeks post inoculation. In panels B and C a Kodak Wratten no. 58 filter was used.

The NS_S protein of TSWV is necessary and sufficient to confer suppression of RNA silencing

To investigate a possible function for the individual TSWV proteins in the suppression of gene silencing, four of its genes, i.e. N, NS_S, G_1G_2 and NS_M were cloned under the control of a 35S promoter and tested. Also clones of HC-Pro of the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) (Mlotshwa *et al.*, 2002) and of 2b of a subgroup I CMV isolate (Chen *et al.*, 2001) were introduced in transgenic *N. benthamiana* leaves expressing the GFP transgene by the *A. tumefaciens*-based delivery system using leaf-infiltration (Johansen & Carrington, 2001; Voinnet *et al.*, 2000). The TSWV gene constructs were injected in leaves together with an *A. tumefaciens* strain carrying the GFP gene to initiate and enhance RNA silencing (Voinnet *et al.*, 1999). Suppression of GFP silencing was monitored during the following days and photographed 6 days after injection

(Fig. 3-3). Only co-infiltration with the NS_S gene lead to suppression of GFP silencing in all of the 16 treated plants (Fig. 3-3D and 3-4). Expression of the TSWV proteins was confirmed by Western blot analysis (data not shown). In none of these cases did the suppression of silencing spread beyond the inoculation focus, indicating the suppressor protein is unable to move from cell-to-cell or induce a mobile silencing suppression signal.

The other TSWV genes, when co-introduced with GFP (approximately 15 plants each) as well as GFP alone did not show GFP fluorescence in the injected areas (Fig. 3-3A, B, C). This indicates that both the original and the *A. tumefaciens* delivered GFP (transient) transgenes were completely silenced and that the TSWV proteins N, NS_M and G_1G_2 are not involved in the observed inhibition of gene silencing during virus infection.

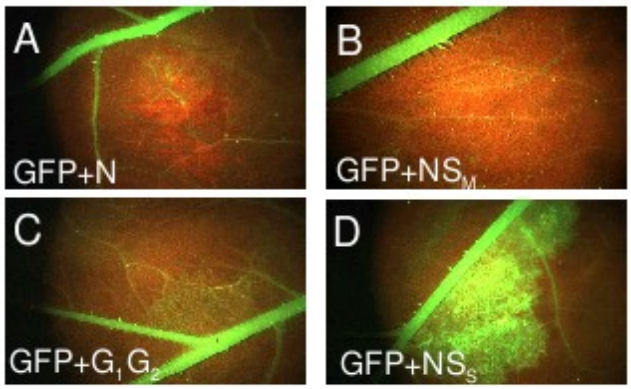

Figure 3-4. *A. tumefaciens* infiltration experiments with different TSWV genes in GFP silenced *N. benthamiana* plants. Agrobacterium strains harboring TSWV genes were co-infiltrated with GFP. Only NS_S suppresses the silencing of GFP (panel D).

The NS3 protein of tenuivirus RHBV is also a suppressor of RNA silencing

Like the NS_S gene of TSWV, the NS3 gene of RHBV is located on the third-largest RNA segment (RNA3), which furthermore encodes the nucleoprotein on the viral complementary strand (Fig. 3-1). So far, no function has been assigned to this protein and it does not contain any protein-sequence homology to NS_S or any other protein in the NCBI database. In order to investigate whether the NS3 protein may also be involved in silencing suppression, its gene was cloned into a plant expression cassette, like its positional tospoviral analogue NS_S. As shown in Fig. 3-4 the transient expression of NS3 reversed the effect of gene silencing, resulting in a phenotype very similar to those obtained with HC-Pro or NS_S.

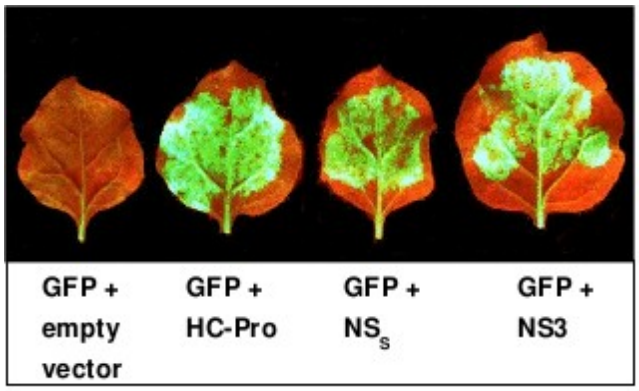

Figure 3-4. RNA silencing suppression activity displayed by HC-Pro (PVY), NS_S (TSWV) and NS3 (RHBV). The picture was taken with a yellow filter 6 days after infiltration.

Both NS_S and NS3 interfere with the initiation of RNA silencing

The efficient reversal of GFP silencing by HC-Pro, NS_S and NS3 in transgenic GFP plants prompted the question whether NS_S and NS3 may be involved in suppressing early stages of the RNA silencing cascade (Mallory *et al.*, 2001). For that purpose untransformed *N. benthamiana* plants were infiltrated with combinations of *A. tumefaciens* strains containing GFP and strains harbouring silencing suppressor genes. The effect of the other TSWV genes was used as a control. When introduced together with the TSWV genes N, G_1G_2 or NS_M, the GFP gene remained silenced, similar to plants where only GFP was infiltrated (not shown). However, when introduced together with the NS_S or NS3 gene-construct local expression of the GFP transgene was significantly boosted yielding a much higher fluorescence (Fig. 3-5A) and enhanced protein content (Fig. 3-5B). Northern blot analysis showed significantly higher levels of GFP messenger RNA when co-expressed with NS_S, NS3 and HC-Pro (Fig. 3-5C). Two CMV 2b genes belonging to different subgroups were also co-infiltrated in non-transgenic plants together with GFP, but showed no markedly enhanced GFP expression compared to the co-infiltration of GFP with the empty control plasmid (Fig. 3-5A).

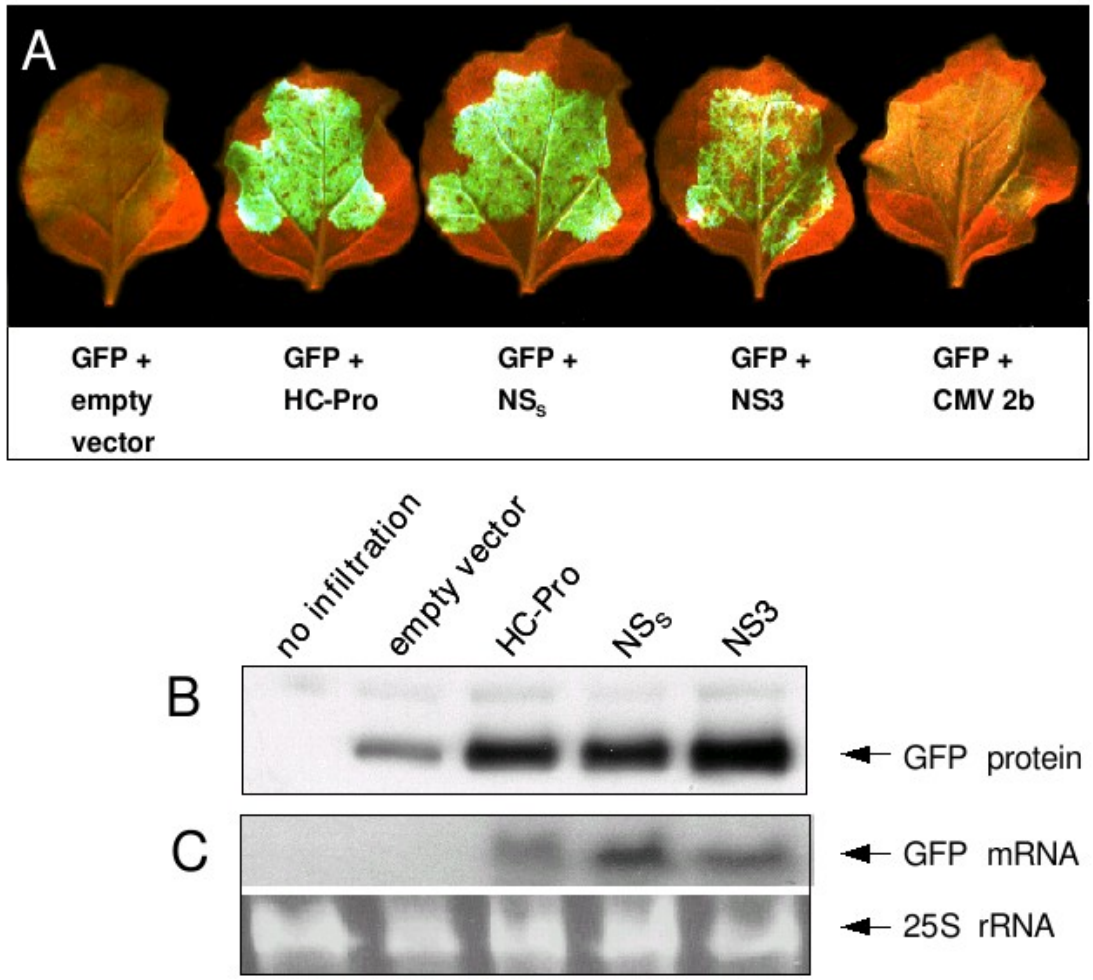

Figure 3-5. NS_S and NS3 interfere with the initiation of RNA silencing. (A) GFP imaging of Agrobacterium infiltrated leaves from non-transgenic plants. GFP expression was visualized by UV light in leaves co-infiltrated with GFP and an empty vector, CABMV HC-Pro, TSWV NS_S, RHBV NS3 and CMV 2b. (B) Total protein was extracted from corresponding infiltrated leaf sectors. Western blot was performed using anti-GFP antibodies. (C) Northern blot analysis of total mRNA extracted from the infiltrated leaf parts. Ethidium bromide staining of the same gel shows the 25S rRNA as a loading control.

Analysis of small interfering RNAs

To further substantiate that reversion of GFP expression in transgenic, silenced plants was based on genuine suppression of RNA silencing, the occurrence of siRNAs in GFP expressing tissues was investigated. RNA was extracted from *A. tumefaciens*-infiltrated GFP expressing leaf sectors and enriched for siRNAs. Subsequently, siRNAs were analysed by RNase protection assays. GFP specific small RNAs were readily detected in GFP infiltrated transgenic plants. As previously reported (Llave *et al.*, 2000; Mallory *et al.*, 2001) the siRNAs did not appear when GFP was co-infiltrated with HC-Pro. Exactly the same was observed when NS_S was co-infiltrated (Fig. 3-6).

The infiltration of NS3 protein results in enhanced GFP fluorescence (Fig. 3-4 and 3-5), due to a

great increase in mRNA levels (Fig. 5C), suggesting a strong RNA silencing suppression. However, unlike NS_S and HC-Pro, NS3 did not prevent the accumulation of small RNAs, indicating that it may have another mode of action compared to HC-Pro or NS_S in *N. benthamiana* plants.

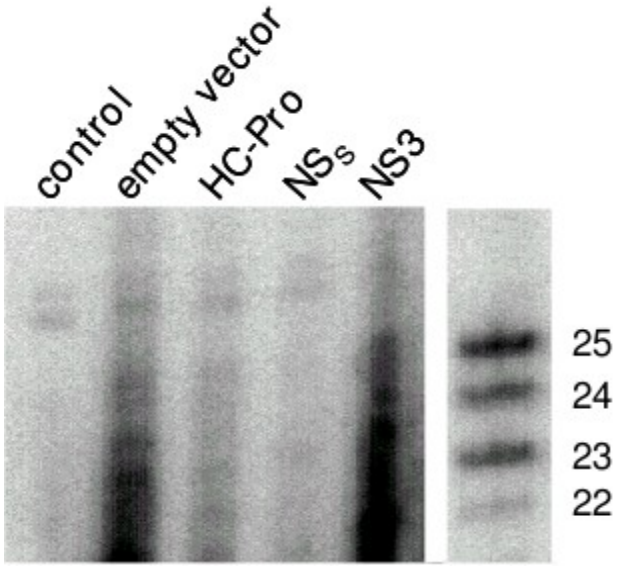

Figure 3-6. Analysis of the GFP siRNAs by RNaseA/T1 protection assay. Small RNA-enriched samples were extracted from Agrobacterium infiltrated sectors of GFP silenced leaves. Non-infiltrated non-transgenic plants were used as a control. All other leaves were co-infiltrated with GFP and (putative) silencing suppressor constructs. Twenty µg of RNA was hybridised to sense GFP RNA transcripts and subsequently treated with the RNases A and T1. Sizes of RNA oligonucleotides are indicated on the right.

Discussion

Upon infection with the tospovirus TSWV, transgenic plants silencing the GFP transgene show a strong recovery of GFP fluorescence, suggesting that RNA silencing is suppressed in the infected plant cells. The suppression of silencing is similar to that observed with potyviruses, resembling suppression capabilities of these viruses both in time as in intensity. Like TSWV, also GRSV and INSV showed suppressor capabilities, indicating the suppression of silencing is a general feature of tospoviruses.

As suppression of GFP silencing closely resembles that of the potyvirus PVY, it is tempting to speculate the underlying mechanism by which tospoviruses suppress RNA silencing may also be similar. PVY is known to counteract silencing by targeting a maintenance step in the gene silencing machinery (Mallory *et al.*, 2001), in contrast to CMV, which targets the initiation of silencing

(Brigneti *et al.*, 1998). The results strongly suggest that tospoviruses mimic the ability of PVY to revert established silencing of a GFP transgene by targeting the maintenance step of the gene silencing machinery.

Transient assays were used to identify the TSWV gene responsible for suppression, making it possible to introduce genes independent of virus or viral vector. Possible complications induced by virus infection or additional suppressors encoded by the viral vector used were circumvented in this way, resulting in a gene function assay free of cytopathogenic interference by the virus. *A. tumefaciens*-mediated introduction of TSWV genes in silenced plants has demonstrated that the non-structural protein NS_S is necessary and sufficient for the silencing suppression. Formally, though, it cannot be excluded that the tospoviral RNA-dependent RNA polymerase (L protein) may play a supporting role, as this large (9 kb) gene was not included in our experiments.

Hitherto, no function of the NS_S protein during the virus infection cycle in plants, or insects, could be assigned. In previous reports it had been shown to be highly expressed in the cytoplasm of infected cells, and was suggested to play a role in the virulence of the virus as more virulent isolates accumulate higher amounts of this protein (Kitajima *et al.*, 1992; Kormelink *et al.*, 1991). In addition, it accumulates to high levels in salivary glands of the thrips, the insect involved in the transmission of the virus, in which the virus also replicates (Wijkamp *et al.*, 1993). Recently, it was demonstrated that the insect virus *Flock house virus* (FHV) also carries a gene which can induce RNA silencing suppression, albeit tested in a plant assay (Li *et al.*, 2002). Hence, the NS_S protein may perform its suppression function in both its plant host and insect vector. High accumulation of this protein in the salivary glands of viruliferous thrips may support this notion (Wijkamp *et al.*, 1993).

The results presented in this paper also demonstrate that RHBV, belonging to another taxon of negative strand RNA viruses (the floating genus *Tenuivirus*), specifies an RNA silencing suppressor. The *A. tumefaciens* infiltration experiments resulted in exactly the same suppressing phenotype for HC-Pro, NS_S and NS3. To dissect the mode of action of these novel RNA silencing suppressors, infiltrated leaf tissues were analysed for the presence of GFP transcripts and for the presence of GFP-specific siRNAs by Northern blot analysis. These experiments show that both HC-Pro and NS_S eliminate GFP-specific siRNAs, consistent with their proposed mode of action. However, NS3 does not eliminate the GFP specific siRNAs, whereas the increased amount of GFP mRNA indicates that the GFP mRNAs are protected from RNA silencing. It can be concluded that NS3 achieves the same strong silencing suppression function as HC-Pro and NS_S, however by another mechanism. This is possibly realized either by directly protecting (a part of) the mRNA

population from degradation or indirectly by sequestering the siRNAs or interfering with the action of RNA silencing complexes, similar to the P19 silencing suppressor of the tombusvirus CymRSV (Silhavy *et al.*, 2002). Another rice virus, *Rice yellow mottle sobemovirus* (RYMV), has been indicated to encode a silencing suppressor protein (P1) using a similar assay in tobacco (Voinnet *et al.*, 1999). Also for P1, the increase of GFP mRNA was indicative for silencing suppression. The boost of transgene expression by NS_S, NS3 and HC-Pro may be achieved by the physical protection of messenger RNAs either directly or by interfering with later phases of the gene silencing machinery such as maintenance causing the suppression of silencing phenomenon. The latter would imply that gene silencing could be very rapidly evoked, even when a transgene is expressed transiently from an Agrobacterium inoculation, as also suggested by Johansen and Carrington (Johansen & Carrington, 2001).

Interestingly, *A. tumefaciens*-mediated introduction of NS_S, NS3 and HC-Pro suppressor proteins, but not CMV 2b, together with GFP in non-transgenic *N. benthamiana* plants resulted in an increased GFP protein expression and fluorescence compared to introduction of GFP alone. A similar observation can be made for the effect of the potyviral HC-Pro on GUS expression in results presented by Llave and co-workers (Llave *et al.*, 2000).

Recent findings in silencing-indispensable host genes indicate that RNA silencing interacts or even participates in gene regulation and plant development (Vance & Vaucheret, 2001). Also the influence of RNA silencing suppressors on the generation of micro RNAs, regulatory RNA elements resembling siRNAs (reviewed in (Schwarz & Zamore, 2002), may play a specific role in the altered regulation of genes in these plants. This is supported by the observation that plants overexpressing HC-Pro show abnormal development (Anandalakshmi *et al.*, 1998; Mlotshwa *et al.*, 2002) and the fact that despite numerous attempts it has not been possible to regenerate plants or even shoots transgenically expressing detectable amounts of NS_S protein (Prins *et al.*, 1996).

In this paper representatives of two different taxa of negative strand plant viruses, TSWV and RHBV, have been shown to encode suppressors of gene silencing in plants, indicating that this feature is present also in negative strand viruses. It will be interesting to investigate whether these proteins also play a role during virus replication in their insect vectors, even more so as RNA silencing has been demonstrated in insects (Pal-Bhadra *et al.*, 1997).

The observation that both genes responsible for silencing suppression are encoded on the same position of the TSWV and RHBV genomes, i.e. on the third largest segment in an ambisense arrangement opposite the N gene (see Fig. 3-1), further underscores the genetic interrelationship between tospo- and tenuiviruses. Whether this analogy can be further extended to related animal-

infecting bunyaviruses that are also transmitted by insects but infect vertebrates remains an open question. Interestingly, similar to TSWV, the NS_S genes of both phleboviruses and orthobunyaviruses have been implicated in virulence of the virus (Bridgen *et al.*, 2001; Vialat *et al.*, 2000). Particularly the NS_S gene of phleboviruses, a genus that shares an ambisense S RNA with the tospoviruses, is an intriguing candidate for further study.

Materials and Methods

Plants and viruses

Transgenic *N. benthamiana* plants harboring a GFP transgene expressed from a 35S promoter–nopaline synthase terminator expression cassette were used (Horsch *et al.*, 1985a). Transgenic lines were selected for strong GFP fluorescence prior to self-pollination. Subsequent S1 plants were scored for gene silencing by checking for GFP expressing in meristematic tissues in otherwise non-expressing (RNA silenced) plants. S2 progenies of these plants were homozygous and all showed a silenced phenotype resulting in silencing of GFP expression in leaf tissue. Complete GFP silencing in veins and stems was reached after several weeks. These S2 plants were used in the inoculation experiments.

TSWV isolate BR-01, GRSV isolate SA-05 and INSV isolate NL-07 were inoculated in series on both GFP silenced and non-transgenic *N. benthamiana* plants acting as controls. For reference also the potyviruses PVY and CABMV (Mlotshwa *et al.*, 2002) as well as two different CMV isolates (CMV-Lily and CMV-Alstroemeria, belonging to subgroup I and II, respectively, (Chen *et al.*, 2001)) were used in these experiments.

Inoculation was performed in the greenhouse in a 4-6 leaf developmental stage. Systemically infected top leaves were homogenized in 10mM sodium phosphate pH 7.2 with 0.1% $NaSO_3$ added using a mortar and pestle. Each plant was inoculated on two leaves using carborundum powder as an abrasive agent. A sponge was used to apply the inocula on the leaf.

A. tumefaciens *clones and* A. tumefaciens-*infiltration*

An expression vector was used harboring an expression cassette consisting of a 35S promoter, *Tobacco mosaic virus* (TMV) 5' untranslated region, multiple cloning site and a nopaline synthase (NOS) terminator. Expression cassettes carried individual TSWV genes: nucleoprotein N, movement protein NS_M, glycoprotein G_1G_2 precursor and NS_S have been previously described (Prins *et al.*, 1996). Also GFP, CABMV HC-Pro protein (Mlotshwa *et al.*, 2002) and CMV (subgroup I) 2b protein were used in similar expression cassettes. The NS3 gene of RHBV was cloned by RT-PCR, using primers containing the respective start and stop codons of the gene plus appropriate restriction sites for cloning. Thus obtained clones were verified by sequence analysis (results not shown). Expression cassettes were cloned in the binary vector pBIN19 and subsequently introduced in *A. tumefaciens* (strain LBA4404) using tri-parental mating. *A. tumefaciens* T-DNA transient expression assays (ATTA) in *N. benthamiana* plants were performed by (co)-infiltrating at least two locations on the basal side of the leaf with *A. tumefaciens* suspensions using a 5 ml syringe without needle. Cultures were grown overnight at 28°C from individual colonies in 2 ml YEB-medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% saccharose, 2 mM $MgSO_4$) containing 20 µg/ml rifampicin and 50 µg/ml kanamycin. 400 µl cell culture was pelleted by centrifugation and

resuspended in 2 ml induction medium (10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 50 µM acetosyringone and 10 mM MES pH5.6). After incubating overnight at 28°C cells were pelleted again and washed in Murashige and Skoog medium containing 10mM MES pH5.6. Cells were resuspended to an OD_{600} of 0.5 in MS-MES including 150 µM acetosyringone. Young fully expanded leaves were used for agro-infiltration and covered with plastic for 2-3 days in the greenhouse. Plants were subsequently monitored for GFP reactivation using a handheld 125W UV lamp. Generally expression reached a maximum level after 3-4 days.

UV photography

Pictures of whole plants (as shown in figure 2A) were made with a digital camera (Kodak DCS professional series) using a handheld 125W ultraviolet lamp (Philips HPW 125W-T) and 30 s exposure time. UV pictures at leaf level were made with 35mm Kodak 200 ASA film using a black box carrying 2 small UV lamps (366nm). Leafs shown in Figure 2B: exposure time 2 min, using a Kodak wratten no. 58 filter. Leafs in Figure 2 were exposed 1 min without filter. Close-up UV pictures as shown in Figure 3 were made with a digital camera (CoolSnap, combined red and green channel) using a binocular stereomicroscope (M3Z, Leica). The GFP imaging photographs of figures 4 and 5A were taken with a yellow 022 B+W filter from Proline. Variable exposure times were used depending on the intensity of the fluorescence.

Molecular analyses

Northern blot analyses were performed using standard protocols using ^{32}P radiolabeled full-length GFP PCR products. Western blot analysis of GFP and NS_S was performed using polyclonal rabbit antiserum. Isolation and enrichment of small RNAs was performed as described by Hamilton and Baulcombe (Hamilton & Baulcombe, 1999). Detection of siRNAs was performed by RNaseA/T1 protection assays according to Sijen et al. (Sijen *et al.*, 2001) with in vitro transcribed GFP RNA probes.

CHAPTER 4

The Influenza A Virus NS1 Protein Binds siRNAs and Suppresses RNA Silencing in Plants*

Abstract

RNA silencing comprises a set of sequence specific RNA degradation pathways that occur in a wide range of eukaryotes, including animals, fungi and plants. A hallmark of RNA silencing is the presence of small interfering RNA molecules (siRNAs). The siRNAs are generated by cleavage of larger double stranded RNAs (dsRNAs) and provide the sequence specificity to degradation of cognate RNA molecules. In plants, RNA silencing plays a key role in developmental processes and in control of virus replication. It has been shown that many plant viruses encode proteins, denoted RNA silencing suppressors, that interfere with this antiviral response. Although RNA silencing has been shown to occur in vertebrates, no relation to inhibition of virus replication has been demonstrated to date. Here we show that the NS1 protein of human influenza A virus has an RNA silencing suppression activity in plants, similar to established RNA silencing suppressor proteins of plant viruses. In addition, NS1 is shown to be capable of binding siRNAs. The data presented here fit with a potential role for NS1 in counter-acting innate antiviral responses in vertebrates by sequestering siRNAs.

*Bucher E. , Hemmes H. , De Haan P. , Goldbach R. , Prins M. (2004). The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* 85: 983-91.

Introduction

Several observations made in plants such as transgene-induced co-suppression of genes (Napoli, 1990; van der Krol *et al.*, 1990), post-transcriptional gene silencing (English *et al.*, 1996), RNA-mediated virus-resistance (Lindbo & Dougherty, 1992; de Haan *et al.*, 1992), virus-induced gene silencing and later in other organisms such as quelling in fungi (Cogoni & Macino, 1997) and RNA interference or RNAi in nematodes, insects and mammals (Elbashir *et al.*, 2001; Fire *et al.*, 1998; Tuschl *et al.*, 1999), turned out to rely on a similar molecular process. This process, now referred to as RNA silencing, is induced by over-expressed and double stranded RNA (dsRNA) molecules and involves sequence-specific RNA degradation in the cytoplasm of eukaryote cells (Sharp, 2001). The degradation products of this process, which is catalysed by an enzyme first identified in flies as DICER (Bernstein *et al.*, 2001) are RNAs of 21-25 nucleotides in length.

Two functional classes of these molecules produced by DICER cleavage have thus far been identified: microRNAs (miRNAs) and small interfering RNAs (siRNAs). The presence of these molecules is regarded as a hallmark of RNA silencing (Hamilton & Baulcombe, 1999). In plants, miRNAs seem to be predominantly involved in targeted mRNA degradation of transcription factors which play a role in development (Llave *et al.*, 2002b; Palatnik *et al.*, 2003), while siRNAs recruit specific proteins to form the RNA induced silencing complex (RISC) and initiate sequence specific degradation of target RNAs, such as viral RNAs (reviewed in (Vaucheret & Fagard, 2001) and (Zamore, 2002)).

The siRNA-mediated RNA silencing machinery has been suggested to play different roles in different organisms. In plants, its major function seems to be providing antiviral defense at the nucleic acid level. Indeed, Arabidopsis mutants exhibiting impaired RNA silencing show enhanced susceptibility to virus infection (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). In nematodes it appears to be stabilizing the genome by inactivating transposable elements (Ketting *et al.*, 1999; Tabara *et al.*, 1999). In fission yeast, RNA silencing plays an important role in the regulation of the chromosome dynamics during cell division (Hall *et al.*, 2003). Interestingly, so far, no naturally occurring siRNAs have been detected in mammalian cells even though they are active in initiating RNA silencing when supplied in trans (McCaffrey *et al.*, 2002), a method generally used to silence endogenous genes (Elbashir *et al.*, 2001). Transfected siRNAs have been shown to efficiently protect mammalian cells against viral infection by providing sequence specific intracellular immunity, suggesting that RNA silencing in mammalian cells can operate as an antiviral mechanism (Gitlin *et al.*, 2002).

To counteract the RNA silencing based defense mechanism in plants, viruses encode specific proteins that have the ability to block various steps of the RNA silencing pathway (Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Llave *et al.*, 2000; Voinnet *et al.*, 1999). Many of these proteins had previously been linked to 'virulence' of the virus. The HC-Pro protein of the plant infecting potyviruses was one of the first such silencing suppressing protein to be identified (Brigneti *et al.*, 1998). Suppression of RNA silencing by this protein is associated with a reduced accumulation of siRNAs, which is important for local RNA silencing (Llave *et al.*, 2000; Mallory *et al.*, 2001). Other viruses, such as *Cucumber mosaic virus* (CMV), express proteins, which have the ability to stop the systemic silencing signal from spreading in the plant (Beclin *et al.*, 1998). Viral silencing suppressors have so far been identified in many positive stranded RNA viruses (Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Voinnet *et al.*, 1999), negative strand RNA viruses (Bucher *et al.*, 2003) and DNA viruses (Voinnet *et al.*, 1999). The identification of RNA silencing suppressors has not remained limited to plant viruses as also the B2 protein of the insect-infecting *Flock house virus* (FHV) was identified as a suppressor of RNA silencing, operating in plants as well as insect cells (Li *et al.*, 2002).

Recently, it was shown that the negative strand RNA plant viruses *Tomato spotted wilt virus* (TSWV) and *Rice Hoja blanca virus* (RHBV) encode RNA silencing suppressors, (Bucher *et al.*, 2003). Like these two plant viruses, related vertebrate viruses also encode non-structural proteins with assigned 'virulence' functions. Many of these proteins were shown to interfere with innate defense responses of which the host interferon α/β- mediated response is the best studied (reviewed by (Garcia-Sastre, 2001)).

One of the most extensively studied proteins with such a 'virulence' function is the NS1 protein of influenza A virus (recently reviewed in (Krug *et al.*, 2003)). For this multi-functional protein three main functional domains have been proposed. On the N-terminal part of NS1 a domain involved in translational enhancement has been mapped between the amino acids 81-113 (Aragon *et al.*, 2000). This domain has been shown to directly interact with the eukaryotic translation initiation factor 4GI (eIF4G) allowing preferential translation of the influenza virus messengers. The C-terminal half constitutes the effector domain, which has been reported to inhibit mRNA processing and nuclear-cytoplasmic transport of host mRNAs (Lu *et al.*, 1995). It has been reported that NS1 interacts with a cleavage and polyadenylation specificity factor (CPSF) and the poly(A)-binding protein II (PAB II) required for 3'-end processing of cellular mRNAs (Chen *et al.*, 1999). This activity is thought to be required to prevent the maturation of host mRNAs encoding proteins with

antiviral activity. Other reports suggest that the C-terminal region of the protein is mainly required for optimal dimerization of NS1 in vivo, which is required for its RNA binding activity (Wang *et al.*, 2002). Additionally, NS1 contains a double-stranded RNA binding domain (dsRBD) located at the very N-terminal part of the protein (Hatada & Fukuda, 1992; Wang *et al.*, 1999). This domain has been reported to be essential for its IFN-α/β antagonistic property (Wang *et al.*, 2000). It was suggested that the mechanism of the NS1 IFN-α/β antagonistic function could be achieved by sequestering dsRNA. This would result in preventing the activation of PKR and NF-κB and thereby in inhibiting the induction of IFN-β. Interestingly, sequestering of dsRNA molecules has also been described as the mode of action of one of the best described plant viral RNA silencing suppressors in plants, i.e. p19 of Tombusviruses which directly associates with siRNAs (Silhavy *et al.*, 2002).

DsRNA play a central role in RNA silencing as well as in the induction of the interferon pathway. Moreover, characteristics of the established RNA silencing suppressors of plant viruses correspond to some of the described properties of influenza virus NS1. Therefore, we aspired to investigate whether the influenza A virus NS1 could play a role in suppressing RNA silencing, analogous to non-structural proteins of negative stranded plant viruses. Additionally we tested if this function of NS1 would be effectuated by sequestering siRNAs.

Results

The influenza virus NS1 protein inhibits RNA silencing in plants

In order to identify RNA silencing suppressor activity of viral proteins, a standard method has been developed which is broadly applied to date (Johansen & Carrington, 2001; Voinnet *et al.*, 2000). The method is based on the infiltration of two *Agrobacterium tumefaciens* strains into leaves of a *Nicotiana benthamiana* plant either a wild-type plant or a plant containing a GFP transgene in a silenced state. One strain carries the GFP silencing initiator reporter gene and the other one the candidate silencing suppressor gene. If indeed the candidate gene possesses silencing suppression activity, it will induce a visible boost of GFP expression in the infiltrated leaf patch. Using this method we were able to identify the first silencing suppressors of plant infecting negative stranded RNA viruses (Bucher *et al.*, 2003).

When infiltrating *A. tumefaciens* strains carrying the GFP reporter gene in combination with the one expressing the NS1 gene product, highly enhanced GFP expression was observed (Fig. 4-1A). The level of GFP protein expression in NS1 expressing leaves was comparable to infiltrations with

the established plant viral silencing suppressor NS_S of TSWV and HC-Pro of the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) (Fig. 4-1B). To verify that high GFP expression was indeed due to mRNA protection rather than an enhanced translation, Northern blot analyses were performed. The high accumulation of GFP mRNAs in plant leaves demonstrate that, like for TSWV NS_S and CABMV HC-Pro, the NS1 protein protects the GFP mRNA from degradation by the RNA silencing machinery (Fig. 4-1C).

To further elucidate the role of NS1 in suppressing RNA silencing, siRNAs were extracted from infiltrated leaves and analysed with a GFP specific probe (Fig. 4-1D). The expression of NS1 protein (Fig. 4-1E) drastically reduces the accumulation of GFP specific siRNAs, indicating that the influenza virus protein directly interacts with siRNAs, or inhibits siRNA production. In both cases NS1 would interrupt the 'degradative PCR' cycle (Lipardi *et al.*, 2001; Sijen *et al.*, 2001) and thus interfere with RNA silencing.

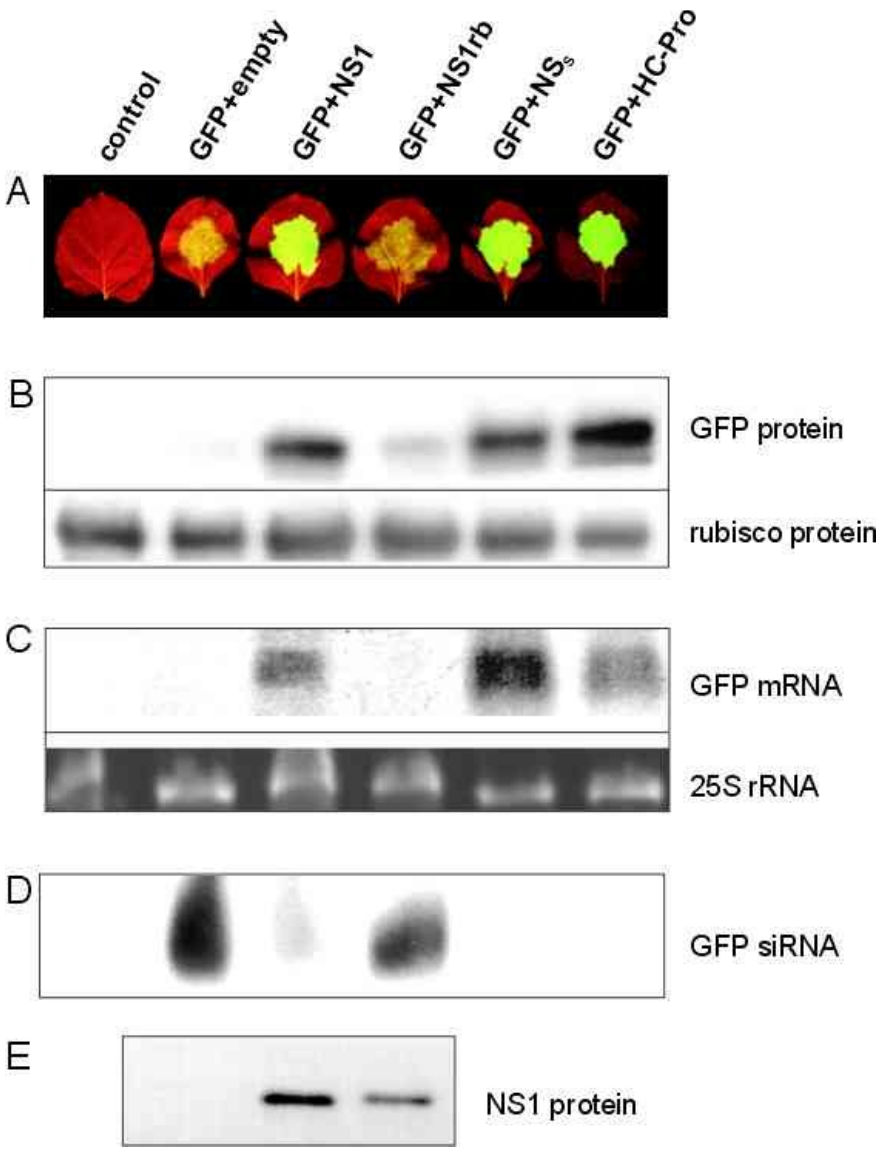

Figure 4-1. The effect of wild type NS1 and mutant NS1rb on RNA silencing of GFP in *N. benthamiana* leaves. (A) UV photography of Agrobacterium infiltrated leaves. From left to right: non-infiltrated wild type and subsequently GFP expression constructs respectively co-infiltrated with an empty binary vector; the influenza virus NS1 gene construct; the NS1rb mutant; HC-Pro and NS_S. (B) Quantitative Western blot analysis performed on total protein extracted from infiltrated sectors of the leaf using anti-GFP antibodies. Rubisco protein abundance in these green leaves was used as a loading control and was visualized using anti-rubisco (SSU) antibodies. (C) Northern blot analyses of mRNA purified from infiltrated leaf sectors, probed with a DIG-labelled GFP specific PCR fragment. Ethidium bromide staining of the same gel shows the 25S rRNA as a loading control. (D) GFP siRNAs extracted from infiltrated leaf sectors and detected using a DIG labelled GFP specific probe. (E) Expression of NS1 and NS1rb protein analysed by Western blot using NS1 specific antibodies.

Expression of NS1 in a PVX virus vector enhances its pathogenicity

In an extensive analysis Brigneti and co-workers (Brigneti *et al.*, 1998) have shown that the expression of viral silencing suppressing proteins in a potato virus X (PVX) vector drastically increases symptom severity of the virus in N. benthamiana plants. To test the influence of NS1 on pathogenicity, we cloned the NS1 gene in sense and anti-sense orientation into the pGR106 PVX vector, kindly provided by the Baulcombe group. Our GFP-silenced transgenic *N. benthamiana* line (Bucher *et al.*, 2003) was then tooth-pick inoculated on a lower leaf with *A. tumefaciens* containing pGR106 with NS1 in sense or anti-sense orientation and monitored for the development of viral symptoms. As shown in figure 4-2A the PVX vector expressing the NS1 protein (as confirmed by western blot analysis shown in Fig. 4-2D) produced severe symptoms while the anti-sense construct typically showed mild, wild type-like symptoms (Fig. 4-2A). To confirm reversal of the silenced state of the GFP transgene, systemically infected (top) leaves were monitored for GFP expression. Indeed, upon expression of NS1 from the PVX genome local GFP expressing patches could be observed (Fig. 4-2B and C).

The influenza virus NS1 protein requires a functional dsRNA binding domain for its activity as a suppressor of RNA silencing

Since the effect of NS1 on RNA silencing could be conferred by its dsRNA binding activity, substitutions of the amino acids R35 and R38, which are essential for the dsRNA binding activity of NS1, with alanine were made resulting in NS1rb (Wang *et al.*, 1999). *A. tumefaciens* infiltration experiments were repeated with this mutant form of NS1 and showed that this protein was unable to enhance GFP expression (Fig. 4-1A and B). Northern blot analysis of the RNA extracted from the infiltrated patch showed that NS1rb does not protect the GFP mRNA from degradation (Fig. 4-1C) indicating that the mutant protein is incapable of inhibiting RNA silencing. Ongoing, active RNA silencing in the presence of mutant NS1 was further demonstrated by the presence of GFP-specific siRNAs (Fig. 4-1D). Expression of NS1 and NS1rb was confirmed by Western blot analysis (Fig. 4-1E).

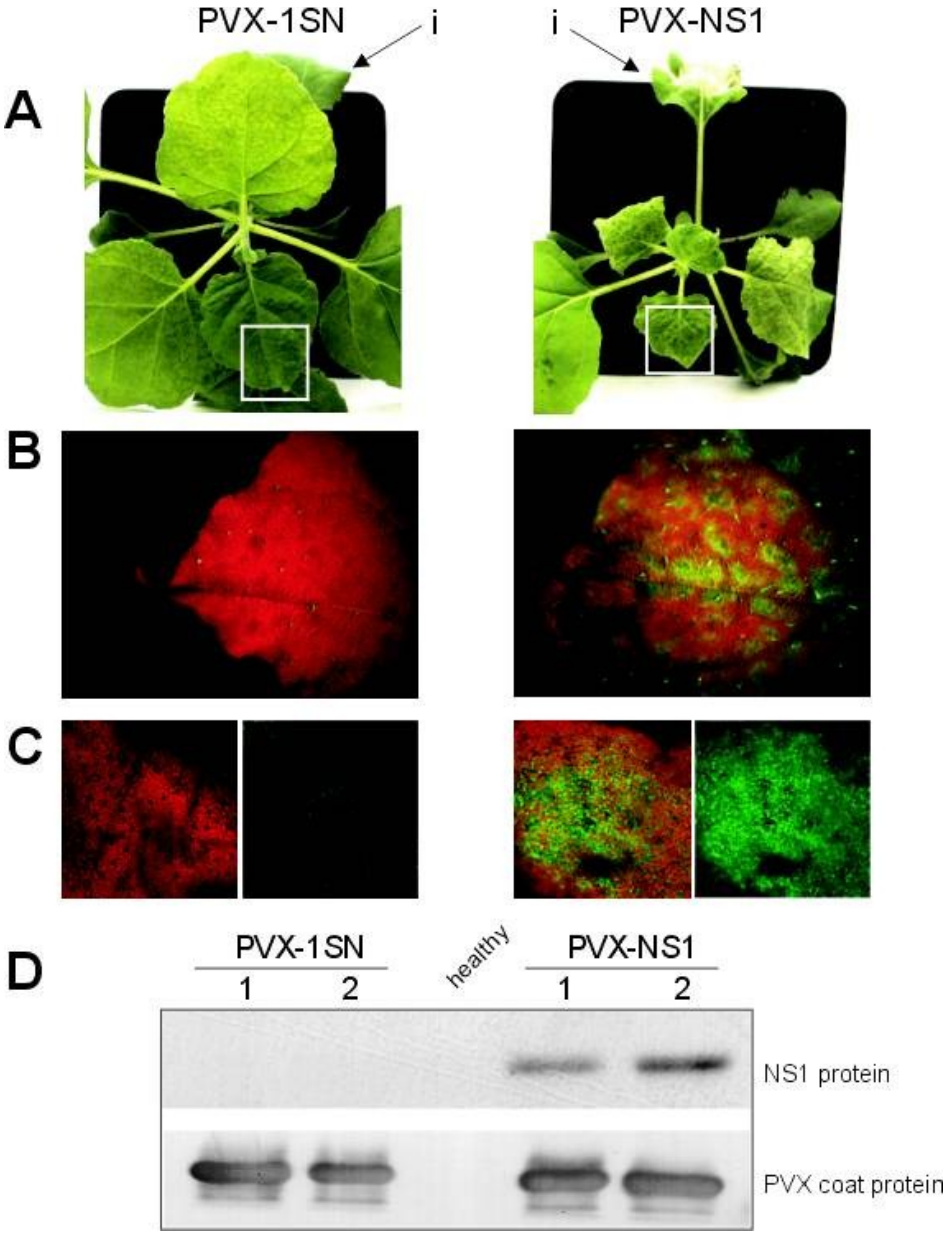

Figure 4-2. Analysis of plants infected with PVX carrying the NS1 gene. (A) Symptoms caused by the infection with PVX-1SN (NS1 cloned in anti-sense orientation) and PVX-NS1 (NS1 cloned in sense orientation) in *N. benthamiana* plants 6 days after inoculation. The inoculation sites are indicated by arrows (i). (B) UV photography of a close up of systemically infected GFP-silenced *N. benthamiana* leaves (section indicated by squares in panel A) either infected with PVX-1SN or PVX-NS1. (C) LSM pictures of a fluorescent patch caused by PVX-NS1 compared to PVX-1SN (left panel: red and green overlay, right panel: green only). The same light intensities were used for both pictures. (D) Western blot analysis of leaves originating from two systemically infected plants using NS1 and PVX coat protein specific antibodies.

The influenza virus NS1 protein binds siRNAs

NS1 has previously been reported to bind dsRNA molecules as short as 50-140 nucleotides (Wang *et al.*, 1999). Combining the observation that NS1 is involved in RNA silencing suppression in plants and that it requires a functional dsRNA-binding domain, we wanted to examine whether NS1 could exert this function by binding siRNAs.

For this purpose gel-shift experiments were performed with purified NS1 protein produced in *E. coli*. As shown in figure 4-3A, the NS1 protein is able to bind radiolabelled synthetic 21 bp siRNAs resulting in band shifting. Similarly, NS1 was capable of binding radiolabelled siRNAs extracted from plants (Fig. 4-3B).

Since the NS1rb lost its capacity to suppress RNA silencing, we tested whether this mutant was deficient in binding siRNAs. His-tagged NS1rb protein was purified from *E. coli* and tested in the same band-shift experiment as wt NS1 (Fig. 4-3A). Indeed it was shown that the NS1 protein with mutations in the RNA binding domain was no longer capable of binding siRNAs, neither synthetically produced nor isolated from plants (Fig. 4-3A and B).

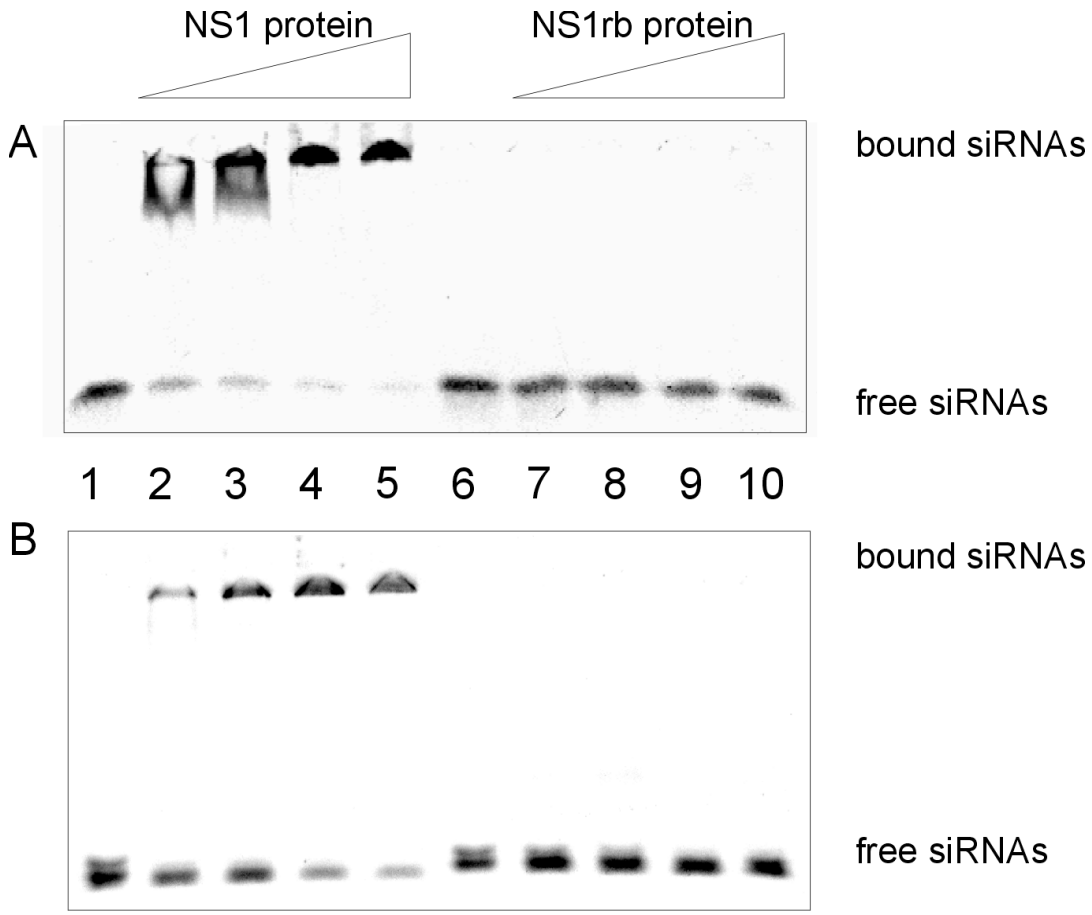

Figure 4-3. Gel retardation studies of siRNAs binding to increasing amounts of NS1 protein. (A) Radiolabelled synthetic siRNAs (2 pM) incubated with 0, 25, 50, 100 and 200 pM of purified NS1 (lanes 1-5) and NS1rb (lanes 6-10) visualized by radiography after native gel electrophoresis. (B) Radiolabelled purified plant siRNAs incubated under the same conditions.

Discussion

Many animal and plant viruses carry genes that have been described to be involved in virulence. Mutation or removal of these genes generally resulted in an attenuated phenotype of the virus (Bergmann *et al.*, 2000; Garcia-Sastre *et al.*, 1998; Qu & Morris, 2002). In plant viruses many of these genes are involved in the suppression of the RNA silencing machinery of the host (reviewed e.g. in (Li & Ding, 2001)). We have recently shown that also negative stranded RNA plant viruses of the Tospovirus and Tenuivirus genera carry such suppressors of RNA silencing (Bucher *et al.*, 2003). Negative strand viruses form a minority among the plant viruses, but are much more common in animal hosts (Van Regenmortel *et al.*, 2000).

We here demonstrate that, like for established plant viral RNA silencing suppressors such as

HC-Pro or NS_S, the influenza A virus protein NS1 enhances expression of an *A. tumefaciens*-delivered reporter protein construct by protecting its mRNAs from degradation. Concomitantly, siRNA production is drastically reduced, which indicates that NS1 interferes with the plant RNA silencing machinery. Additionally, here and in Delgadillo *et al.* (Delgadillo *et al.*, 2004) it was shown that, expression of NS1 from a PVX viral vector results in strong enhancement of symptoms, a phenomenon often observed when expressing RNA silencing suppressors in plants (Brigneti *et al.*, 1998). Additionally, we show the expression of NS1 in a PVX background leads to some local suppression of an established RNA silencing state in transgenic GFP silenced plants.

The siRNA binding capability of NS1 indicates that it may sequester free siRNAs during infection. By doing so it prevents the siRNAs from being incorporated into the RISC complex and take part in the generation of longer dsRNA molecules and subsequent DICER/RISC mediated degradative PCR of (viral) RNAs. In previous research, NS1 has been proven to bind longer dsRNAs (Wang *et al.*, 1999), although it is less efficient in binding to dsRNAs than other cellular dsRNA binding proteins (Krug *et al.*, 2003). Here we show that NS1 has a high affinity for siRNAs. It would be interesting to investigate whether NS1 has a higher affinity for the larger class of siRNAs (24-26 nt), since independent research by Delgadillo et al. (Delgadillo *et al.*, 2004) shows that this size class is most affected by NS1 action. In plants, the smaller size class of siRNA (21-22 nt) has been implicated in suppression of local RNA silencing (RISC action) while the larger size class seems involved in systemic silencing and methylation status of homologous DNA (Hamilton *et al.*, 2002). As shown in Fig. 4-1, NS1 has a clear effect on local RNA silencing leading to re-emergence of GFP expression, while Delgadillo et al (Delgadillo *et al.*, 2004) also show an effect on the suppression of systemic silencing.

It can be argued that specific siRNA binding by NS1 merely reflects its capacity to generally bind any dsRNA. Indeed a recent report showed that several double stranded RNA binding proteins (dsRBPs) were capable of acting as RNA silencing suppressors in plants (Lichner *et al.*, 2003). It was suggested that they act by sequestering (larger) dsRNA molecules and hiding them from the silencing machinery. Next to the NS1 protein we have expressed the NSP5 protein of Rotavirus, also a viral dsRNA binding protein (Vende *et al.*, 2002), in the *A. trumefaciens* infiltration assay. Expression of this protein however did not result in RNA silencing suppression (results not shown) and may suggest that RNA silencing suppression is not a general feature of dsRNA binding proteins.

Recent elegant work by the Burgyàn group has demonstrated that the P19 protein of the plant-infecting Tombusviruses suppresses RNA silencing in plants by sequestering siRNAs through direct

binding (Silhavy *et al.*, 2002). Here we show that, beside its previously demonstrated activity to bind larger dsRNA molecules, NS1, like P19, efficiently binds siRNAs, either synthesised synthetically or isolated from plants. The dsRNA-binding motif in NS1 is required for binding siRNAs as it is for its RNA silencing suppression activity in plants.

It may be assumed that, like in plants, NS1 binds siRNAs and longer dsRNAs in its natural host. In plants the involvement of siRNAs is essential for virus inhibition through RNA silencing. In animals, larger dsRNA molecules (Williams, 1999) as well as siRNAs (Bridge *et al.*, 2003; Sledz *et al.*, 2003) have been demonstrated to be involved in the sequence unspecific initiation of the interferon-mediated innate antiviral response. It has been demonstrated that dsRNA triggers the secretion of interferons, which subsequently induce the production of PKR, 2'-5'-oligoadenylate synthetases (OAS) and many more proteins with proposed antiviral activities (Kaufman, 1999; Rebouillat & Hovanessian, 1999; Williams, 1999). Until recently it was assumed that only longer stretches of dsRNA could induce the interferon response. However, recent reports suggest that short hairpin RNAs and siRNAs, designed to initiate RNA interference, can also trigger the interferon response (Bridge *et al.*, 2003; Sledz *et al.*, 2003).

As yet, it can not be excluded that the function of NS1 in mammalian cells may be limited to sequestering siRNA and larger dsRNA molecules from detection by the IFN-α/β response and PKR (Garcia-Sastre, 2001). However, siRNA molecules, when transfected to mammalian cells, have been demonstrated to inhibit virus replication in a sequence specific manner (Andino, 2003; Gitlin *et al.*, 2002), suggesting an active sequence specific RNA silencing machinery, which, like in plants and insects, can act as an antiviral response in mammalian cells. Taking this assumption further this would imply that, next to or underlying the IFN-α/β response, RNA silencing may play a role in the antiviral defense in mammals. It is very interesting to note that the amino-acids which are involved in the interferon antagonistic properties of NS1 in mammalian cells coincide with the ones essential for RNA silencing suppression in plants.

Taken all together, we have demonstrated here that NS1 suppresses RNA silencing in plants by binding siRNAs. As a result our data suggests that RNA silencing may represent an important inhibitory function of virus infection that is counteracted by specific viral proteins, not only in plants, where it is well established, but also in animals. Clearly, to prove this final assumption, it will be of great importance to investigate the effects of NS1 on siRNA and dsRNA-induced RNA silencing in mammalian systems using the recently established experimental protocols (Gitlin *et al.*, 2002; McCaffrey *et al.*, 2002).

Materials and Methods

Protein expression clones,* A. tumefaciens-*infiltration and photography

The Agrobacterium clones containing the GFP, NS_S and HC-Pro expression cassettes were described earlier (Bucher *et al.*, 2003). The NS1 ORF of Influenza A/PR/8/34 virus was cloned by PCR from the pRF462 plasmid provided by Ron Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. The expression cassette was cloned in the binary vector pBIN19 and subsequently introduced into Agrobacterium tumefaciens (strain LBA4404). In the mutant form of NS1, NS1rb, the R35A and R38A mutations were inserted by PCR using primers EB08: 5'-**GC**GCTTCGC**G**CAGATCAGAAATCCC-3' and EB11: 5'-ATCAAGGAATGGGGCATCACCTAG-3' (Bold characters indicate the point mutations). *A. tumefaciens*-infiltration experiments and GFP imaging photography were performed as presented previously (Bucher *et al.*, 2003). The laser scanning (LSM) photography pictures were taken using a Zeiss LSM510 microscope.

Isolation of siRNAs, Northern blot and non-radioactive detection

Transgenic leaf material was ground in liquid nitrogen and resuspended in 1.3 ml of 2% Sarkosyl, 5M NaCl per gram of leaf material. After phenol extraction, polysaccharide contaminants were precipitated with 1 Vol. 3M Ammonium acetate pH 5.2 (Sharma *et al.*, 2003). The water phase was ethanol precipitated and resuspended in TE. To remove larger RNA molecules a PEG precipitation was performed using 5% PEG8000 / 0.5M NaCl final concentration (Hamilton & Baulcombe, 1999). The supernatant, containing the siRNAs was precipitated with ethanol. Twenty µg of total siRNAs per sample and 10 µg total RNA of the PEG8000 precipitate were analysed by Northern blot using a GFP specific DIG labelled (Boehringer) PCR product to detect the GFP siRNAs and mRNAs respectively.

Western blot analysis, NS1 and NS1rb protein production, purification and siRNA binding studies

Total protein was extracted from infiltrated leaves and quantified by Bradford assay (Biorad). 10 µg total protein was analysed by Western blot using anti-GFP and anti-rubisco (SSU) antibodies. The NS1 and NS1rb proteins were expressed in an N-terminally his-tagged form using the pQE31 vector system (Qiagen). The proteins were purified on TALON CellThru affinity columns (BD Biosiences). Synthetic double stranded luciferase GL3 siRNAs were purchased from Qiagen. The 5' phosphate groups of the siRNAs were removed by phosphatase treatment and replaced by ^{33}P radiolabelled phosphate groups. Plant siRNAs were enriched, radiolabelled and purified from an 8% PAGE gel using the radiolabelled luciferase siRNAs as marker. For the band-shift studies different concentrations of NS1 or NS1rb protein were mixed with labelled siRNAs and incubated on ice for 20 min. A native 5% PAGE gel was used for sample analysis (Wang *et al.*, 1999). Radiolabelled siRNAs were detected by autoradiography.

CHAPTER 5

Profiling Changes in the *Nicotiana benthamiana* Transcriptome upon Expression of Viral RNA Silencing Suppressor Proteins

Abstract

RNA silencing processes in plants are involved in gene regulation, but have also been recognised as an important antiviral defense mechanism. Whereas gene regulation by RNA silencing is guided by plant-encoded microRNAs (miRNAs), antiviral activity is specified by small interfering RNAs (siRNA) derived from the virus. To counteract, what can be regarded as an siRNA based immune system of the plant, viruses have evolved proteins that can inhibit this line of defense. In previous studies, some of these viral RNA silencing suppressor proteins have been shown to also interfere with the activity of miRNA guided gene regulation. These studies, mainly based on expression of RNA silencing suppressor proteins in transgenic plants have shown that the expression levels of a multitude of transcription factors can be influenced by RNA silencing. Since miRNAs play an important role in development the disturbing activity exhibited by viral silencing suppressors make them important determinants in the symptom manifestation of the host. As an alternative to the often-used microarray technology, cDNA-AFLP provides a powerful tool to perform such transcriptome analyses. This technology has advantages especially in plant species of which the complete genome sequence is still lacking. The aim of the research described in this chapter is to study the changes in the plant transcriptome at large caused by 6 different viral RNA silencing suppressors in the plant virus model organism *Nicotiana benthamiana*.

Introduction

RNA silencing is a sequence specific RNA degradation mechanism that can be induced by double-stranded RNA, which is now generally regarded as a major defense mechanism of plants against pathogenic nucleic acids. These include positive and negative stranded RNA viruses, DNA viruses and transposons (Baulcombe, 2004; Sijen & Plasterk, 2003; Voinnet, 2001). The initiator dsRNA can be produced by plant-encoded RNA dependent RNA polymerases for example from over-expression of a specific RNA or aberrant RNA, or perhaps viral intermediate RNAs of double stranded nature, such as replication complexes (Baulcombe, 2004). The key molecules of RNA silencing are the siRNAs which are produced from dsRNA by an RNase III domain-containing enzyme termed DICER. These siRNAs are then incorporated into the RNA induced silencing complex (RISC) which uses the siRNAs as guides to identify mRNAs for cleavage.

Plant and animal infecting viruses cope with RNA silencing mediated defense activity by encoding specific RNA silencing suppressor proteins, which can either modulate or even completely block the RNA silencing machinery of the host. Almost all plant viruses encode suppressors of RNA silencing (Goldbach *et al.*, 2003; Roth *et al.*, 2004). Intriguingly, these proteins show no protein sequence homology. This indicates that different suppressors might act at different steps of the RNA silencing pathway. Indeed such differences have been found in the case of the well studied suppressors. For instance, P19 of the tombusviruses has been shown to specifically bind small interfering RNAs (siRNAs) (Silhavy *et al.*, 2002). Therefore P19 can interfere with the siRNA dependent steps of the pathway, such as the siRNA guided mRNA cleavage. The HC-Pro protein of the potyviruses, for example, appears to act differently. It was reported to interact with an endogenous suppressor of RNA silencing, rgs-CaM, and transgenic plants expressing HC-Pro show developmental defects (Anandalakshmi *et al.*, 1998; Anandalakshmi *et al.*, 2000; Brigneti *et al.*, 1998).

Interestingly, RNA silencing has recently been shown to be also involved in processes in the cell other than antiviral defense. Of these processes the influence of RNA silencing-mediated activity on development has by far shown to have the greatest impact. Rather that processing siRNAs from larger dsRNA molecules of, e.g. viral origin, the effector molecules of RNA silencing in development are miRNAs, similar in structure to siRNAs, but processed from larger host-encoded precursor molecules (Bartel, 2004; Carrington & Ambros, 2003).

The transgenic expression of silencing suppressors and their effect on the expression of some plant genes have been studied in several reports (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004;

Kasschau *et al.*, 2003; Mallory *et al.*, 2002). It was found that the expression of i.e. HC-Pro could interfere with several RISC mediated functions such as miRNA-mediated cleavage of SCARECROW-like mRNAs resulting in increased levels of these regulatory proteins. In general, miRNAs play an important role in directing the transcript levels of regulatory proteins and therefore a disturbance in their production or activity due to silencing suppression can result in deviating phenotypes in (transgenic) plants. At the biochemical level, HC-pro was suggested to inhibit the proper unwinding and insertion of miRNAs into RISC, thereby preventing their action (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003).

An in depth study regarding the effect of silencing suppressors on the plant transcriptome was reported on AC2 of the *African cassava mosaic virus* and the *Mungbean yellow mosaic virus*-Vinga (Trinks *et al.*, 2005). It was found that the transactivation activity of the AC2 suppressor protein of this DNA virus was capable of inducing the transcription of a certain set of genes of the host plant. Some of theses genes were shown to act as endogenous suppressors of RNA silencing such as Werner Exonuclease 1. The aforementioned rgs-CaM identified using a protein-protein interaction screen with HC-Pro was also implicated as such (Anandalakshmi *et al.*, 2000). These proteins would then function in the endogenous negative regulation of RNA silencing. It can be envisaged that such proteins could be required in certain developmental stages where RNA silencing mediated regulation of endogenous genes by i.e. miRNAs needs to be down-regulated.

The work presented here aims to shed more light on how a series of viral silencing suppressors act at the plant transcriptome level. Thus far, research on the effect of RNA silencing suppressor proteins has concentrated on *A. thaliana* plants, because genomic tools in this model are well established. Most viruses, however, do not naturally occur in *A. thaliana*. Moreover, studies thus far were based on constitutive expression of the proteins. As these proteins interfere with normal development of the plant this may present an unwanted selection of transgenic plant lines. *Nicotiana benthamiana* plants can be infected by nearly all viruses including positive and negative sense RNA viruses and DNA viruses. Furthermore, we chose to use a transient expression system allowing normal development of the plant prior to expression of the suppressor proteins which is in better agreement with the natural occurrence of these proteins. As no microarrays are available for *N. benthamiana* as yet, we chose to set up a cDNA-AFLP assay for monitoring gene expression. The advantage of this system is that it can be used without prior knowledge on the host genome. With this set-up information will be generated on similarities and differences in gene expression patterns changing due to transient expression of RNA silencing suppressors. Resemblances in the activities of a range of 6 silencing suppressors and their effects on the host will be discussed.

Results

Experimental setup

The *A. tumefaciens* mediated transient expression assay is a commonly used method in the study of silencing suppressors (Johansen & Carrington, 2001; Voinnet *et al.*, 2000). *N. benthamiana* plants are most often used in these assays, since they are easy to infiltrate with *A. tumefaciens* suspension cultures and, in contrast to *A. thaliana*, are susceptible to infection with numerous viruses (Van Regenmortel *et al.*, 2000). Since many researchers have already used *A. tumefaciens* infiltration assays to identify new silencing suppressor proteins and to determine how different silencing suppressors act, we aspired to use this approach for a range of established RNA silencing suppressor proteins. To this end 6 different silencing suppressors, representing viruses from different classes (positive- and negative-stranded RNA viruses) were expressed and their effect on the plant transcriptome was analysed. The following silencing suppressors were used: The 2b proteins of the lily isolate of *Cucumber mosaic virus*-lily, subgroup 1 (2b-lil) (Chen *et al.*, 2001; Palukaitis *et al.*, 1992) and CMV-alstroemeria, subgroup 2 (2b-als) (Chen *et al.*, 2001). 2b-lil and 2b-als share 38% identity and 48% positives at protein level. These two were included so that also two closely related silencing suppressors could be compared. HC-Pro of *Cowpea aphid borne mosaic virus* (CABMV) (Mlotshwa *et al.*, 2002)), NS_S of *Tomato spotted wilt virus* (TSWV) (Bucher *et al.*, 2003), NS3 of *Rice hoja blanca virus* (RHBV) (Bucher *et al.*, 2003) and NS1 of *Influenza A virus* (Bucher *et al.*, 2004). NS1 is the only protein used in this study which does not originate from a plant virus. However, it was included since it was shown to act as a suppressor of RNA silencing in plants (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004).

All silencing suppressors were cloned in a binary vector and transformed into *A. tumefaciens*. The *A. tumefaciens* strains (LBA4404) were grown in culture and infiltrated as described previously (Bucher *et al.*, 2003). *A. tumefaciens* strains containing a binary vector lacking a transgene or a binary vector containing GFP were included as controls. Young *N. benthamiana* plants (4-6 leaf stage) were infiltrated with *A. tumefaciens* suspensions. Leaves were collected for RNA extraction after six days, this time was previously determined to result in the maximum RNA silencing effect (results not shown and Johansen & Carrington, 2001). All silencing suppressors were co-infiltrated with GFP as an internal control.

For genomic approaches, the use of *A. thaliana* has advantages over *N. benthamiana*. The *A. thaliana* genome is completely sequenced and commercial microarray chips are available. At present, little sequence information is available on *N. benthamiana*, though it is currently being

sequenced as part of the the Tobacco Genome Initiative (TGI, North Carolina State University). On the other hand, *N. benthamiana* is the most used model organism for plant virology since it is susceptible to a broad range of viruses. As an equivalent replacement of the microarray chips technology cDNA-amplified fragment length polymorphism (cDNA-AFLP) can be applied (Volkmuth *et al.*, 2003). The advantage of cDNA-AFLP, also in this case, is that no prior knowledge of the genome sequence of the model organism is required. Furthermore, the *A. tumefaciens* infiltration assay allows the transient local expression of genes of interest in fully developed tissue. To analyse the transriptome changes in such an assay, cDNA-AFLP analyses were carried out at Keygene N.V., 6700 AE Wageningen, The Netherlands, as described in (Vos *et al.*, 1995) and (Volkmuth *et al.*, 2003). The analysis was carried out using the *Taq*I and *Mse*I restriction enzymes and +2/+3 nucleotides in the amplification step. The number of primer combinations used is sufficient to obtain an overall coverage of approximately 50% (25,000 transcripts) of the transcribed mRNAs of *N. benthamiana*.

Differential analysis and annotation

Quantitative analyses of the cDNA-AFLP gels were carried out using the MegaXtractor and Xdiff software packages (Keygene). Differentials were calculated from band intensity differences between AFLP fragments from plants infiltrated with mixed *A. tumefaciens* suspensions containing GFP plus empty vector or GFP plus the different RNA silencing suppressor expression vectors. Differentials were only considered as significant when no difference was observed between infiltration with empty vector only and GFP plus empty vector. Thus excluding possible differentials that could be caused by GFP expression. Approximately 25,000 cDNA-AFLP fragments were detected covering an estimated 50% of the *N. benthamiana* transcriptome. 362 cDNA-AFLP fragments were scored as significant (changes higher then +/-3 fold; see Tab. 5-1.), isolated from the original gels, re-amplified and sequenced. The amplified sequences usually ranged from 100-500 nucleotides in length. Of these 362 cDNA fragments, 294 differentials were analysed in more detail. Based on similarities with other sequenced plant genes present in databases, 227 differentially expressed sequences could be annotated. Sequence annotation was performed by blasting against several public databases (NCBI, TIGR and TAIR). All annotated AFLP fragments are shown in table 5-1 All annotated and non-annotated fragments with additional information are provided in a supplementary Microsoft Access or OpenOffice.org database file (see Supplementary Files 1 and 2, available online: http://www.dpw.wau.nl/viro/research/aflp.html). The database also contains blast results of the cDNA-AFLP fragments against the ASRP small RNA database

(http://asrp.cgrb.oregonstate.edu/db/) (Gustafson *et al.*, 2005). Blast results in the TIGR database would sometimes result in a significant hit with a yet non-annotated gene. In such cases, these blast hits were used to obtain additional sequence information surrounding the region homologous to the AFLP fragment and these sequences were re-blasted to obtain additional information.

Cluster analysis of the expression profiles

In order to get a general overview on how the silencing suppressors affect the plant transrciptome and to globally compare them a cluster analysis was performed. To group the effects of the different silencing suppressors by expression pattern, hierarchical average cluster analysis was performed as initially described by (Eisen *et al.*, 1998) (Fig. 5-1). The first and foremost observation is that the vast majority of differentially expressed transcripts are down regulated. Clearly, NS_S and HC-Pro had the most outstanding effect on the plant transcriptome compared to the other RNA silencing suppressors used in this study. In part this was expected, as these proteins are known to have a strong effect on RNA silencing as opposed to the weaker suppressors such as 2b. Though in the *A. tumefaciens* infiltration experiment the effect of NSs and HCpro share several similarities, a significant difference in gene expression change caused by NS_S and HC-Pro can be observed. The total overlap of genes that are regulated in the same way by these silencing suppressors is around 30%, while many other genes are either affected only by NS_S or by HCpro. As was to be expected the two silencing suppressors of both CMV subgroups, 2b-lil and 2b-als cluster together. These two suppressors appear to affect only a few transcripts, which might reflect the relatively weak effect on GFP silencing inhibition these suppressors exhibit when compared to the other suppressors (Bucher *et al.*, 2003). The control experiment where no *A. tumefaciens* infiltration was performed also clusters in that section.

The hierarchical average cluster analysis allows the grouping of genes with similar expression patterns. The AFLP fragments could be grouped into three major clusters (Fig. 5-1). Cluster A contains most of the genes being up-regulated by all silencing suppressors used. Cluster B contains mainly genes down-regulated by HC-Pro only. Similarly, genes mostly down-regulated by NS_S are clustered in cluster C.

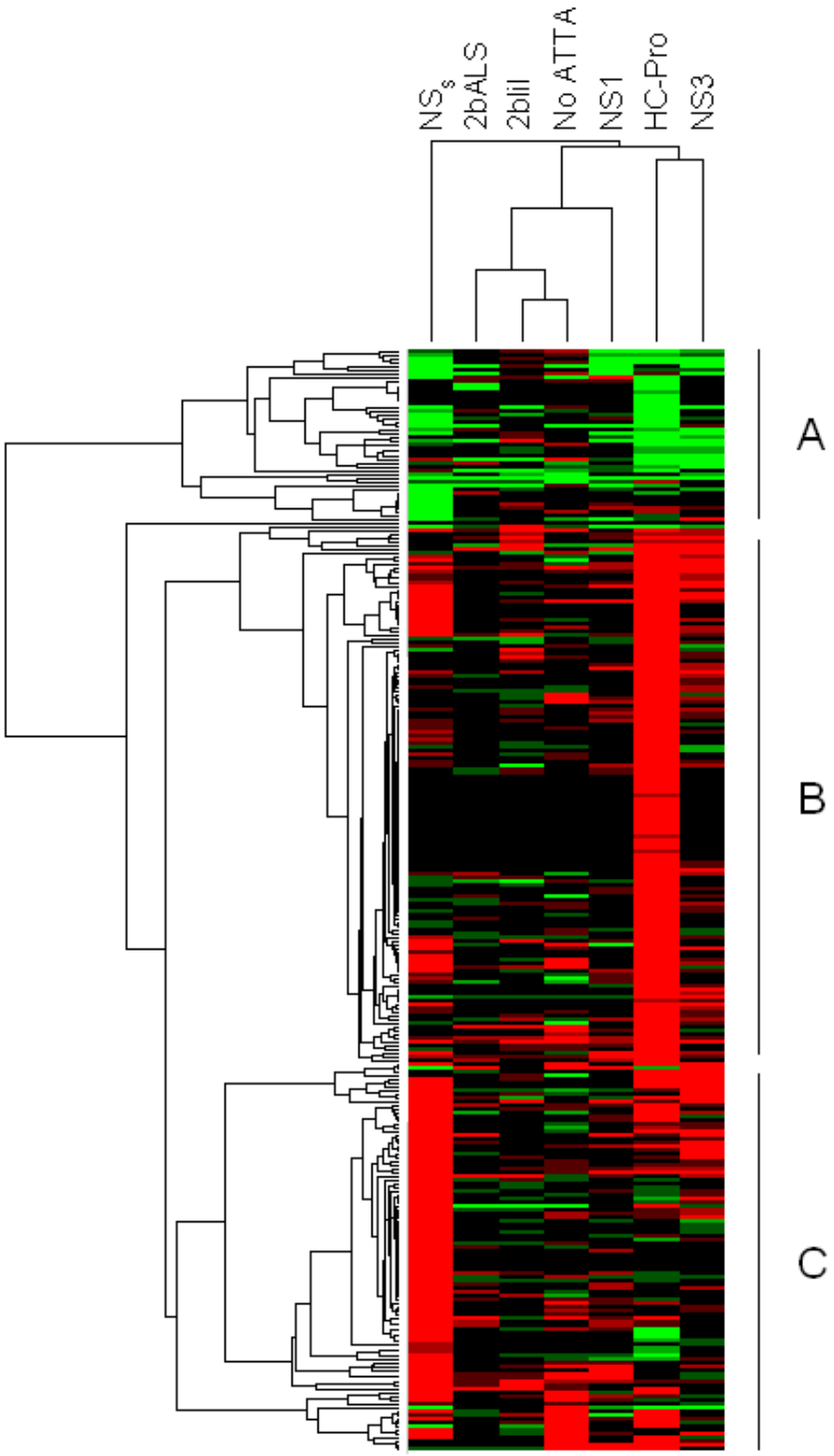

Figure 5-1. Cluster analysis of the cDNA-AFLP analysis. Red is down- and green is up-regulation.

Gene families affected by silencing suppressors

Where possible, annotated cDNA-AFLP fragments were grouped by their (putative) function (Tab. 5-1.). This was done in order to identify which pathways within the plant transcriptome were most affected by RNA silencing suppression. The main functional groups are: Metabolism, chloroplast-related, defense response, signalling, transcription factors, RNA processing and stress response. Interestingly cluster B contains 11 out of the 14 transcription factors found, confirming the previously indicated influence of HC-pro on transcription factors (Kasschau *et al.*, 2003). Other functional groups do not seem to specifically cluster together with the expression of a specific suppressor protein.

Controls and significance

A first confirmation of the objectivity of cDNA-AFLP was given when genes were found that would have been expected to be detected as differentials. A very clear differential was the co-infiltrated GFP gene that was included in all experiments. As expected, all strong local silencing suppressors (NS_S, HCpro, NS3, NS1) enhanced the GFP mRNA levels 3 to 22 fold compared to the control. Furthermore differentials were retrieved for the expression of the suppressor proteins themselves (section controls in Tab. 5-1). Theoretically, numerous differentials caused by *A. tumefaciens*, which affects defense responses in plants, can be expected (Ditt *et al.*, 2005). However, these are filtered out of our dataset since all experiments were compared to controls such as the co-infiltration of bacteria containing an empty plasmid. Transcriptome alterations caused by both the silencing suppressors and *A. tumefaciens* are sometimes reflected when differentials are observed in comparison with the "No ATTA" experiment.

Alteration of the expression of genes previously reported to be influenced by virus infections

Further confirming the validity of the data obtained, a number of genes found here have been reported earlier to be affected during virus infection. An interesting group consists of the chaperone proteins $P58^{IPK}$ and HSP70. To ensure reproducibility, these differentials were confirmed in an independent repeat cDNA-AFLP analysis (data not shown). $P58^{IPK}$ was shown twice to be induced by NS_S and NS1. $P58^{IPK}$ has been shown to have a function in viral pathogenesis during *Tobacco mosaic virus* (TMV) infection (Bilgin *et al.*, 2003). It was reported to directly interact with the helicase proteins of TMV. Interestingly, $P58^{IPK}$ was proposed to be an endogenous suppressor of RNA silencing (R. Marathe, group of S.P. Dinesh-Kumar, poster presented at the Keystone Symposium 2004: siRNAs and miRNAs). It could be argued that the silencing suppression observed

with e.g. NS_S or NS1 is caused only by the fact that they enhance the expression of $P58^{IPK}$. This, however, does not seem to be the case since the overall changes in expression patterns due to NS1 and NS_S differ significantly. The effects of $P58^{IPK}$ itself on the plant transcriptome could possibly be attributed to the overlapping differentials of NS1 and NS_S. Future studies on $P58^{IPK}$ will help elucidate its role in the RNA silencing pathway. The other interesting chaperon protein found was annotated as "luminal binding protein 3 (BiP3)". This transcript contains an HSP70 domain signature and is induced by all strong local silencing suppressors, NS_S, HC-pro, NS3 and NS1. This expression pattern can be compared to the finding that increase of HSP70 expression is a general response to non-folded protein accumulation (Aparicio *et al.*, 2005), in this case caused by the over-accumulation of GFP protein. However the data presented here is also supported by the observation that HSP70 and polyubiquitin (also found to be up-regulated by HC-pro) were both found to be cooperatively induced by *Pea seed-borne mosaic virus* (Aranda *et al.*, 1996). It is interesting to note that some viruses, such as closteroviruses encode proteins homologous to HSP70 which have been shown to be involved in virion assembly, transport (Alzhanova *et al.*, 2001) and regulation of cell to cell movement (reviewed in (Boevink & Oparka, 2005)). This suggests that up-regulation of HSP70-like genes is beneficial for the virulence and movement of viruses. A further notable observation is that NS1 up-regulates both $P58^{IPK}$ and HSP70 in plants. It has been shown previously that influenza activates $P58^{IPK}$ and thereby modulates HSP70 activity in mammalian cells (Melville *et al.*, 1999). This could indicate that some parts of either the antiviral pathways or activities performed by viral silencing suppressors could be conserved between plants and mammals.

Discussion

To better understand direct and indirect effects of viral RNA silencing suppressor proteins on the gene expression pattern of a host plant, we undertook a comprehensive analysis of changes in the *N. benthamiana* transcriptome induced by these proteins. This was done using a novel set-up by combining *A. tumefaciens* mediated transient expression, a broadly used tool in the analysis of viral silencing suppressors (Johansen & Carrington, 2001; Voinnet *et al.*, 2000), and cDNA-AFLP analysis. cDNA-AFLP was the tool of choice in this set-up, since little genome sequence information is obtainable, and no microarray chip is available for *N. benthamiana*, a host plant susceptible to the majority of plant viruses. For the application of cDNA AFLP, no prior knowledge of the genome of interest is required. Furthermore, since cDNA-AFLP is a PCR based technology,

this makes it more sensitive then hybridization based techniques. cDNA-AFLP is an unbiased technique which does not require assumptions on which genes are expected to show differential expression. In this study approximately 25,000 cDNA-AFLP fragments were detected covering about 50% of the *N. benthamiana* genome. Of these differentials, 227 could be annotated based on database comparisons. Interestingly, a series of these differentially regulated genes were previously described to be affected during viral infections. Clustering of the data by silencing suppressors or gene expression pattern allows a comprehensive interpretation of the data. This work gives new insights into the activities of silencing suppressors. The most interesting observations are discussed below.

Silencing suppressors affect a broad range of genes

It has recently become clear that miRNAs play a major role in gene regulation in numerous organisms (Ambros, 2004; Baulcombe, 2004). Many genes were recently found to be regulated by miRNAs and using bioinformatics approaches new genes which are potentially regulated by miRNAs are being identified (Adai *et al.*, 2005; Rhoades *et al.*, 2002). While it was initially believed that mainly transcription factors are regulated by miRNAs, it appears that miRNAs regulate genes involved in all kinds of pathways (Adai *et al.*, 2005). Furthermore, several reports demonstrated that RNA silencing suppressor proteins do not only interfere with the antiviral activity of RNA silencing, they can also affect the accumulation and functions of endogenous small RNAs such as the miRNAs. For instance HC-Pro has been reported to interfere with miRNA activity in plants (Chapman *et al.*, 2004; Kasschau *et al.*, 2003; Mallory *et al.*, 2002). That being the case, it should be expected that the expression of numerous genes has to be affected by the silencing suppression. Indeed this is reflected in dataset produced here. The cDNA-AFLP analysis presented shows that HC-Pro affects a broad range of pathways such as transport, metabolism, chloroplast related genes, defense response, RNA processing, transcription factors and signalling. It can be envisaged that many changes observed in the expression patterns are the result of the indirect effect the suppressor proteins have on the expression of these proteins by influencing regulatory gene expression. If i.e. a transcription factor is affected by silencing suppression, this can affect all genes regulated by that factor. Interesting is the fact that a vast majority of genes is down-regulated and only a small proportion (about 20%) are up-regulated. Considering the possibility that most differentials could be caused by disturbed miRNA activity it seems that HC-Pro and NS_S enhance the activity of miRNAs in plants. This could be similar to observations made on the turnip yellow mosaic virus p69 silencing suppressor, which has been shown to suppress the siRNA pathway but to

promote the miRNA pathway in *A. thaliana* (Chen *et al.*, 2004). Furthermore, Chen and co-workers (Chen *et al.*, 2004) have reported that p69 might be mechanistically related to NS_S. It is possible that such a broad inhibition of host gene expression could be compared to host shut-off as it can be found in mammalian virus infections. Such a mechanism in plants has already been proposed before by (Aranda *et al.*, 1996). Finally, a certain bias in the data presented here cannot be excluded since in miRNA-guided cleavage the 3' end of the target mRNA decays slower then the 5' end (Llave *et al.*, 2002b; Souret *et al.*, 2004) and data presented here is based on polyadenylated RNA.

Comparison of HC-Pro and NS_S

Based on their effect on mRNA stability and disappearance of siRNAs, we initially proposed that HC-Pro and NS_S might act in a similar way on the RNA silencing pathway (Bucher *et al.*, 2003), since both enhance GFP expression in the *A. tumefaciens* infiltration assay and prevent the production of siRNAs. This study however, shows that this effect is not reflected in the mRNA profile of the host. A minority of only 20% of the differentially expressed genes are regulated in the same way by HC-Pro and NS_S. This can have several reasons: 1. Both proteins originate from different virus families, CABMV being a positive stranded RNA and TSWV a negative strand RNA virus. This might be important since the intermediary RNA and dsRNA products of these viruses are different during virus replication. For instance the genome of the positive strand RNA virus *Cymbidium ringspot virus* has secondary structures that are recognized by the RNA silencing machinery (Molnar *et al.*, 2005). The genome of TSWV however is completely packaged into nucleocapsid and is therefore less accessible for RNA silencing. This means that HC-Pro and NS_S have to play different roles in order to efficiently protect viral replication and transcription products. 2. The biochemical differences are displayed also by the fact that HC-Pro can suppress RNA silencing induced by dsRNA (Johansen & Carrington, 2001), while NS_S can not (Takeda *et al.*, 2002). That NS_S and HC-Pro showed the same effects on the siRNA levels in the *A. tumefaciens* infliltrations reported earlier (Bucher *et al.*, 2003) might indicate that transient sense silencing can be suppressed by both proteins but that the other remaining suppression activities do not overlap.

Silencing suppressor mediated modulation of host defense response?

A large proportion of the observed differentials (23) are of genes related to defense response and disease resistance. It has been reported earlier that HC-Pro causes perturbations in the salicylic acid (SA) related and unrelated defense pathways, thereby inducing resistance to certain pathogens (Pruss *et al.*, 2004). Possibly, HC-Pro (and NS_S) influences the balance between SA mediated

response and other defense pathways. Further supporting this hypothesis, another interesting differential found in this respect are two SNF1-related kinase complex anchoring protein differentials. We found two independent fragments derived from this gene, each being down-regulated by NS_S and by HC-Pro. SNF1 kinase, a global regulator of metabolism, has been implicated in silencing suppression by geminivirus proteins AL2 and L2. These proteins can inhibit SNF1 activity by direct interaction hence enhancing the susceptibility of the plant, while on the other hand over-expression of SNF1 enhances resistance (Hao *et al.*, 2003). Many of the observed differentials are related to metabolism (Tab. 5-1). This might also be the result of a disturbed regulation of the expression of SNF1 kinase. It will be interesting to investigate whether the balance between different defense pathways is directly mediated by RNA silencing. This especially since RNA silencing has been shown to be a defense pathway by itself (Voinnet, 2001).

Selected interesting genes

An interesting cDNA-AFLP differential that was observed was DCL1-like (annotated as CAF protein-like in Tab. 5-1) potentially directly linking the silencing suppressors to RNA silencing itself. DCL1-like is one of the very few genes being up-regulated in our experimental set-up. Although it was not confirmed whether this DCL-1 homologue is indeed the functional miRNA generating enzyme of *N. benthamiana*, this would confirm previous observations showing that HC-Pro and p69 silencing suppression activity leads to an increase of DCL1 mRNA level. This is due to the fact that several components of the RNA silencing machinery, such as AGO1 and DCL1 are autoregulated by miRNAs themselves (Vaucheret *et al.*, 2004; Xie *et al.*, 2003). Interfering with miRNA regulation indeed could result in an over-expression of such components. This remarkable observation may indicate how complex and intimately linked the regulation of the RNA silencing components and RNA silencing suppressors are.

An auxin responsive protein, member of the auxin response factor (ARF) gene family most closely related to IAA8 of *A. thaliana* was found to be strongly down-regulated by both NS_S and HC-Pro. This and the fact that we did not find any ARF homologue being up-regulated seems to contradict previous reports (Chapman *et al.*, 2004). However, while hitherto all studies on silencing suppressors and their effect on miRNA mediated cleavage were done in transgenic *A. thaliana* plants, the system used here relies on the transient expression of the silencing suppressors, which more closely resembles the timing of a viral infection. In the set-up used here the plant does not go through embryonic development while expressing a protein that interferes with developmental gene regulation, which could possibly results in long-term effects. Therefore this study represents a

comprehensive complementation to the previous studies based on a completely different approach.

Final conclusions

The work presented here demonstrates the power of cDNA-AFLP combined with a new approach for studying complicated processes in the transcriptome of a plant species of which limited genetic data is available. It provides new tools to study the effects of a single gene being transiently expressed in a plant. This was so far only achieved using transfection of protoplasts.

We show that silencing suppressors cause broad changes in the expression patterns of many gene families. These numerous effects add to the complexity of the virus-host interaction. It appears that silencing suppressors not only protect the transcription and replication-derived RNA products of the virus from the defense activities of the RNA silencing machinery. They also influence other defense responses of the plant, either directly by protein-protein interactions or indirectly by disturbing the miRNA production or activity.

A vast majority of genes appeared to be down-regulated by most silencing suppressors studied, an effect which could function as a host shut-off. Furthermore, the silencing suppressor proteins modulate the plant transcriptome by weakening plant defenses. The techniques presented here provide the possibility to create fingerprints of different silencing suppressors and will allow their grouping based on function. This will be very helpful since the amino acid sequences of these proteins show no homologies at all. This, however does not exclude functional overlaps. While clearly the data presented here raises more questions than it answers, it provides a good starting point for more in-depth studies.

Table 5-1 Annotated summary of *N. benthamiana* cDNA AFLP fragments differentially expressed due to the activity of silencing suppressors. The numbers indicate fold changes compared to the control experiment. Positive numbers represent an up-regulation and negative numbers a down-regulation. Up-regulations higher then 500 times indicate that the band was over-exposed.

Function	*Desciption*	2b lil	2b ALS	HC-Pro	NS_S	NS3	NS1	no inf.
	AAA-type ATPase family protein			-5	-10			
	aconitase			2	2		2	
	acyl-peptide hydrolase like				-48			
	apospory-associated protein C			-11	0	-2		
	ARV1-like family protein			-20				
	benzoquinone reductase			-20				
	C3HC4-type RING finger			-158	-10	-2826		-9
	calcium homeostasis regulator			-3		-2		
	calmodulin binding protein, probable			2		2		
	CGI-144-like protein			-17				
	coumarate-CoA ligase				-3			
	expressed protein, DUF149			-4	-4			
	expressed protein COG1196, Smc		-3	2	3	-665	-6	-9
	FAT domain-containing protein			2	-19	-3		
	glutaredoxin-like			-23		-2		-5
	H1 histone gene flanking region		2	-19	-42	-2		2
	H ATPase inhibitor				-3			
	kinesin motor family protein			-38	-35	-2		
	large subunit rRNA gene			2	-2			
	leaf senescence related protein-like				-8	-4		
	meprin and TRAF domain-containing protein			3	4	3	2	-10
	NC domain-containing protein			-63	-2			
	nectarin 5	-2		-2		-2		
	pentatricopeptide (PPR) repeat			-47				
	pentatricopeptide repeat-containing protein			-21				
	peptidyl-tRNA hydrolase			-26		0	-2	
	pollen-specific protein homolog			-171		-4		
	Potyvirus VPg interacting protein				-2			
	Predicted membrane protein COG4243	-20			-21			
	ribosomal RNA intergenic spacer region			5	-20			
	rRNA			5	-20			
	sulfotransferase				-12			
	transcribed rRNA spacer			2	-3		2	
	VQ motif-containing protein		-2		-24			
	zinc finger (C3HC4-type RING finger)				-30	-3		-77

Function	***Desciption***	**2b lil**	**2b ALS**	**HC-Pro**	**NS_S**	**NS3**	**NS1**	**no inf.**
	family protein							
	zinc finger (FYVE type) family protein			-52				
	zinc finger protein-related, RING finger	-2		-10		-2	-2	
cell division	cell division cycle family protein			4	2			
	Chromosome segregation ATPases, smc, COG1196	2		-6		-6	2	-2
chloroplast-related	ATP-dependent protease			-12	-3			-58
	ATP-dependent Clp protease regulatory subunit CLPX	2	2	-5				
	chlorophyll a/b binding protein	-2		-2		-2		
	chlorophyll a/b binding protein			-2				
	chlorophyll a/b binding protein			-4	-2	-3		
	chlorophyll a/b-binding protein	-2		-2		-2		
	chlorophyll a/b-binding protein			-7	-2			
	chloroplast genome DNA			-17	-9	-12		10
	chloroplast outer membrane translocon subunit, putative				-4			
	expressed protein				-21	-42		
	expressed protein DUF561			-9	-3	-2	-6	
	gamma-glutamylcysteine synthetase				-6			
	oxygen evolving enhancer protein, putative			-4	-2	-3		
	Rieske Fe/S protein of cytochrome b6/f complex			-3				
	signal peptidase I familiy protein			-3				
	similar to cell death suppressor protein LLS1			-3		-2		
	transketolase 2	-12	-20	-14	-24			
	ZIP			-2				
control	GFP			12	22	18	3	
	GFP			19	8	5	18	
	HC-Pro		3	34				
	HC-Pro		3	34				
	NS1						1000	
	NS_S				1000			
	NS_S				1000			
defense response	alcohol NADP+ oxidoreductase			-3				
	Avr9/Cf-9 rapidly elicited protein 36			-5		2		
	Avr9/Cf-9 rapidly elicited protein 36			2	2	3		
	Avr9/Cf-9 rapidly elicited protein 216				-13			
	CGI-144-like protein	4	4	-3	-47			3
	cyclophilin		-9		-83	-35	-11	
	cyclophilin-like			-20	-20			

Function	*Desciption*	2b lil	2b ALS	HC-Pro	NS_S	NS3	NS1	no inf.
	expressed protein pfam00597			-6				
	leucine-rich repeat family protein			2	-2			
	peroxidase			-24	-5		3	-3
	prohibitin			-12				
	prohibitin				-3			
	resistance protein SlVe1 precursor			-2	-14			-4
	copine III-like				4	2		
	lipoxygenase 3				-33	-12		
	peroxidase			-8		-13		3
	remorin family protein			-4	-8			
	serine/threonine protein phosphatase			-14				
development	auxin-responsive protein			-20	-19			
	auxin-responsive family protein	-2		-8				
	auxin-regulated protein-like			-3				
	CLAVATA1 receptor kinase like			-69				
	no apical meristem	-3		-4		-3		
	seven in absentia (SINA1)			-24	-2			
	similar to nodule inception protein			0	-6	-2	-5	-3
development / signaling	farnesyltransferase beta subunit			-5				
disease resistance	disease resistance homolog			-45	-45	-2	-3	-6
	disease resistance protein (TIR-NBS-LRR class)				-36	2		
	disease resistance protein (LRR)				-3			-12
	thioredoxin				3			
	thioredoxin protein			-2				4
DNA repair	type II CPD photolyase PHR1				5	-3	3	2
metabolism	ACT domain-containing protein	2			2	2		3
	aminotransferase			-3	-4			-90
	AMP-activated protein kinase			-7	-22			-17
	beta-galactosidase			-45				
	betaine-aldehyde dehydrogenase			-3	-2	-2		
	calcium/calmodulin-dependent protein kinase (CCaMK)	7	0	23	15	12		
	cis-prenyltransferase			-20				
	gamma-glutamyltransferase homolog			-9	-11	-18		
	glucose-6-phosphate isomerase (GPI)			-33		-2		
	glucosyltransferase	3	2	-102				
	glycine hydroxymethyltransferase				-33	-2		-2
	lactoylglutathione lyase, putative			5				
	nitrate reductase			-32	-6	-3	0	
	PAPS reductase like	-18	-11	-7		-10	-17	
	phosphoribosyl pyrophosphate synthase			-2	-6			2

Function	***Desciption***	**2b lil**	**2b ALS**	**HC-Pro**	**NS_S**	**NS3**	**NS1**	**no inf.**
	tRNA pseudouridine synthase				-4			
	UDP-sulfoquinovose synthase			-24	-5	-6		
	galactosyltransferase			4	-11			-2
	ARP protein			-28				
	Isoleucyl-tRNA synthetase				-3			
	nucleotide diphosphate kinase			-20				
mitochondria	mitochondrial DNA				-92		-2	
protein kinase	MAP3K protein kinase-like			-10				
protein-protein interaction	BTB/POZ domain protein			-60				
	expressed protein, ARM repeats				-7			
	F-box family protein				-27			
	F-box family protein	-10		-25	2			
regulatory protein	CBS domain-containing protein				-5			
RNA/DNA binding	zinc knuckle (CCHC-type) zinc finger			3	-5			
RNA processing	ATP-dependent RNA helicase	-9		-36				
	ATP-dependent RNA helicase, putative			-25				
	CAF protein-like	-51	1	5	12	2	3	1
	DNA-directed RNA polymerase I 190K chain-like protein			-4				
	heterogeneous nuclear ribonucleoprotein, putative			-41		-2		
	KH domain-containing protein			-20				
	La domain-containing protein	-5	-3		-17	-2		
	nuclear RNA-binding protein, putative			-5	-3	-3	-3	
	Ribonuclease 1				-13	-3		
	RNA-binding protein G5BF, putative			7	4			
	RNA binding protein-like		-2		-5076		-2	
	tRNA isopentenyltransferase 9			-38				
signalling	CBS domain-containing protein			-102				
	leucine-rich repeat family protein			4		3		-2
	leucine-rich repeat transmembrane protein kinase, putative	3		-138		-2	-2	
	LSTK-1-like kinase	-2	-2	-20			-2	-6
	MAP kinase				-8			
	mitogen-activated protein kinase			-9		-2		
	phospholipase C2, phosphodiesterase		-2	3		3		2
	protein kinase family protein			-20		-2	-3	
	protein phosphatase 2C, putative			1	-5			
	putative phototropic-responsive protein	-12		-19	-14			
	Ser/Thr protein kinase			-100				
	Serin/Threonin kinase domain			-5				

Function	*Desciption*	2b lil	2b ALS	HC-Pro	NS_S	NS3	NS1	no inf.
	TIP41-like protein			-3				
	receptor lectin kinase family protein			-25	-6			
stress response	ACC synthase			-4	-4			
	AKIN beta3			-14				
	annexin			-4	-10	-10		
	C2 domain-containing protein	2		-4				
	luminal binding protein (BiP), HSP-70-like		-2	2	393			
	luminal binding protein 3 (BiP3), HSP-70-like			5	3	3	5	
	$P58^{IPK}$				4		5	
	polyubiquitin			-14	-2			3
	polyubiquitin	-2		0	22			
	salt induced protein			1	-9			
	SNF1-related kinase complex anchoring protein			-67				3
	SNF1-related kinase complex anchoring protein SIP1 homologue				-49		-2	
	wound inducive gene			-21				
structural protein	Cell wall protein-like			-6	-14			
	dynein light chain			6	5		-5	5
structure	actin-depolymerizing factor		8		5	14	39	7
transcription factor	AG-motif binding protein	-2		-20	-2	-3		
	ascorbate oxidase promoter-binding protein				-18	-18		2
	basic helix-loop-helix (bHLH) family protein			-47		2		
	DNA-binding protein 4 (WRKY4)			6				
	Dof-type zinc finger domain-containing protein			-3				
	Dof-type zinc finger domain-containing protein		-2	-6	-12			
	E4/E8 binding protein-1			-24			-2	
	heat shock factor			-38				-5
	homeobox-leucine zipper protein			-53		2		
	Myb DNA binding protein			-4				-17
	Myb-like DNA-binding domain, putative			-116	-40	-2		-3
	plus-3 domain-containing protein			-6				
	scarecrow-like transcription factor 1 (SCL1)			-2				
	WRKY transcription factor			-27			-3	-47
transport	ABC1 family protein			-2	-5	-2		
	ABC transporter			-26	-2			

Function	***Desciption***	**2b lil**	**2b ALS**	**HC-Pro**	**NS_S**	**NS3**	**NS1**	**no inf.**
	ABC transporter				-59			
	ABC transporter family protein			4		2		
	ATP synthase protein I -related				-4			
	cyclic nucleotide-gated channel			2	-56			
	expressed protein DUF258			-17				
	integral membrane protein, putative			-3	-30	-5	-2	
	iron transport protein			-5				-2
	microtubule associated protein			-12		-2		
	mitochondrial substrate carrier family protein			-1	-8		-2	-8
	Na+/H+ antiporter			-10				
	nitrate transporter			-37			-2	-8
	nodulin-like protein			-20	-20	-20		
	no exine formation-1			-10		-48		
	non-cell-autonomous protein pathway1				-3			
	potassium antiporter	2		5	4			
	potassium channel			-8	-4	-2		-2
	purine permease			-49				-104
	purine permease family protein			-1	-38	-25		
	Ras-related GTP-binding protein			-6				
	SEC14 cytosolic factor family protein			-19	-20	-8		
	transmembrane amino acid transporter protein			-1	-53	-4		-2
	vacuolar protein sorting-associated protein			-10				
	exocyst complex component			-1	-23	-2		
transposon	putative polyprotein			-8				3
	putative polyprotein			2		3		
	putative retroelement pol polyprotein				-500			
	transposable element, activator-like				-7			-9

Materials and Methods

A. tumefaciens *infiltration setup*

Young *N. benthamiana* plants, at the 4-6 leaves stage, were infiltrated with *A. tumefaciens* cultures. This allowed an easier sampling since the whole leave could be used for further analysis instead of just using small patches. To reduce the number of unspecific differentials, several measures were taken: All plants were grown at the same site under equal light conditions. To diminish time dependent changes in expression patterns, the sampling took approx 30min, and to avoid plant specific effects four plants per infiltrated construct were sampled. Finally the four samples per construct were pooled and RNA was extracted.

RNA extraction and cDNA-AFLP analysis

The cDNA-AFLP analysis was performed basically as described in (Vos *et al.*, 1995) and (Volkmuth *et al.*, 2003). Polyadenylated RNA was extracted using biotin labeled oligo-dT primers and magnetic Dynal Streptavidin beads. The RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and turned into dsDNA with E. coli DNA ligase, E. coli DNA polymerase I and RNase-H. The dsDNA was then purified using the Qiagen Qiaquick PCR purification kit and digested with the enzymes *Taq*I and *Mse*I. Taq and Mse adapters were ligated to the digestion products and a 10 times dilution of the obtained product used for the first pre-amplification. The pre-amplified products were diluted 600 times and amplified in a radioactive reaction with selective primer-combinations. The radiolabeled fragments were separated on a 4.5% denaturing gel and scanned. Band intensities and differentials were calculated using the Keygene N.V. software packages MegaXtractor and Xdiff. MegaXtractor measures individual band intensities and corrects for differences in the total lane intensity. Xdiff was used for normalisation and the calculation of the differentials.

Annotation and Cluster Analysis

Cluster analysis was performed as described by (Eisen *et al.*, 1998) using the cluster 3.0 software from (de Hoon *et al.*, 2004) and visualised on Java TreeView from (Saldanha, 2004).

CHAPTER 6

General Discussion

Introduction

During the past 10 to 15 years RNA silencing has gained much attention. Having first been observed in transgenic petunia plants in 1990, impressive advances have been made in understanding the underlying mechanisms until today. A considerable number of enzymes and co-factors involved in the RNA silencing pathway have now been identified, manly due to the progress that has been achieved using genetic screens in *Caenorhabditis elegans*. A part of our understanding of the role(s) of RNA silencing in living organisms comes from the study of viral proteins that have the ability to suppress RNA silencing in many different ways. In this thesis it is shown that also negative strand RNA viruses (both plant- and mammal-infecting) encode such proteins. A broad study to better understand the general response of plants to silencing suppressors was performed.

In all, this thesis touches several aspects of the interplay between RNA silencing as a tool to interfere with virus infection and the virus encoded suppressor proteins that antagonize this process.

Virus resistance

In Chapter 2 it is shown that RNA silencing is an excellent tool to obtain multiple virus resistance at – moreover – very high frequency. Exploiting this natural defense mechanism in transgenic plants is demonstrated to result in complete immunity against four related viruses. This finding now raises the question whether the ultimate solution for plant virus problems has been found. Could RNA silencing-mediated virus resistance be a kind of plant vaccination? Or will plant viruses be able to adapt to these transgenic plants and overcome the resistance with time, as (most) human viruses overcome vaccinations? The double-stranded RNA (dsRNA)-based approach, shown here to work for four viruses simultaneously, can easily be extended to include even more viruses, depending on the regional abundance of viruses to be controlled. While this approach appears to be broadly applicable, it has to be noted that the selected gene used in the hairpin construct can be of importance. Considering that the viral genome of negative strand RNA viruses itself may not be prone to RNA silencing (Prins *et al.*, 1997) it would generally be a good choice to select the nucleocapsid gene to be targeted by RNA silencing. Limiting the supply of this protein will reduce the replicational activity of the RNA dependent RNA polymerase (RdRP) of negative strand RNA viruses since it is dependent on the (free) nucleocapsid protein concentration (Meyer *et al.*, 2002). Furthermore the reduction of N protein levels could result in a more accessible form of the viral

genomic RNA to the silencing machinery. Possibly, the RdRP itself is a poor target since it is only required in catalytic quantities. Transient RNA silencing approaches can't achieve 100% shut off (i.e. (Elbashir *et al.*, 2001)), meaning that the remaining leaky expression could still be enough to drive virus transcription and replication. Otherwise the viral RdRP would be an ideal target since it is the most conserved gene of negative strand RNA viruses (van Poelwijk *et al.*, 1997) allowing broader resistance against multiple viruses. Since our approach to produce virus resistant plants was so successful the question arises whether this strategy can also be applied in mammalian systems. An interesting approach would be to produce transgenic cells stably expressing long viral dsRNA constructs and test whether they also provoke virus resistance. It has been demonstrated that long dsRNAs efficiently silence genes in embryonic and some somatic cells (Billy *et al.*, 2001; Paddison *et al.*, 2002). A remaining problem in the application of dsRNAs in mammals is the induction of the interferon pathway by long dsRNAs, possibly interfering with the strategy. An alternative is the direct delivery of siRNAs into living animals. However this technique still represents a major challenge since in mice it involves either high pressure injection of immense amounts of siRNAs into the tail, or the direct injection into the liver (McCaffrey *et al.*, 2002). Also there have not been any reports about virus induced gene silencing in mammalian systems raising the question whether RNA silencing actually has an antiviral function. This has been shown so far only for invertebrates, i.e. *Drosophila melanogaster* (Li *et al.*, 2002) and *Caenorhabditis elegans* (Wilkins *et al.*, 2005), but not for vertebrates which have evolved a protein-based immune system which at first sight seems to be completely different to the one employed by plants and invertebrates. Since Chapter 4 and 5 of this thesis provide evidence that there might be more similarities between plants and vertebrates then initially thought this point will be discussed later. The results obtained in Chapter 2 could have interesting applications in mammalian systems. Since siRNAs are effective in rendering cells immune to HIV (Lee *et al.*, 2002) and other viruses it would be of great interest to test whether long dsRNA combining different viruses could provide broad resistance in mammals, too. Furthermore the simultaneous targeting of several sequences of an incoming virus can be useful to prevent adaptation of the virus for instance to a single siRNA as has been described for HIV (Das *et al.*, 2004). An alternative way to render cells resistant against viruses is to silence viral receptors and other host components required by the virus (Novina *et al.*, 2002). Also in this case the technology developed in Chapter 2 would allow the knocking-down of several host components at once, increasing the chances of obtaining immunity. Such a set-up is more challenging since endogenous genes of the host have to be knocked-down in a controlled temporary manner, so as not to disturb the normal functioning of these genes. These problems could be circumvented by the use

of virus- or drug- inducible promoters.

Silencing suppressors of negative stranded RNA viruses

The observation that viruses have evolved proteins capable of suppressing the RNA silencing defense mechanisms has helped to better understand certain aspects of the plant-virus interaction. They are a final proof that in plants indeed RNA silencing acts as an antiviral defense mechanism, and since they have also been found in mammalian viruses are highly indicative that this is also the case in mammals. Also silencing suppressor proteins significantly contribute to the numerous side effects in host plants during infection. Suppressors of RNA silencing play a central role in defining the virulence of a certain virus and thereby greatly affect how a virus behaves. For instance, silencing suppressors influence the symptom severity (Brigneti *et al.*, 1998) and they can influence the speed of systemic virus spread (Kasschau & Carrington, 2001). An interesting example of how silencing suppressors influence virulence are mixed virus infections. During mixed infections two viruses can exhibit synergistic effects, meaning that one virus (for instance *Potato virus Y*, PVY) has the capability of supporting the replication of another virus (for instance *Potato virus X*, PVX) (Pruss *et al.*, 1997). HC-Pro of potyvirus PVY was first identified to be the protein which enhances PVX replication, while later this protein was shown to suppress RNA silencing (Anandalakshmi *et al.*, 1998) and that the PVX/PVY synergism is linked to this activity.

Recent findings indicate that the NS_S and NS3 silencing suppressors are also active in insect cells (H. Hemmes, unpublished data). It is now known that viruses infecting insect cells are also targeted by the silencing machinery and that they need a functional suppressor of RNA silencing for replication (Li *et al.*, 2002). If the silencing suppression activity is performed by the same protein in plants and in insects, such as it is most probably the case for NS_S and NS3, it is tempting to suggest that the suppressing activity of these proteins takes place at a step in the pathway which is conserved between plants and insects.

As mentioned earlier, silencing suppressors can have strong effects on the symptoms caused by the virus. This is due to the fact that these proteins interfere with the natural development of plants. Most of these effects can be linked to disturbances of the miRNA levels, as it has been demonstrated for HC-Pro for instance (Kasschau *et al.*, 2003; Mallory *et al.*, 2002). It can be questioned whether these effects actually are beneficial for the virus or not. The human *Epstein-Barr virus* actually codes for miRNAs itself and has found a way to benefit of such a disturbance

(Pfeffer *et al.*, 2004). It is also possible that a broad disturbance in miRNA functions leads to a general host shut-down, as suggested by the results obtained in Chapter 5. This would then negatively influence virus replication. This assumption however requires that a specific set of antiviral response genes are directly or indirectly regulated by miRNAs so that the plant can react on unwanted silencing suppression. Taken together it is very likely that for a virus it is not helpful to just cause a complete shut-down of the RNA silencing machinery which would otherwise limit the production of host factors that are required for virus infection. Therefore, silencing suppressors are important modulators of the interplay between the virus and the host. Their fine-tuning shifts the scales in favor of the virus or the host in the complex virus-host interaction.

Silencing suppression by a mammalian infecting virus

The work on influenzaviral NS1 presented in Chapter 4 provides the very first evidence that RNA silencing might also act as an antiviral defense mechanism in mammals. It is shown that NS1 can suppress RNA silencing in a plant-based assay and that it possibly does so by binding small interfering RNAs (siRNAs). An important remaining question at the end of this thesis is whether this protein indeed is a genuine suppressor of RNA silencing in its homologous (mammalian) system and whether this function is required for efficient virus replication. An independent report confirmed the data presented here that NS1 suppresses RNA silencing in plants (Delgadillo *et al.*, 2004).

Furthermore, NS1 has also been reported to suppress RNA silencing in mosquito cells (Li *et al.*, 2004). While this finding indicates activity in an animal (albeit insect) system, this still does not prove its biological significance in mammals. During this thesis research, multiple attempts to demonstrate NS1 silencing suppressor activity in mammalian cells were performed (data not shown in any of the previous chapters). For instance, human (HeLa) and hamster (BHK) cells were transfected with expression vectors containing NS1 and silencing was induced either directly by siRNAs or through a short hairpin RNA transcribed from a co-transfected plasmid. These silencing-inducing molecules were designed to target a reporter gene either stably expressed from a transgene or transiently from a co-transfected vector. Whichever set-up was used the outcome was always that NS1 could not prevent RNA silencing induced by siRNAs (Fig. 6-1). This is surprising considering that Chapter 4 of this thesis indicates that NS1 can bind siRNAs and should therefore be able to interfere with their activity. A critical aspect though, which has not yet been addressed, is whether

NS1 binds siRNAs preferentially over longer dsRNA molecules and whether NS1 does so *in vivo*. Another critical factor in these experiments is the amount of NS1 protein actually produced upon transfection. It is known that NS1 binds to dsRNA as a dimer (Wang *et al.*, 1999), and therefore it may be assumed that the same holds for short dsRNAs such as siRNA molecules. That means that to efficiently suppress RNA silencing at least two NS1 proteins are required to bind to one siRNA. To address this stoichiometric assumption during this Ph. D. research extensive work was done without any clear (and therefore unpublishable) result. For instance, in the BHK transfection experiments the amount of transfected siRNAs was reduced to the absolute detectable minimum, but still no suppression of RNA silencing could be observed. Another issue that was not tested is the induction of RNA silencing in mammalian cells by long double stranded RNAs (dsRNAs) transcribed from the nucleus. If NS1 preferentially binds longer dsRNA molecules it might show a better activity in that case, especially since it has been reported that NS1 localises in the nucleus. Another finding which, at this moment, still questions the biological relevance of mammalian virus-encoded silencing suppressors is the so far complete lack of evidence for virus derived siRNAs during virus infection of mammalian cells. Such molecules accumulate to high amounts in infected plants (i.e. (Hamilton *et al.*, 2002; Molnar *et al.*, 2005)).

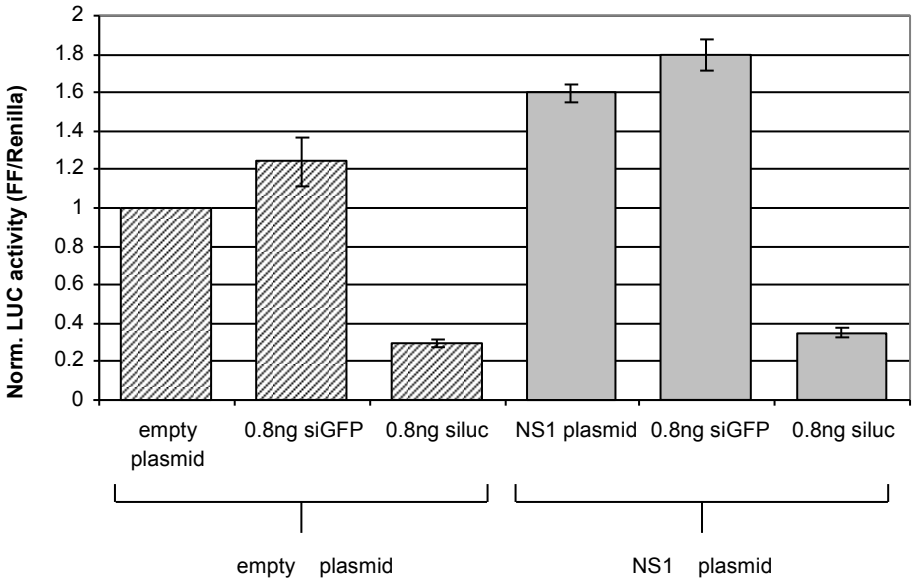

Figure 6-1. Silencing suppression activity of Influenza NS1 in mammalian cells. These experiments are based on the dual-luciferase assay, all cells were transformed with firefly and renilla luciferase genes. The firefly luciferase gene was silenced using specific siRNAs (siluc). GFP specific siRNAs (siGFP) were used as a negative control and the renilla luciferase was used for normalisation. Addition of NS1 expression plasmid (grey bars) did not influence the silencing efficiency compared to the controls (hatched bars).

On the other hand, several recent reports are supportive for the idea that mammalian infecting viruses also encode suppressors of RNA silencing. The NS_S of the *La Crosse virus*, which shares 30% homology to the NS_S of TSWV, has recently been shown to suppress RNA silencing in mammalian cells (Soldan *et al.*, 2005). While carrying out the experiments of Chapter 3, in which the silencing suppressor function of TSWV NS_S was investigated, it was also tested whether NS_S of *Rift valley fever virus* (RVFV) could suppress RNA silencing in plants. The outcome was negative, but expression of this protein could not be verified due to the lack of specific antibodies (data not shown). Furthermore, a recent paper shows that the Tat protein, the viral gene transcription activator of *human immunodeficiency virus I* (Strebel, 2003), is also capable of suppressing RNA silencing (Bennasser *et al.*, 2005).

Taken together the following role of RNA silencing during virus infection of a mammalian host can be postulated. Upon entry of a virus the first line of defense is the protein-based antiviral system. The virus will be targeted by the immune system, antibodies recognizing the viral antigens, will label the virus and macrophages will eliminate them by phagocytosis. However, upon infection with a new virus, the host is still naive with respect to its immune system and therefore does not possess antibodies against this specific virus. The virus can therefore evade this line of defense and infect a cell. The next line of defense will then be the intracellular RNA silencing machinery. At least at the primary infection site RNA silencing is the only available counter measure, in this model. This hypothesis is supported by the finding that small dsRNAs, such as virus-derived siRNAs, can cause abnormal expression of the major histocompatibility complex turning normal cells into antigen presenting cells (Suzuki *et al.*, 1999). The cellular defense response is then supported by the activation of the interferon pathway which is induced by double stranded RNAs that can be product of either the silencing machinery or active virus replication.

Is there a plant PKR like pathway?

Interestingly, all silencing suppressor proteins of mammalian viruses found so far have previously been implicated as interferon antagonists at the PKR step (NS1 (Bucher *et al.*, 2004), E3L (Li *et al.*, 2004), Tat (Bennasser *et al.*, 2005)). A number of viral proteins known to inhibit the interferon pathway at the PKR step are listed in table 6-1. An imminent question that can now be raised is: Does a link between RNA silencing and interferon response exist? An intriguing fact is that for both antiviral defense pathways, the central molecule is dsRNA. In this respect, the work presented in

Chapter 5 found an interesting gene being up-regulated by the silencing suppression activity of NS_S and NS1. The chaperon protein $P58^{IPK}$ has a function in viral pathogenesis during *Tobacco mosaic virus* (TMV) infection (Bilgin *et al.*, 2003). Furthermore it has been suggested to act as an endogenous suppressor of RNA silencing (R. Marathe, group of S.P. Dinesh-Kumar, poster presented at the Keystone Symposium 2004: siRNAs and miRNAs). So what is the role of $P58^{IPK}$ induction *in planta*? Does $P58^{IPK}$ inhibit a plant PKR homologue like it does in mammals and what would be the role of such a plant or mammalian PKR in RNA silencing? A simple blast at the National Center for Biotechnology Information (NCBI) of the human PKR protein sequence against the *A. thaliana* genome reveals that there are many proteins sharing homologies with the human PKR (best hit: AT3G59410.1, E=7e-29). If indeed a plant PKR would exist then this would hint to the exciting possibility that the PKR is the last antiviral defense pathway step evolutionary conserved among mammals and plants. In mammals the dsRNA induced PKR leads to the activation of the interferon response which is absent in plants. Such a potential plant PKR should therefore activate other antiviral defense mechanisms. The PKR would then be a sort of cytoplasmic control agent surveying the dsRNA levels and inducing defense mechanisms should a certain threshold level of dsRNA be exceeded.

Virus	*Mechanism of action/inhibition*
Adenovirus	Produces VA RNA that binds to but fails to activate PKR
Baculovirus	PK2 binds eIF2α kinases, including PKR, and blocks their activities
Epstein–Barr virus	Produces EBER RNA that binds to but fails to activate PKR
Hepatitis C virus	NS5A binds to and inhibits PKR ; E2 also interacts with PKR and may inhibit its activity
Herpes simplex virus	ICP 34.5 redirects protein phosphatase 1 to dephosphorylate (re-activate) elF2α ; US11 blocks PKR activity
Human immunodeficiency virus	Down-regulates PKR by unknown mechanism ; Tat and short Tat-responsive region RNA inhibit PKR
Influenza virus	NS1 binds dsRNA and PKR to inhibit its activity. Influenza virus also induces cellular inhibitor of PKR (p58IPK)
Poliovirus	Induces the degradation of PKR
Poxviruses (many)	Example : vaccinia virus E3L binds dsRNA and PKR; K3L binds PKR; σ3 binds dsRNA and thus inhibits PKR (and 2h–5h oligoadenylate synthetase)
Rotavirus	NSP3 binds dsRNA and thus inhibits PKR (and 2h–5h oligoadenylate synthetase)

Table 6-1. IFN-induced antiviral enzymes at the PKR step and their viral countermeasures. Taken from (Goodbourn *et al.*, 2000).

Outlook

During the research presented in this thesis the suppressors of RNA silencing of 2 plant-infecting negative strand RNA viruses have been disclosed. As one of these viruses (TSWV) is closely related to animal-infecting viruses the question came into mind whether animal-infecting viruses would also encode such suppressor proteins, which in turn would indicate that the meantime demonstrated existence of the RNAi mechanism in animals would have an antiviral function. This possibility was further explored by analysing the NS1 "virulence protein" of *Influenza A virus*, for its potential to block RNA silencing. Using the same, plant-based assay, this turned out to be the case (Chapter 4). Chapter 5 then analysed the effects of viral silencing suppressor proteins on the plant hosts in more detail. However, Chapter 5 also raises more questions than answers due to the complexity of the plant response to the silencing suppressors. An obvious follow-up would therefore be the biochemical characterisation of the silencing suppressor proteins with respect to their interaction with host proteins and RNA molecules such as already done for HC-Pro (Anandalakshmi *et al.*, 2000) and P19 (Silhavy *et al.*, 2002). Furthermore it will be interesting to investigate the role of NS_S in the tospoviral insect vector (thrips) to establish whether its silencing

suppression activity plays an essential role during the insect life cycle of the virus, thereby again demonstrating an antiviral function of RNAi in insects.

Last but not least, Chapter 2 describes a relatively complete story on how to efficiently obtain broad transgenic resistance to viruses in plants and it can be hoped that the achievements presented there will lead to useful industrial applications.

References

Adai A., Johnson C., Mlotshwa S., Archer-Evans S., Manocha V. et al. (2005). Computational prediction of miRNAs in Arabidopsis thaliana. *Genome Res* 15: 78-91.

Ahlquist P. (2002). RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science* 296: 1270-3.

Al-Kaff N. S., Covey S. N., Kreike M. M., Page A. M., Pinder R. et al. (1998). Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* 279: 2113-5.

Alzhanova D. V., Napuli A. J., Creamer R., Dolja V. V. (2001). Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. *Embo J* 20: 6997-7007.

Ambros V. (2004). The functions of animal microRNAs. *Nature* 431: 350-5.

Anandalakshmi R., Marathe R., Ge X., Herr, J M, Jr, Mau C. et al. (2000). A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* 290: 142-4.

Anandalakshmi R., Pruss G. J., Ge X., Marathe R., Mallory A. C. et al. (1998). A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci U S A* 95: 13079-84.

Andino R. (2003). RNAi puts a lid on virus replication. *Nat Biotechnol* 21: 629-30.

Aparicio F., Thomas C. L., Lederer C., Niu Y., Wang D. et al. (2005). Virus induction of heat shock protein 70 reflects a general response to protein accumulation in the plant cytosol. *Plant Physiol* 138: 529-36.

Aragon T., de la Luna S., Novoa I., Carrasco L., Ortin J. et al. (2000). Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. *Mol Cell Biol* 20: 6259-68.

Aranda M. A., Escaler M., Wang D., Maule A. J. (1996). Induction of HSP70 and polyubiquitin expression associated with plant virus replication. *Proc Natl Acad Sci U S A* 93: 15289-93.

Aukerman M. J., Sakai H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* 15: 2730-41.

Bartel D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-97.

Baulcombe D. (1999). Viruses and gene silencing in plants. *Arch Virol Suppl* 15: 189-201.

Baulcombe D. (2004). RNA silencing in plants. *Nature* 431: 356-63.

Baulcombe D. C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol* 32: 79-88.

Beclin C., Berthome R., Palauqui J. C., Tepfer M., Vaucheret H. (1998). Infection of tobacco or Arabidopsis plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. *Virology* 252: 313-7.

Beclin C., Boutet S., Waterhouse P., Vaucheret H. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Curr Biol* 12: 684-8.

Bender J. (2004). Chromatin-based silencing mechanisms. *Curr Opin Plant Biol* 7: 521-6.

Bennasser Y., Le S. Y., Benkirane M., Jeang K. T. (2005). Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity* 22: 607-19.

Bergmann M., Garcia-Sastre A., Carnero E., Pehamberger H., Wolff K. et al. (2000). Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* 74: 6203-6.

Bernstein E., Caudy A. A., Hammond S. M., Hannon G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409: 363-6.

Bilgin D. D., Liu Y., Schiff M., Dinesh-Kumar S. P. (2003). P58(IPK), a plant ortholog of double-stranded RNA-dependent protein kinase PKR inhibitor, functions in viral pathogenesis. *Dev Cell* 4: 651-61.

Billy E., Brondani V., Zhang H., Muller U., Filipowicz W. (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci U S A* 98: 14428-33.

Boevink P., Oparka K. J. (2005). Virus-host interactions during movement processes. *Plant Physiol* 138: 1815-21.

Bohmert K., Camus I., Bellini C., Bouchez D., Caboche M. et al. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. *Embo J* 17: 170-80.

Boutet S., Vazquez F., Liu J., Beclin C., Fagard M. et al. (2003). Arabidopsis HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr Biol* 13: 843-8.

Bridge A., Pebernard S., Ducraux A., Nicoulaz A., Iggo R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 34: 263-4.

Bridgen A., Weber F., Fazakerley J. K., Elliott R. M. (2001). Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc Natl Acad Sci U S A* 98: 664-9.

Brigneti G., Voinnet O., Li W. X., Ji L. H., Ding S. W. et al. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. *Embo J* 17: 6739-46.

Brummelkamp T. R., Bernards R., Agami R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296: 550-3.

Bucher E., Hemmes H., De Haan P., Goldbach R., Prins M. (2004). The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* 85: 983-91.

Bucher E., Sijen T., De Haan P., Goldbach R., Prins M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J Virol* 77: 1329-36.

Carrington J. C., Ambros V. (2003). Role of microRNAs in plant and animal development. *Science* 301: 336-8.

Chapman E. J., Prokhnevsky A. I., Gopinath K., Dolja V. V., Carrington J. C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* 18: 1179-86.

Chen J., Li W. X., Xie D., Peng J. R., Ding S. W. (2004). Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microrna in host gene expression. *Plant Cell* 16: 1302-13.

Chen Y. K., Derks A. F., Langeveld S., Goldbach R., Prins M. (2001). High sequence conservation among cucumber mosaic virus isolates from lily. *Arch Virol* 146: 1631-6.

Chen Z., Li Y., Krug R. M. (1999). Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. *Embo J* 18: 2273-83.

Chen, Y K, Lohuis, D, Goldbach, R, Prins, M (2004). High frequency induction of RNA-mediated resistance against Cucumber mosaic virus using inverted repeat constructs. *Molecular Breeding* 14: 215 - 226.

Cogoni C., Macino G. (1997). Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. *Proc Natl Acad Sci U S A* 94: 10233-8.

Cogoni C., Macino G. (2000). Post-transcriptional gene silencing across kingdoms. *Curr Opin Genet Dev* 10: 638-43.

Crete P., Vaucheret H. (1999). Expression and sequence requirements for nitrite reductase co-suppression. *Plant Mol Biol* 41: 105-14.

Dalmay T., Hamilton A., Rudd S., Angell S., Baulcombe D. C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101: 543-53.

Das A. T., Brummelkamp T. R., Westerhout E. M., Vink M., Madiredjo M. et al. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* 78: 2601-5.

Dawe R. K. (2003). RNA interference, transposons, and the centromere. *Plant Cell* 15: 297-301.

De Avila A. C., de Haan P., Smeets M. L. L., Resende R. D. O., Kormelink R. et al. (1992). Distinct levels of relationships between tospovirus isolates. *Arch. Virol* : 211-227.

de Haan P., Gielen J., Prins M., Wijkamp I., Van Schepen A. et al. (1992). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic plants. *Bio/Technology* 10: 1133-1137.

de Haan P., Kormelink R., de Oliveira Resende R., van Poelwijk F., Peters D. et al. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *J Gen Virol* 72: 2207-16.

de Haan P., Wagemakers L., Peters D., Goldbach R. (1990). The S RNA segment of tomato spotted wilt virus has an ambisense character. *J Gen Virol* 71: 1001-7.

de Hoon M. J., Imoto S., Nolan J., Miyano S. (2004). Open source clustering software. *Bioinformatics* 20: 1453-4.

de Miranda J., Hernandez M., Hull R., Espinoza A. M. (1994). Sequence analysis of rice hoja blanca virus RNA 3. *J Gen Virol* 75: 2127-32.

de Miranda J. R., Munoz M., Wu R., Hull R., Espinoza A. M. (1996). Sequence of rice hoja blanca tenuivirus RNA-2. *Virus Genes* 12: 231-7.

Delgadillo M. O., Saenz P., Salvador B., Garcia J. A., Simon-Mateo C. (2004). Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J Gen Virol* 85: 993-9.

Diallo M., Arenz C., Schmitz K., Sandhoff K., Schepers U. (2003). Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures. *Oligonucleotides* 13: 381-92.

Ditt R. F., Nester E., Comai L. (2005). The plant cell defense and Agrobacterium tumefaciens. *FEMS Microbiol Lett* 247: 207-13.

Dong X., van Wezel R., Stanley J., Hong Y. (2003). Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. *J Virol* 77: 7026-33.

Dunoyer P., Lecellier C. H., Parizotto E. A., Himber C., Voinnet O. (2004). Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* 16: 1235-50.

Dunoyer P., Pfeffer S., Fritsch C., Hemmer O., Voinnet O. et al. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J* 29: 555-67.

Eisen M. B., Spellman P. T., Brown P. O., Botstein D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95: 14863-8.

Elbashir S. M., Harborth J., Lendeckel W., Yalcin A., Weber K. et al. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-8.

English J. J., Mueller E., Baulcombe D. C. (1996). Suppression of Virus Accumulation in Transgenic Plants Exhibiting Silencing of Nuclear Genes. *Plant Cell* 8: 179-188.

Falk B., Tsai J. (1998). Biology and Molecular Biology of viruses in the genus Tenuivirus. *Annu. Rev. Phytopathol* 36: 139-163.

Filippov V., Solovyev V., Filippova M., Gill S. S. (2000). A novel type of RNase III family proteins in eukaryotes. *Gene* 245: 213-21.

Finnegan E. J., Margis R., Waterhouse P. M. (2003). Posttranscriptional gene silencing is not compromised in the Arabidopsis CARPEL FACTORY (DICER-LIKE1) mutant, a homolog of Dicer-1 from Drosophila. *Curr Biol* 13: 236-40.

Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E. et al. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391: 806-11.

Garcia-Sastre A. (2001). Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology* 279: 375-84.

Garcia-Sastre A., Egorov A., Matassov D., Brandt S., Levy D. E. et al. (1998). Influenza A virus lacking the NS1 gene replicates in interferon- deficient systems. *Virology* 252: 324-30.

Ge Q., McManus M. T., Nguyen T., Shen C. H., Sharp P. A. et al. (2003). RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc Natl Acad Sci U S A* 100: 2718-23.

Gitlin L., Karelsky S., Andino R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 418: 430-4.

Goldbach R., Bucher E., Prins M. (2003). Resistance mechanisms to plant viruses: an overview. *Virus Res* 92: 207-12.

Goldbach R., Peters D. (1994). Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* 5: 113-120.

Goodbourn S., Didcock L., Randall R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J Gen Virol* 81: 2341-64.

Guo H. S., Ding S. W. (2002). A viral protein inhibits the long range signaling activity of the gene silencing signal. *Embo J* 21: 398-407.

Gustafson A. M., Allen E., Givan S., Smith D., Carrington J. C. et al. (2005). ASRP: the Arabidopsis Small RNA Project Database. *Nucleic Acids Res* 33: D637-40.

Hall I. M., Noma K., Grewal S. I. S. (2003). RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc Natl Acad Sci U S A* 100: 193-8.

Hamilton A., Voinnet O., Chappell L., Baulcombe D. (2002). Two classes of short interfering RNA in RNA silencing. *Embo J* 21: 4671-9.

Hamilton A. J., Baulcombe D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286: 950-2.

Hammond S. M., Bernstein E., Beach D., Hannon G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404: 293-6.

Hao L., Wang H., Sunter G., Bisaro D. M. (2003). Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell* 15: 1034-48.

Hatada E., Fukuda R. (1992). Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol* 73 (Pt 12): 3325-9.

Heinze C., Maiss E., Adam G., Casper R. (1995). The complete nucleotide sequence of the S RNA of a new Tospovirus species, representing serogroup IV. *Phytopathology* : 683-690.

Himber C., Dunoyer P., Moissiard G., Ritzenthaler C., Voinnet O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *Embo J* 22: 4523-33.

Horsch R., Fry J., Hoffman N., Eichholtz D., Rogers S. et al. (1985a). A simple and general method for transfering genes into plants. *Science* 227: 1229-1231.

Horsch R. B., Rogers S. G., Fraley R. T. (1985b). Transgenic plants. *Cold Spring Harb Symp Quant Biol* 50: 433-7.

Jackson A., Goodin M., Moreno I., Johnson J., Lawrence D. (1999). Rhabdoviruses (Rhabdoviridae): plant rhabdoviruses. In: A. Granoff and R.G. Webster (eds). *Encyclopedia of Virology, Academic Press, San Diego* : 1531-1541.

James C. (2003). *International Service for the Acquisition of Agri-Biotech Applications Briefs* 30.

Jan F. J., Fagoaga C., Pang S. Z., Gonsalves D. (2000a). A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *J Gen Virol* 81: 2103-9.

Jan F. J., Fagoaga C., Pang S. Z., Gonsalves D. (2000b). A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. *J Gen Virol* 81: 235-42.

Ji L. H., Ding S. W. (2001). The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. *Mol Plant Microbe Interact* 14: 715-24.

Johansen L. K., Carrington J. C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiol* 126: 930-8.

Juarez M. T., Kui J. S., Thomas J., Heller B. A., Timmermans M. C. (2004). microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature* 428: 84-8.

Kalantidis K., Psaradakis S., Tabler M., Tsagris M. (2002). The occurrence of CMV-specific short Rnas in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. *Mol Plant Microbe Interact* 15: 826-33.

Kasschau K. D., Carrington J. C. (2001). Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* 285: 71-81.

Kasschau K. D., Xie Z., Allen E., Llave C., Chapman E. J. et al. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev Cell* 4: 205-17.

Kaufman R. J. (1999). Double-stranded RNA-activated protein kinase mediates virus-induced apoptosis: a new role for an old actor. *Proc Natl Acad Sci U S A* 96: 11693-5.

Kennerdell J. R., Carthew R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95: 1017-26.

Ketting R. F., Fischer S. E., Bernstein E., Sijen T., Hannon G. J. et al. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev* 15: 2654-9.

Ketting R. F., Haverkamp T. H., van Luenen H. G., Plasterk R. H. (1999). Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 99: 133-41.

Kidner C. A., Martienssen R. A. (2004). Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature* 428: 81-4.

Kitajima, E. W., De Avila, A. C., Resende, R. de O., Goldbach, R. W., Peters, D. (1992). Comparative cytological and immunogold labelling studies on different isolates of tomato spotted wilt virus. *J. Submisosc. Cytol. Pathol* 24: 1-14.

Kormelink R., de Haan P., Meurs C., Peters D., Goldbach R. (1992). The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. *J Gen Virol* 73: 2795-804.

Kormelink R., Kitajima E. W., De Haan P., Zuidema D., Peters D. et al. (1991). The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells. *Virology* 181: 459-68.

Krug R. M., Yuan W., Noah D. L., Latham A. G. (2003). Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* 309: 181-9.

Lakatos L., Szittya G., Silhavy D., Burgyan J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *Embo J* 23: 876-84.

Lee N. S., Dohjima T., Bauer G., Li H., Li M. J. et al. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 20: 500-5.

Lee R. C., Feinbaum R. L., Ambros V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75: 843-54.

Lee Y., Ahn C., Han J., Choi H., Kim J. et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425: 415-9.

Lee Y. S., Nakahara K., Pham J. W., Kim K., He Z. et al. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117: 69-81.

Li H., Li W. X., Ding S. W. (2002). Induction and suppression of RNA silencing by an animal virus. *Science* 296: 1319-21.

Li W. X., Ding S. W. (2001). Viral suppressors of RNA silencing. *Curr Opin Biotechnol* 12: 150-4.

Li W. X., Li H., Lu R., Li F., Dus M. et al. (2004). Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc Natl Acad Sci U S A* 101: 1350-5.

Lichner Z., Silhavy D., Burgyan J. (2003). Double-stranded RNA-binding proteins could suppress RNA interference- mediated antiviral defences. *J Gen Virol* 84: 975-80.

Lindbo J. A., Dougherty W. G. (1992). Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology* 189: 725-33.

Lindbo J. A., Silva-Rosales L., Proebsting W. M., Dougherty W. G. (1993). Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *Plant Cell* 5: 1749-1759.

Lipardi C., Wei Q., Paterson B. M. (2001). RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* 107: 297-307.

Liu Q., Rand T. A., Kalidas S., Du F., Kim H. E. et al. (2003). R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* 301: 1921-5.

Llave C., Kasschau K. D., Carrington J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc Natl Acad Sci U S A* 97: 13401-6.

Llave C., Kasschau K. D., Rector M. A., Carrington J. C. (2002a). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* 14: 1605-19.

Llave C., Xie Z., Kasschau K. D., Carrington J. C. (2002b). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. *Science* 297: 2053-6.

Lohmann J. U., Endl I., Bosch T. C. (1999). Silencing of developmental genes in Hydra. *Dev Biol* 214: 211-4.

Lu R., Martin-Hernandez A. M., Peart J. R., Malcuit I., Baulcombe D. C. (2003). Virus-induced gene silencing in plants. *Methods* 30: 296-303.

Lu Y., Wambach M., Katze M. G., Krug R. M. (1995). Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. *Virology* 214: 222-8.

Lucy A. P., Guo H. S., Li W. X., Ding S. W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *Embo J* 19: 1672-80.

Mallory A. C., Ely L., Smith T. H., Marathe R., Anandalakshmi R. et al. (2001). HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *Plant Cell* 13: 571-83.

Mallory A. C., Reinhart B. J., Bartel D., Vance V. B., Bowman L. H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc Natl Acad Sci U S A* 99: 15228-33.

Matzke M., Aufsatz W., Kanno T., Daxinger L., Papp I. et al. (2004). Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim Biophys Acta* 1677: 129-41.

McCaffrey A. P., Meuse L., Pham T. T., Conklin D. S., Hannon G. J. et al. (2002). RNA interference in adult mice. *Nature* 418: 38-9.

Meister G., Tuschl T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* 431: 343-9.

Melville M. W., Tan S. L., Wambach M., Song J., Morimoto R. I. et al. (1999). The cellular inhibitor of the PKR protein kinase, P58(IPK), is an influenza virus-activated co-chaperone that modulates heat shock protein 70 activity. *J Biol Chem* 274: 3797-803.

Mette M. F., Aufsatz W., van der Winden J., Matzke M. A., Matzke A. J. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *Embo J* 19: 5194-201.

Meyer B. J., de la Torre J. C., Southern P. J. (2002). Arenaviruses: genomic RNAs, transcription, and replication. *Curr Top Microbiol Immunol* 262: 139-57.

Mitter N., Sulistyowati E., Dietzgen R. G. (2003). Cucumber mosaic virus infection transiently breaks dsRNA-induced transgenic immunity to Potato virus Y in tobacco. *Mol Plant Microbe Interact* 16: 936-44.

Mlotshwa S., Verver J., Stihole-Niang I., Prins M., Van Kammen A. et al. (2002). Trangenic plants expressing HC-Pro show enhanced virus sensitivity while silencing of the transgene results in resistance. *Virus Genes* 15: 45-57.

Molnar A., Csorba T., Lakatos L., Varallyay E., Lacomme C. et al. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J Virol* 79: 7812-8.

Morris K. V., Chan S. W., Jacobsen S. E., Looney D. J. (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 305: 1289-92.

Mourrain P., Beclin C., Elmayan T., Feuerbach F., Godon C. et al. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101: 533-42.

Muangsan N., Beclin C., Vaucheret H., Robertson D. (2004). Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. *Plant J* 38: 1004-14.

Napoli C. (1990). Introduction of chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* 2: 279-289.

Ngo H., Tschudi C., Gull K., Ullu E. (1998). Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. *Proc Natl Acad Sci U S A* 95: 14687-92.

Noma K., Sugiyama T., Cam H., Verdel A., Zofall M. et al. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* 36: 1174-80.

Novina C. D., Murray M. F., Dykxhoorn D. M., Beresford P. J., Riess J. et al. (2002). siRNA-directed inhibition of HIV-1 infection. *Nat Med* 8: 681-6.

Okamura K., Ishizuka A., Siomi H., Siomi M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* 18: 1655-66.

Paddison P. J., Caudy A. A., Hannon G. J. (2002). Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A* 99: 1443-8.

Pal-Bhadra M., Bhadra U., Birchler J. A. (1997). Cosuppression in Drosophila: gene silencing of Alcohol dehydrogenase by white-Adh transgenes is Polycomb dependent. *Cell* 90: 479-90.

Palatnik J. F., Allen E., Wu X., Schommer C., Schwab R. et al. (2003). Control of leaf morphogenesis by microRNAs. *Nature* 425: 257-63.

Palukaitis P., Roossinck M. J., Dietzgen R. G., Francki R. I. (1992). Cucumber mosaic virus. *Adv Virus Res* 41: 281-348.

Pang S. Z., Jan F. J., Gonsalves D. (1997). Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. *Proc Natl Acad Sci U S A* 94: 8261-6.

Pfeffer S., Dunoyer P., Heim F., Richards K. E., Jonard G. et al. (2002). P0 of beet Western yellows virus is a suppressor of posttranscriptional gene silencing. *J Virol* 76: 6815-24.

Pfeffer S., Zavolan M., Grasser F. A., Chien M., Russo J. J. et al. (2004). Identification of virus-encoded microRNAs. *Science* 304: 734-6.

Plisson C., Drucker M., Blanc S., German-Retana S., Le Gall O. et al. (2003). Structural characterization of HC-Pro, a plant virus multifunctional protein. *J Biol Chem* 278: 23753-61.

Prins M., Goldbach R. (1998). The emerging problem of tospovirus infection and nonconventional methods of control. *Trends Microbiol* 6: 31-5.

Prins M., Kikkert M., Ismayadi C., de Graauw W., de Haan P. et al. (1997). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NS(M) gene sequences. *Plant Mol Biol* 33: 235-43.

Prins M., Resende R. D. O., Anker C., Van Schepen A., De Haan P. et al. (1996). Engineered RNA-mediated resistance to tomato spotted wilt virus in sequence specific. *Molecular Plant-Microbe Interactions* 9: 416-418.

Pruss G., Ge X., Shi X. M., Carrington J. C., Bowman Vance V. (1997). Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9: 859-68.

Pruss G. J., Lawrence C. B., Bass T., Li Q. Q., Bowman L. H. et al. (2004). The potyviral suppressor of RNA silencing confers enhanced resistance to multiple pathogens. *Virology* 320: 107-20.

Qu F., Morris T. J. (2002). Efficient infection of Nicotiana benthamiana by Tomato bushy stunt virus is facilitated by the coat protein and maintained by p19 through suppression of gene silencing. *Mol Plant Microbe Interact* 15: 193-202.

Ramirez B. C., Lozano I., Constantino L. M., Haenni A. L., Calvert L. A. (1993). Complete nucleotide sequence and coding strategy of rice hoja blanca virus RNA4. *J Gen Virol* 74: 2463-8.

Ramirez B. C., Macaya G., Calvert L. A., Haenni A. L. (1992). Rice hoja blanca virus genome characterization and expression in vitro. *J Gen Virol* 73: 1457-64.

Ratcliff F., Harrison B. D., Baulcombe D. C. (1997). A similarity between viral defense and gene silencing in plants. *Science* 276: 1558-1560.

Ratcliff F. G., MacFarlane S. A., Baulcombe D. C. (1999). Gene silencing without DNA. RNA-mediated cross-protection between viruses. *Plant Cell* 11: 1207-16.

Rebouillat D., Hovanessian A. G. (1999). The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. *J Interferon Cytokine Res* 19: 295-308.

Reinhart B. J., Slack F. J., Basson M., Pasquinelli A. E., Bettinger J. C. et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403: 901-6.

Rhoades M. W., Reinhart B. J., Lim L. P., Burge C. B., Bartel B. et al. (2002). Prediction of plant microRNA targets. *Cell* 110: 513-20.

Ritzenthaler C. (2005). Resistance to plant viruses: old issue, news answers?. *Curr Opin Biotechnol* 16: 118-22.

Roth B. M., Pruss G. J., Vance V. B. (2004). Plant viral suppressors of RNA silencing. *Virus Res* 102: 97-108.

Saldanha A. J. (2004). Java Treeview--extensible visualization of microarray data. *Bioinformatics* 20: 3246-8.

Sanchez Alvarado A., Newmark P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc Natl Acad Sci U S A* 96: 5049-54.

Schauer S. E., Jacobsen S. E., Meinke D. W., Ray A. (2002). DICER-LIKE1: blind men and elephants in Arabidopsis development. *Trends Plant Sci* 7: 487-91.

Schwarz D. S., Hutvagner G., Haley B., Zamore P. D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Mol Cell* 10: 537-48.

Schwarz D. S., Zamore P. D. (2002). Why do miRNAs live in the miRNP?. *Genes Dev* 16: 1025-31.

Sharma A. D., Gill P. K., Singh P. (2003). RNA isolation from plant tissues rich in polysaccharides. *Anal Biochem* 314: 319-21.

Sharp P. A. (2001). RNA interference--2001. *Genes Dev* 15: 485-90.

Shi B. J., Palukaitis P., Symons R. H. (2002). Differential virulence by strains of Cucumber mosaic virus is mediated by the 2b gene. *Mol Plant Microbe Interact* 15: 947-55.

Shi X. M., Miller H., Verchot J., Carrington J. C., Vance V. B. (1997). Mutations in the region encoding the central domain of helper component-proteinase (HC-Pro) eliminate potato virus X/potyviral synergism. *Virology* 231: 35-42.

Sijen T., Fleenor J., Simmer F., Thijssen K. L., Parrish S. et al. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107: 465-76.

Sijen T., Plasterk R. H. (2003). Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. *Nature* 426: 310-4.

Silhavy D., Molnar A., Lucioli A., Szittya G., Hornyik C. et al. (2002). A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *Embo J* 21: 3070-80.

Sledz C. A., Holko M., de Veer M. J., Silverman R. H., Williams B. R. (2003). Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 5: 834-9.

Smith N. A., Singh S. P., Wang M. B., Stoutjesdijk P. A., Green A. G. et al. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* 407: 319-20.

Soldan S. S., Plassmeyer M. L., Matukonis M. K., Gonzalez-Scarano F. (2005). La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *J Virol* 79: 234-44.

Souret F. F., Kastenmayer J. P., Green P. J. (2004). AtXRN4 degrades mRNA in Arabidopsis and its substrates include selected miRNA targets. *Mol Cell* 15: 173-83.

Storms M. M., Kormelink R., Peters D., Van Lent J. W., Goldbach R. W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* 214: 485-93.

Strebel K. (2003). Virus-host interactions: role of HIV proteins Vif, Tat, and Rev. *Aids* 17 Suppl 4: S25-34.

Sunkar R., Zhu J. K. (2004). Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* 16: 2001-19.

Suzuki K., Mori A., Ishii K. J., Saito J., Singer D. S. et al. (1999). Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A* 96: 2285-90.

Tabara H., Sarkissian M., Kelly W. G., Fleenor J., Grishok A. et al. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* 99: 123-32.

Takeda A., Sugiyama K., Nagano H., Mori M., Kaido M. et al. (2002). Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. *FEBS Lett* 532: 75-9.

Tenllado F., Llave C., Diaz-Ruiz J. R. (2004). RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res* 102: 85-96.

Trinks D., Rajeswaran R., Shivaprasad P. V., Akbergenov R., Oakeley E. J. et al. (2005). Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J Virol* 79: 2517-27.

Tuschl T., Zamore P. D., Lehmann R., Bartel D. P., Sharp P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* 13: 3191-7.

Vaistij F. E., Jones L., Baulcombe D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* 14: 857-67.

van der Krol A. R., Mur L. A., Beld M., Mol J. N., Stuitje A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2: 291-9.

van der Vlugt R. A., Ruiter R. K., Goldbach R. (1992). Evidence for sense RNA-mediated protection to PVYN in tobacco plants transformed with the viral coat protein cistron. *Plant Mol Biol* 20: 631-9.

van Poelwijk F., Prins M., Goldbach R. (1997). Completion of the impatiens necrotic spot virus genome sequence and genetic comparison of the L proteins within the family Bunyaviridae. *J Gen Virol* 78 (Pt 3): 543-6.

Van Regenmortel M. H. V., Fauquet C. M., Bishop D. H. L., Carstens E. B., Estes M. K. et al. (2000). Seventh report of the International Committee on Taxonomy of Viruses. *Virus taxonomy, Academic Press, San Diego* : .

Vance V., Vaucheret H. (2001). RNA silencing in plants--defense and counterdefense. *Science* 292: 2277-80.

Vanitharani R., Chellappan P., Fauquet C. M. (2003). Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proc Natl Acad Sci U S A* 100: 9632-6.

Vanitharani R., Chellappan P., Pita J. S., Fauquet C. M. (2004). Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J Virol* 78: 9487-98.

Vargason J. M., Szittya G., Burgyan J., Tanaka Hall T. M. (2003). Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* 115: 799-811.

Vaucheret H., Fagard M. (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends Genet* 17: 29-35.

Vaucheret H., Vazquez F., Crete P., Bartel D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18: 1187-97.

Vazquez F., Gasciolli V., Crete P., Vaucheret H. (2004). The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr Biol* 14: 346-51.

Vazquez Rovere C., del Vas M., Hopp H. E. (2002). RNA-mediated virus resistance. *Curr Opin Biotechnol* 13: 167-72.

Vende P., Taraporewala Z. F., Patton J. T. (2002). RNA-binding activity of the rotavirus phosphoprotein NSP5 includes affinity for double-stranded RNA. *J Virol* 76: 5291-9.

Verdel A., Jia S., Gerber S., Sugiyama T., Gygi S. et al. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 303: 672-6.

Vialat P., Billecocq A., Kohl A., Bouloy M. (2000). The S segment of rift valley fever phlebovirus (Bunyaviridae) carries determinants for attenuation and virulence in mice. *J Virol* 74: 1538-43.

Voinnet O. (2001). RNA silencing as a plant immune system against viruses. *Trends Genet* 17: 449-59.

Voinnet O., Lederer C., Baulcombe D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. *Cell* 103: 157-67.

Voinnet O., Pinto Y. M., Baulcombe D. C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc Natl Acad Sci U S A* 96: 14147-52.

Volkmuth W., Turk S., Shapiro A., Fang Y., Kiegle E. et al. (2003). Technical advances: genome-wide cDNA-AFLP analysis of the Arabidopsis transcriptome. *Omics* 7: 143-59.

Volpe T., Schramke V., Hamilton G. L., White S. A., Teng G. et al. (2003). RNA interference is required for normal centromere function in fission yeast. *Chromosome Res* 11: 137-46.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T. et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23: 4407-14.

Wang H., Hao L., Shung C. Y., Sunter G., Bisaro D. M. (2003). Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* 15: 3020-32.

Wang W., Riedel K., Lynch P., Chien C. Y., Montelione G. T. et al. (1999). RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. *RNA* 5: 195-205.

Wang X., Basler C. F., Williams B. R., Silverman R. H., Palese P. et al. (2002). Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains. *J Virol* 76: 12951-62.

Wang X., Li M., Zheng H., Muster T., Palese P. et al. (2000). Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol* 74: 11566-73.

Wassenegger M., Heimes S., Riedel L., Sanger H. L. (1994). RNA-directed de novo methylation of genomic sequences in plants. *Cell* 76: 567-76.

Waterhouse P. M., Helliwell C. A. (2003). Exploring plant genomes by RNA-induced gene silencing. *Nat Rev Genet* 4: 29-38.

White S. A., Allshire R. C. (2004). Loss of Dicer fowls up centromeres. *Nat Cell Biol* 6: 696-7.

Wijkamp I., van Lent J., Kormelink R., Goldbach R., Peters D. (1993). Multiplication of tomato spotted wilt virus in its insect vector, Frankliniella occidentalis. *J Gen Virol* 74: 341-9.

Wilkins C., Dishongh R., Moore S. C., Whitt M. A., Chow M. et al. (2005). RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. *Nature* 436: 1044-7.

Williams B. R. (1999). PKR; a sentinel kinase for cellular stress. *Oncogene* 18: 6112-20.

Xie Z., Johansen L. K., Gustafson A. M., Kasschau K. D., Lellis A. D. et al. (2004). Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* 2: E104.

Xie Z., Kasschau K. D., Carrington J. C. (2003). Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. *Curr Biol* 13: 784-9.

Yang S. J., Carter S. A., Cole A. B., Cheng N. H., Nelson R. S. (2004). A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by Nicotiana benthamiana. *Proc Natl Acad Sci U S A* 101: 6297-302.

Yu D., Fan B., MacFarlane S. A., Chen Z. (2003). Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. *Mol Plant Microbe Interact* 16: 206-16.

Zamore P. D. (2002). Ancient pathways programmed by small RNAs. *Science* 296: 1265-9.

Zhang H., Kolb F. A., Jaskiewicz L., Westhof E., Filipowicz W. (2004). Single processing center models for human Dicer and bacterial RNase III. *Cell* 118: 57-68.

Zilberman D., Cao X., Jacobsen S. E. (2003). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* 299: 716-9.

Samenvatting

Het in dit proefschrift beschreven onderzoek betreft het mechanisme van "RNA silencing" in planten in relatie tot virusinfecties. Ondermeer wordt aangetoond hoe dit mechanisme kan worden uitgebuit om virusresistente planten te produceren. Gebaseerd op de activiteit van RNA silencing, dat in planten een belangrijke verdedigingslinie tegen virussen is, kunnen transgene planten worden voorgeprogrammeerd om zogenaamde "small interfering RNAs" (siRNAs) te produceren die specifiek het RNA genoom of de boodschapper RNA's van inkomende virussen kunnen herkennen en daarmee hun afbraak initiëren.

RNA silencing werkt in principe net als een anti-virus softwarepakket in een computer, waarbij de identificatie van computervirussen gebeurt aan de hand van specifieke stukken codetekst. Ieder inkomend dossier (potentieel virus) wordt gescand op de aanwezigheid van specifieke codes. Een siRNA doet in feite hetzelfde bij inkomend viraal RNA.

Om brede resistentie tegen verschillende virussen tegelijk te verkrijgen werd in Hoofdstuk 2 de efficiënte silencing activiteit van dubbelstrengs RNA (dsRNA) gecombineerd met een fusiestrategie waarin korte segmenten van de nucleocapside (N) genen van vier verschillende tomateninfecterende tospovirussen aan elkaar werden gekoppeld. De aldus gefuseerde virale gensequenties werden vervolgens als een geinverteerde repetitie in de plant tot expressie gebracht, leidend tot de productie van een lang dsRNA. Transgene planten die stabiel deze dsRNA's tot expressie brachten bleken virusspecifieke siRNAs te produceren, resulterend in een hoge frequentie van meervoudige virusresistentie. De op deze wijze verkregen hoge frequentie kan van belang zijn bij het verkrijgen van virusresistentie in gewassen waarbij lage transformatie- en regeneratiefrequenties verwacht worden.

Aangezien RNA silencing een natuurlijke antivirale afweer van planten is, dienen virussen tijdens hun infectieproces deze hindernis te nemen. Zij doen dit door eiwitten te coderen die in staat zijn om het RNA silencing mechanisme te blokkeren. Bij de aanvang van het hier beschreven onderzoekproject waren dergelijke RNA silencing "suppressoreiwitten" slechts bekend bij een aantal plus-strengs RNA virussen. In Hoofdstuk 3 is aangetoond dat ook min-strengs RNA virussen, zoals het Tomatenbronsvlekkenvirus (TSWV) en Rijst "hoja blanca" virus (RHBV), dergelijke eiwitten coderen (NSS en NS3, respectievelijk). Terwijl de suppressoreiwitten in beide onderzochte virale genomen op analoge posities worden gecodeerd, tonen zij nauwelijks moleculaire verwantschap en lijken bovendien een verschillende suppressoractiviteit te bezitten.

Vervolgens is in Hoofdstuk 4 onderzocht of ook min-strengs RNA virussen van (zoog)dieren dergelijke suppressoreiwitten coderen. Dit was een logische volgende stap om twee redenen. Ten eerste behoort TSWV tot de Bunyaviridae, waarvan de meeste soorten zoogdieren infecteren, en ten tweede repliceren veel bunyavirussen, waaronder ook TSWV zelf, in hun insectenvectoren, en van insecten was inmiddels aangetoond dat deze eveneens een RNA silencing mechanisme bezitten. Aldus werd de mogelijkheid getest of het goed gekarakteriseerde NS1 eiwit van Influenzavirus A RNA silencing suppressoractiviteit zou bezitten. Inderdaad kon worden aangetoond dat NS1 RNA silencing in planten kan onderdrukken, mogelijk door siRNAs te binden. Hiermee in overeenstemming kon tevens worden vastgesteld dat NS1 de virulentie van aardappelvirus X (PVX) versterkt. Chimeer PVX/NS1 virus veroorzaakte heftiger ziektesymptomen en bleek in staat bestaande RNA silencing van een reportergen (GFP) in de geïnfecteerde plant ongedaan te maken. Of NS1 ook RNA silencing activiteit vertoont in zoogdiercellen werd weliswaar onderzocht, maar kon (nog) niet bewezen worden. Interessant is wel dat NS1 een remmer van de interferonroute is, een antiviraal mechanisme in zoogdiercellen waarin, net als in RNA silencing, dsRNA een belangrijke rol speelt. Dit zou erop kunnen wijzen dat de interferonroute en RNA silencing op enigerlei wijze onderling gekoppeld zijn en wellicht gecoördineerd werken. Nochtans kon niet worden uitgesloten dat het NS1 eiwit dsRNA bindt louter om het voor de interferonroute onzichtbaar te maken en dat de waargenomen suppressie van RNA silencing in planten hiervan een bijwerking is.

Een deel van dit proefschrift richtte zich op de mogelijke invloed van virale RNA silencing suppressoreiwitten op de expressie van de gastheergenen. De gebruikte suppressoreiwitten waren de bovengenoemde NSS, NS3, NS1 en ook het (potyvirale) HC-Pro eiwit en het (cucumovirale) 2b eiwit. Voor deze analyse werd gebruikgemaakt van cDNA-AFLP. Deze techniek maakt een zeer brede analyse van transcriptionele veranderingen in (planten)weefsels mogelijk. De virale suppressoreiwitten werden steeds transient in volgroeide bladeren tot expressie gebracht met behulp van Agrobacterium tumefaciens. Uit ongeveer 25.000 geteste RNA fragmenten (ongeveer de helft van alle genen vertegenwoordigend) werden er 362 gevonden die een substantieel veranderd expressiepatroon vertoonden als gevolg van de RNA silencing suppressor activiteit. Gevonden werd dat de expressie van de grote meerderheid van deze genen (80%) onderdrukt werd. Het feit dat een redelijk groot contingent (enige honderden) genen verandering van expressie zouden ondergaan was niet onverwacht, aangezien RNA silencing processen ook een belangrijke rol spelen bij het reguleren transcriptieniveaus d.m.v. zogenaamde “micro RNAs” (miRNAs). Verstoringen in de activiteit van miRNAs kunnen complexe veranderingen in de expressie van het transcriptoom

veroorzaken, zoals al eerder voor HC-Pro was aangetoond.

Concluderend kan worden gesteld dat de interacties tussen de antivirale RNA silencing en de maatregelen die virussen hebben ontwikkeld om dit proces te frustreren enerzijds deel uitmaken van een belangrijk thema binnen de virologie en anderzijds een krachtig aanknopingspunt bieden voor doorbraken in andere wetenschapsgebieden zoals de functionele genomica en de ontwikkelingbiologie. In de toepassingssfeer heeft RNA silencing ons in staat gesteld een efficiënte en brede virusresistentie in planten te ontwikkelen.

Summary

The research described in this thesis centres around the mechanism of RNA silencing in relation to virus-host interaction, an area of increasing importance. It shows how this recently disclosed mechanism can be used to produce virus-resistant plants. Based on the activity of the RNA silencing machinery, which is an important line of defense of plants against viruses, plants can be pre-programmed to produce so-called small-interfering RNAs (siRNAs) that specifically target the genome or transcripts of incoming viruses. This principle works like an antivirus software package on a computer, in which signatures recognizing specific viruses are included. Any incoming file (potential virus) is scanned for the recognition sequence much like an siRNA does on an incoming viral RNA.

In order to produce multiple virus resistance the work presented in this thesis combined the very high silencing activity of double stranded RNA (dsRNA) with a fusion strategy involving small, 150 basepairs long segments of the nucleocapsid (N) gene of four different tomato-infecting tospoviruses. The virus-derived sequences were arranged in an inverted repeat leading to the production of dsRNA. Transgenic plants stably expressing these dsRNAs were shown to process them into siRNAs thereby pre-programming the plants to fight incoming tospoviruses. Indeed, these transgenic plants showed multiple virus resistance at a high frequency. The high frequency is important for the production of transgenic plants that are difficult to transform.

As RNA silencing is an important antiviral defense mechanism of plants, viruses have to overcome this hurdle and they do this by encoding proteins capable of blocking this pathway at certain steps. At the onset of this research project a number of such ("RNA silencing suppressor") proteins had been characterized for positive strand RNA viruses and DNA viruses. In Chapter 3 it is shown that also negative strand RNA viruses, i.e. *Tomato spotted wilt virus* (TSWV) and *Rice hoja blanca virus* (RHBV), encode suppressors of RNA silencing (NS_S and NS3, respectively). Interestingly, while the silencing suppressors are encoded on analogous genomic positions, they show different silencing suppression activities.

Having identified the first negative strand RNA viral silencing suppressors the next step was to investigate whether also mammalian negative strand RNA viruses could also encode such proteins. This was a logical next step to take for two reasons. 1. TSWV is a member of the Bunyaviridae, a virus family of which most other members infect mammals, and 2. viruses of this family are propagated in their insect vectors while it has been shown that silencing suppressors can be active in insects. To test this possibility the well characterized NS1 protein of the *Influenza A virus* was

chosen to be tested. Indeed, it could be demonstrated that NS1 is capable of suppressing RNA silencing in plants, possibly by binding siRNAs. These findings were strengthened by the observation that NS1 enhances the virulence of *Potato virus X* when it was incorporated into its genome. The chimeric PVX/NS1 virus induced more pronounced disease symptoms, the NS1 still being capable of suppressing RNA silencing when expressed from that viral vector. Whether NS1 also has RNA silencing suppressor activity in mammalian cell systems though, still awaits to be confirmed. An intriguing fact is that NS1 is an inhibitor of the interferon pathway, a mammalian antiviral mechanism in which – again – dsRNA plays a key role. This may indicate that in mammalian cells the interferon response and RNA silencing are somehow interlinked to act in concert. It is also possible that NS1 binds long dsRNA to hide it from the interferon response and that the resulting silencing suppression in plants is only a side effect caused by that activity.

A further aim of this thesis was to better understand the interactions between viral silencing suppressors and the host. The suppressors of RNA silencing used were the previously mentioned NS_S, NS3, NS1 and additionally HC-Pro of *Cowpea aphid borne mosaic virus* and 2b of *Cucumber mosaic virus*. For the analysis, cDNA-amplified fragment length polymorphism (AFLP) technology was used. This technique allows a very broad analysis of transcriptional changes in plant tissues upon certain treatments. This combined with the *A. tumefaciens* mediated transient gene expression allowed monitoring of transcription profile changes caused by the expression of viral silencing suppressors. Out of approximately 25,000 mRNAs tested 362 were found to be differentially expressed due to the activity of silencing suppressors. Interestingly a large majority of genes (80%) was down-regulated. The fact that quite a number of genes were differentially expressed was to be expected since RNA silencing also plays a role in regulating certain mRNA levels via the activity of so-called micro-RNAs (miRNAs). Any disturbances in this processes will produce complex transcriptional changes for instance by directly inhibiting the activity of miRNAs (as it has been shown for HC-Pro for instance).

To conclude it can be stated that the interactions between antiviral RNA silencing and the countermeasures viruses have evolved to frustrate such process are on one hand a very important topic in virology and on the other hand a strong starting point for breakthroughs in other fields of research such as functional genomics and development. In an application environment, RNA silencing has allowed us to develop efficient and broad virus resistance in plants.

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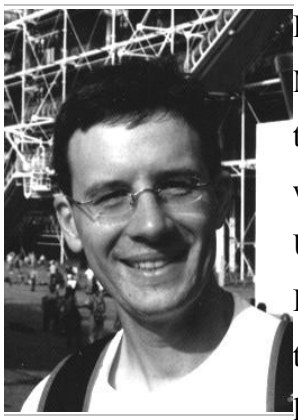
Finally, I want to thank my family for all their support during all that time in Wageningen. Danijela, without you I would never have achieved all of this. First you came with my to this (at least culturally) lost place called “Wageningen”. Then all the time you supported me morally and any time when I was down you could put me up again. You are the wind in my sails. And thank you for all the love. Florence, Marie-Anne and Erich, thank you for all your financial and moral support, it is really great to have a family that believed that I could get this far.

Curriculum vitae



Etienne Bucher was born the 13.4.1975 in St. Gallen, Switzerland. He did his Matura at the Mathematisch Naturwissenschaftlichen Gymnasium in Basel in the year 1995. In the same year he had to join the Swiss army for 15 weeks, which was his least interesting experience by far. Just after that he went to University to study Biology. In 2001 he finished his MSc thesis in the lab of Barbara Hohn at the Friedrich Miescher Institut in Basel, where he was trying to develop gene targeting in plants. Then he felt like he had to go abroad to learn something new. From 2002 to 2005 he was working on his PhD thesis at the Laboratory of Virology in Wageningen, The Netherlands, under the supervision of Prof. Dr. Rob Goldbach and Dr. Marcel Prins. There he learned how to use RNA silencing in order to obtain RNA virus resistance in plants. He is currently working in the lab of Dr. Antonius Matzke and Dr. Marjori Matzke in the Gregor Mendel Institute of Molecular Plant Biology in Vienna, Austria. There he is trying to discover new factors involved in RNA mediated DNA methylation. His great hope for the future is to apply his new knowledge to efficiently fight plant DNA viruses.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Etienne C. Bucher
Date: 15 May 2006
Group: Laboratory of Virology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Indentification of the silencing suppressor of TSWV	11Apr 2002
► **Writing or rewriting a project proposal**	
High frequency of resistance against multiple viruses in transgenic crop plants	22 May 2002
► **Writing a review or book chapter**	
RNA silencing: A natural resistance mechanism in plants	15 Nov 2004
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe handling of radioactive isotopes	2002
Subtotal Start-up Phase	*15 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
Wageningen University	24 Jan 2002
Utrecht University	27 Mar 2003
Free University of Amsterdam	03 Jun 2004
► **EPS theme symposia**	
► **NWO Lunteren days and other National Platforms**	
ALW Lunteren	Apr 2002
ALW Lunteren	Apr 2003
► **Seminars (series), workshops and symposia**	
Dutch-German Plant Virology Meeting	Mar 2004
► **Seminar plus**	
► **International symposia and congresses**	
ICV Paris, France	27 Jul -01 Aug 2002
EMBO Workshop "Genomic approaches in plant virology", Keszthely, Hungary	28-31 May 2003
Keystone Meeting "siRNAs and miRNAs", Colorado, USA	14-19 Apr 2004
► **Presentations**	
Poster 'Silencing Suppressors', ICV Paris, France	2002
Poster 'NS1 of Influenza as a silencing suppressor', PhD students day	2003
Poster 'Multiple virus resistance', Colorado, USA	2004
Oral, ALW Lunteren	2002
Oral, ALW Lunteren	2003
Oral, EMBO workshop, Keszthely, Hungary	2003
Oral, Syngenta, UK	2003
Oral, FMI, Basel, Switzerland	2004
Oral, John Innes Centre, UK	2005
Oral, GMI, Vienna	2005
► **IAB interview**	04 Jun 2004
► **Excursions**	
Subtotal Scientific Exposure	*14.9 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Disease Resistance in Plants	14-16 Oct 2002
► **Journal club**	
Literature discussion with Virology group	2002-2005
► **Individual research training**	
AFLP training at Keygene B.V.	Feb-Mar 2005
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development	date
► **Skill training courses**	
Course on Presentation Skills (40 hours)	Mar-Apr 2002
► **Organisation of PhD students day, course or conference**	
Member Organ. Committee 'Workshop on Molecular Aspects of Germination and Dormancy' (Wageningen)	23-24 May 2004
Member Organ. Committee '3rd Intern. Symp. Plant Dormancy: From Mol. Level to the whole Plant (Wageningen)	25-28 May 2004
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*1.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**38.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

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