TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. AII OSA - TOM. 219

BIOLOGICA - GEOGRAPHICA - GEOLOGICA

RNA Silencing and its Inhibition in Transgenic Tobacco Plants

by

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Turku 2007

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ISBN 978-951-29-3448-5 (PRINT)
ISBN 978-951-29-3449-2 (PDF)
ISSN 0082-6979
Painosalama Oy – Turku, Finland 2007

It is He Who has spread out the earth for the creatures, Therein are fruits and date-palms, producing sheathed fruit-stalks (enclosing dates); And also grain with stalks and fragrant herbs, Then which of the Blessings of your Lord will you deny?

Ar-Rehman 55: 10-14.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are refferred to, in the text, by their Roman numerals.

I **Siddiqui, S. A., Sarmiento, C., Valkonen, S., Truve, E. & Lehto, K**. (2007). Suppression of infectious TMV genomes expressed in young transgenic tobacco plants. *MPMI*, 20: 1489-1494.

II **Siddiqui, S. A., Sarmiento, C., Truve, E., Lehto, H. & Lehto, K.** (2008). Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic *Nicotiana benthamiana* and *N. tabacum*. *MPMI*, in press.

III **Siddiqui, S. A., Sarmiento, C., Kiisma, M., Koivumäki, S., Lemmetty, A., Truve, E. & Lehto, K.** (2007). Effects of viral silencing suppressors on *tobacco ringspot virus* infection in two *Nicotiana* species. Submitted to *J. Gen. Virol.*

IV **Siddiqui, S. A. & Lehto, K.** (2007). Interference of different viral silencing suppressors with the infections of homologous and heterologous viruses in two different *Nicotiana* hosts. Manuscript.

ABBREVIATIONS

A. thaliana	*Arabidopsis thaliana*
ABA	Abscisic acid
ACLSV	*Apple chlorotic leaf spot virus*
ACMV	*African cassava mosaic virus*
AGO	ARGONAUTE
AP2	APETALA2
APS	ATP-sulfurylase
ARF	AUXIN RESPONSE FACTOR
BNYVV	*Beet necrotic yellow vein virus*
BSMV	*Barley stripe mosaic virus*
BWYV	*Beet western yellows virus*
BYSV	*Beet yellow stunt virus*
BYV	*Beet yellows virus*
BYVMY	*Bhendi yellow vein mosaic virus*
C. elegans	*Caenorhabtitis elegans*
CABYV	*Cucurbit aphid-borne yellows virus*
caf	CARPEL FACTORY
CfMV	*Cooksfoot mottle virus*
ChlH	Chlorophyll subunit H
CMV	*Cucumber mosaic virus*
CP	Coat protein
CPMV	*Cowpea mosaic virus*
CSD	COPPER SUPEROXIDE DISMUTASE
crTMV	Crucifer TMV
CTAB	Cetrimonium bromide
CTV	*Citrus tristeza virus*
CUC	CUP shaped cotyledons
CymRSV	*Cymbidium ringspot virus*
DCL	Dicer-like
DIG	Digoxigenin
DNA	Deoxyribose nucleic acid
dpi	Days post inoculation
Dr	Doctor
DRB	dsRNA-binding protein
DRD	Defective in RNA-directed DNA methylation
ds	Double stranded
E2-UBC	E2 ubiquitin-conjugating protein
EDTA	Ehtylenediamine tetraacetic acid
GFP	Green fluorescence protein
GRF	GROWTH REGULATING FACTOR
GVA	*Grapevine virus A*
h	Hour
HASTY	Protein exporting miRNAs from nucleus to cytoplasm
HCl	Hydrochloric acid

HcPro	Helper component protease
HCRSV	*Hibiscus chlorotic ringspot virus*
hc	Heterochromatin
HD-ZIPIII	Gene targeted by miRNAs
HEN1	a dsRNA methylase
HYL1	dsRNA binding protein
kb	Kilo bases
kDa	Kilo dalton
Km	Kanamycine
LTRs	Long terminal repeats
M	Molar
MADS	Transcription factors gene family
Me	Methylated
miRNAs	MicroRNAs
mM	Mili molar
MS	Murashige-Skoog
MYB	Myeloblastosis
MYMV	*Mungbean yellow mosaic virus*
N. benthamiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
NAC	Transcription factor gene family
NaCl	Sodium chloride
nat-siRNAs	Natural antisence transcripts siRNAs
nt	Nucleotides
^{o}C	Degree centigrade
P	Phosphorous
PAZ	Piwi/Argonaute/Zwille
PCR	Polymerase chain reaction
PCV	*Peanut clump virus*
PDS	Phytoene desaturase
PHB	PHABULOSA
PHV	PHAVOLUTA
PME	Pectin methylesterase
Pol IV	Polymerase IV
PoLV	*Pothos latent virus*
Pre	Precursor
Pri	Primary
PSLV	*Poa semilatent virus*
PSRP1	RNA binding protein in phloem
PTGS	Post transcriptional gene silencing
PVX	*Potato virus X*
PVY	*Potato virus Y*
RBS	Small subunit of Rubisco
RCNMV	*Red clover necrotic mosaic virus*
RdDM	RNA directed DNA methylation
RDR	RNA dependent RNA polymerase

RdRP	RNA dependent RNA polymerase
RDV	Rice *dwarf virus*
REV	REVOLUTA
RHBV	*Rice hoja blanca virus*
RISC	RNA-induced silencing complex
RITS	Transcriptional silencing complex
RMD	RNA mediated defense
RNA	Ribose nucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RYMV	*Rice yellow mottle virus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Shizosaccharomyces pombe*
SBP	SQUAMOSA-promoter binding protein
SBWMV	*Wheat mosaic virus*
SCL	SCARECROW-LIKE
SDE1	Silencing Defective1
SGS2	Suppressor of Gene Silencing 2
sin1	Short integuments
siRNAs	Short interfering RNAs
SLCMV	*Srilankan cassava mosaic virus*
SPCSV	*Sweet potato chlorotic stunt virus*
ss	Single stranded
sus1	SUC synthase 1 mutants
ta-siRNAs	Transacting siRNAs
TAV	*Tomato aspermy virus*
TBSV	*Tomato bushy stunt virus*
TCP	Gene family targeted by miRNA
TCV	*Turnip crinkle virus*
TEV	*Tobacco etch virus*
TGS	Transcriptional gene silencing
TMV	*Tobacco mosaic virus*
ToMV	*Tomato mosaic virus*
TRSV	*Tobacco ring spot virus*
TRV	*Tobacco rattle virus*
TSWV	*Tomato spotted wilt virus*
TuMV	*Turnip mosaic virus*
TYLCV- C	*Tomato yellow leaf curl virus*-China
TYLCV-Is	*Tomato yellow leaf curl virus*-Israel
TYMV	*Turnip yellow mosaic virus*
VIGS	Virus induced gene silencing
WCMV	*White clover mosaic virus*
wt	Wild type
μ mol m^{-2} s^{-1}	micro mol per meter square per second
μ g	Microgram
2-PGK	2-phophoglycerate kinase

ABSTRACT

The primary and secondary host responses associated with natural virus infections and symptom development are complex, and difficult to resolve and dissect. Symptoms of different plant virus diseases may be caused by one or more of the virus-encoded proteins, by virus-induced host defense reactions or by the virus-encoded counter-defense reaction, by general stress, or by all of these. The roles of the separate protein products, encoded by intact, wild type (wt) virus in disease phenotype are difficult to identify, because deletion or mutation of any viral gene leads to severe impairment of the targeted virus function, and thus of the whole viral life cycle. However, the functions of individual viral genes can be effectively studied by using transgenic plants, which express either the intact or differently modified versions of virus genomes. Such transgenic plants allow expression of different combinations of viral proteins and RNA, independently from the virus spread or encapsidation. In present studies, I have examined these virus-host interactions by expressing the wt *tobacco mosaic virus* (TMV) genome, and its mutated derivative in transgenic plants.

In most cases plants can activate sequence specific RNA-silencing pathways to defend themselves against viral infections. To counter-act these defense mechanism, plant viruses produce various silencing suppressors. The silencing suppressors encoded by different viruses have no sequence similarity, and they also appear to function in different fashion with one another, and many of them may target different steps in the silencing pathways. To analyze the effects of various silencing suppressors on the host phenotypes, and on different heterologous virus infection, I have produced a set of transgenic *Nicotiana benthamiana* and *N. tabacum* plants, each expressing a silencing suppressor protein derived from seven different plants viruses.

Results obtained from the transgenic plants expressing the infectious TMV indicated that the disease phenotype, i.e. the plant response to the endogenous virus infection changed during the growth in transgenic plants. During the first weeks of growth the plants were resistant against TMV replication and symptom induction. This resistance reaction caused a significant stress to the plants, indicated by a stunted growth of the plants, but its functional mechanism could not be identified, as RNA silencing appeared not to play the major role in it.

Transgenic *Nicotiana tabaccum* and *N. benthamiana* lines expressing different viral silencing suppressors showed distinct phenotypes in the two tobacco species. The reactions of the silencing suppressor-expressing transgenic plants to five different virus infections showed that these suppressors affected the accumulation and spread of these homologous and heterologous viruses differently in two *Nicotiana* species. Phenotypes and viruses behaviour in these transgenic plants indicated that the silencing suppressors interfere in different ways with the silencing and that the interference is specific, at least to some extent, in these two *Nicotiana* species.

TABLE OF CONTENETS

1 INTRODUCTION

Different RNA molecules are known to carry out multiple functions in the molecular cell biology. mRNA molecules carry the translatable information from DNA to the translational machinary and rRNAs and tRNAs form essential components of this machinary. RNA also has essential role as component of some RNA-processing or DNA repair enzymes (e.g., Rnase P, snoRNAs and telomerases). In recent years, our knowledge of repertoire of RNA-mediated functions has been hugely increased, with the discovery of small non-coding RNAs which play a central part in a process called RNA silencing. RNA silencing, induced by double-stranded RNAs (dsRNA) and targeted to homologous RNA and DNA sequences, is a complex surveillance and regulatory process. It mediates the post-transcriptional repression of the target gene expression and represses the proliferation and expression of different invading nucleic acids, such as viruses, viroids, transposons or transgenes. Its functional components and mechanisms have been intensively studied in different organisms, such as *Caenorhabtitis elegans*, *Drosophila melanogaster*, and vertebrates including humans, *Neurospora* fungi and *Saccharomyces pombe* yeast and in plants, using *in vitro* assays and sequence comparisons (Bartel & Bartel, 2003; Jones-Rhoades & Bartel, 2004; Lewis *et al.,* 2003; Moss & Tang, 2003; Pasquinelli *et al.,* 2000; Rhoades *et al.,* 2002; Tang *et al.,* 2003). In animals this phenomenon has been named RNA interference (RNAi) (Fire *et al.,* 1998; Hammond *et al.,* 2000), in fungi it is called quelling, and in plants, co-suppression or post-transcriptional gene silencing (PTGS) (Catalanotto *et al.,* 2000; Cogoni & Macino, 1999; Fagard *et al.,* 2000; Fulci & Macino, 2007 and reference therein; Van der Krol *et al.,* 1990; Vaucheret *et al.,* 1998). Specifically, when induced by replicating viruses, the RNA silencing is called RNA-mediated defense (RMD), and when virus-vectors are used as tools to target silencing to an inserted sequence, the process is called virus-induced gene silencing (VIGS) (Baulcombe, 1999; Covey *et al.,* 1997; Lu *et al.,* 2003; Ratcliff *et al.,* 1999). The silencing research has been thoroughly reviewed in several recent articles (e.g. Aravin & Tuschl, 2005; Bisaro, 2006; Bonnet *et al.,* 2006; Brodersen & Voinnet, 2006; Dunoyer & Voinnet, 2005; Fulci & Macino, 2007; Huettel *et al.,* 2007; Li & Ding, 2006; MacDiarmid, 2005; Ruiz-Ferrer & Voinnet, 2007; Vaucheret, 2006; Voinnet, 2005 a & b; Xie & Guo, 2006).

RNA silencing phenomenon was first observed in petunia, when over expression of dihydroflavonol-4-reductase reduced the flower pigmentation and caused flower phenotypes. The phenomenon was called co-suppression (Van der Krol *et al.,* 1990) but its underlying mechanism was not known yet at that time. The short RNAs were first discovered by Lee *et al.* (1993) and their role in RNA silencing was first reported in *C. elegans*, where small regulatory RNAs *lin-4* and *let-7*, of about 22 nucleotides, were found to prevent translation of their homologous target mRNAs (Fire *et al.,* 1998). Soon thereafter small RNAs homologous to *let-7*, and also multiple other small RNAs of similar size and properties were found in different animal species (Lagos-Quintana *et al.,* 2002; Lau *et al.,* 2001; Lee & Ambros, 2001; Pasquinelli *et al.,* 2000), in fungi and yeast (Chicas *et al.,* 2004; Cogoni & Macino, 1999; Reinhart and Bartel, 2002), and in plants (Lleave *et al.,* 2002 a & b; Mette *et al.,* 2000; Park *et al.,* 2002; Reinhart *et al.,* 2002). These RNAs

were found to be involved in many central aspects of genetic regulation, being essential for controlled tissue development and differentiation (Brennecke *et al.,* 2003; Carrington & Ambros, 2003; Kasschau *et al.,* 2003; Park *et al.,* 2002; Pasquinelli and Ruvkun, 2002; Reinhart *et al.,* 2000). This group of small regulatory RNAs is called microRNAs (miRNAs).

The miRNAs are very similar with another class of small regulatory RNAs, the short interfering RNAs (siRNAs), which are induced in plants, as well as in other eukaryotes as a defense mechanism against invading nucleic acids (Hamilton & Baulcombe, 1999; Lleave *et al.,* 2002a; Voinnet, 2001). The siRNAs can be induced by over-expressed transgenes, transposons and viral pathogens, and suppress the expression of the corresponding RNAs, usually by sequence-specific cleavage of the target. They also induce methylation of homologous genomic DNA sequences, and heterochromatin formation (reviewed in Bartel & Bartel, 2003; Matzke *et al.,* 2004).

Silencing-related factors have been identified in various organisms using genetic analysis of different developmental mutants, by inducing silencing with endogenous and exogenous triggers, and by analyzing how silencing is related to virus resistance and transposon inactivation. The results indicate that the miRNA and siRNA mediated silencing pathways are closely related, but differ in terms of the production of the regulatory small RNAs. At least in plants, they also appear to be only partially overlapping in the sense that they are distinctly induced and regulated, apparently via separate alleles of the effector enzymes and accessory proteins (Dunoyer *et al.,* 2004; Lecellier & Voinnet, 2004; Xie *et al.,* 2004). However, the key components and mechanisms between the miRNA and siRNA mediated silencing processes are highly similar, indicating that they have evolved from the same predecessor pathways. The effector molecules and mechanisms of these pathways are also conserved between different classes of eukaryotic organisms, indicating that they are evolutionarily very old (Moss & Tang, 2003; Reinhart *et al.,* 2002; Zamore, 2002).

Specific steps and mechanisms of the silencing process are discussed below. A particular emphasis is given to the silencing processes occurring in plants, on their function as a defense system against viral pathogens, and on their interference with virus-encoded silencing suppressor counter defense mechanisms.

1.1. Components of RNA silencing machinery

1.1.1. The trigger

dsRNAs trigger the silencing system by inducing in eukaryotic cells a nuclease activity, which degrades homologous RNAs in regions of identity with the inducing dsRNA (Elbashir *et al.,* 2001b). Silencing can be effectively triggered also by single stranded (ss) RNAs, which are recognized by the cell as aberrant or abnormal, and converted to ds-form via cellular RNA-dependent RNA polymerase (RdRP) activity. It is not fully clear what features of an ssRNA are recognized as aberrant or defective, and thus target it for elimination by the cell. At least truncated, non-polyadenylated transcripts can induce RNA-silencing *in vivo* (Han & Grierson, 2002).

Also RNAs which are expressed in over-abundance *in vivo* may induce RNA-silencing, as was first recognized in connection with co-suppressed transgenes and homologous endogenes (Napoli *et al.,* 1990; Smith *et al.,* 1990; Van der Krol *et al.,* 1990). Using a wheat germ extract as an *in vitro* system, Tang *et al*. (2003) showed that also exogenous ssRNAs, when applied in high amounts are copied by endogenous RdRP into dsRNA form, and cleaved by the dicer enzyme. In these cases the initial binding interaction between RdRP and the RNA may be achieved through high abundance of the target RNA (Tang *et al.,* 2003).

Although the silencing process is sequence specific, it can also spread from the initial target site, along the RNA molecule either in the 5' direction, or both into 5' and 3' directions (Himber *et al.,* 2003; Vaistij *et al.,* 2002). This spreading is apparently mediated by synthesis of dsRNA from RNA templates, which have been truncated and made aberrant by the initial dicer cleavage.

1.1.2. The mediating factors: miRNAs and siRNAs

The small RNA fragments (21-30 nt long), homologous to the target RNA sequences, are the hallmark of activated silencing systems (reviewed in Bartel, 2004; Dugas and Bartel, 2004). The small endogenous miRNAs were first reported from animals by Lee *et al.,* (1993) and in plants, they were first discovered in 1999 (Hamilton and Baulcombe). Since then, various miRNAs and siRNAs, have been reported from number of plants, animals, yeast and fungi (Brodersen & Voinnet, 2006; Cogoni and Macino, 1999; Elbashir *et al.,* 2001 a & b; Li & Ding, 2006; Lleave *et al.,* 2002 a & b; Reinhart & Bartel, 2002; Vaucheret *et al.,* 2006). The small RNAs are called either miRNAs or siRNA, depending on their mode of synthesis. miRNAs are derived from local double-stranded hairpin structures, formed by specific precursor transcripts (Fig. 1). siRNAs, on the other hand, are derived from any dsRNA structures, produced from aberrant transcripts of transgenes, viroids, transposons or heterochoromatic DNA, or from double-stranded replication intermediates of RNA-viruses (Fig. 1). Consequently, the miRNAs are composed of one specific ssRNA fragment, derived from one arm of the precursor hairpin loop, while siRNAs are composed of a set of multiple RNA fragments, cleaved from both strands over the whole length of the inducing dsRNA. miRNAs are targeted to a specific target RNAs, while the siRNAs are targeted to any RNA sequences which are homologous to the initial inducing sequence (reviewed in Bartel & Bartel; 2003, Dugas & Bartel, 2004; Mallory & Vaucheret, 2004; Zamore, 2002).

Recently, some short RNAs other than classical si- and miRNAs have been found. e.g. natural antisence transcript siRNAs (nat-siRNAs) and trans-acting siRNAs (ta-siRNAs) (reviewed in Arvin & Tuschl, 2005; Brodersen & Voinnet, 2006; Li & Ding, 2006; Vaucheret *et al,* 2006). 24 nt nat-siRNAs are produced from dsRNAs formed by transcription of the complementary strand of the target gene (Borsani *et al.,* 2005), and the 21 nt ta-siRNAs are produced from non-coding, single stranded pri-tasiRNAs, similar to the miRNA precursors, via conversion to ds-form by RNA-dependent RNA polymerases (Allen *et al.,* 2005; Ronemus *et al.,* 2006; Xie *et al.,* 2005). Not much is known about these two newly found small regulatory RNAs yet except that both ta- and nat-siRNAs use the

same complex molecular machinery what is required for the silencing of other ssRNAs. These components of the silencing machinary are described in Fig. 1.

Interestingly, both ta-siRNA and miRNAs can guide degradation of homologous target sequences of different members of the same gene family. So far, ta-siRNA generating loci (TAS1-3) have been identified only in *Arabidopsis* (reviewed in Aravin & Tuschl, 2005; Brodersen & Voinnet, 2006; Vaucheret, 2006).

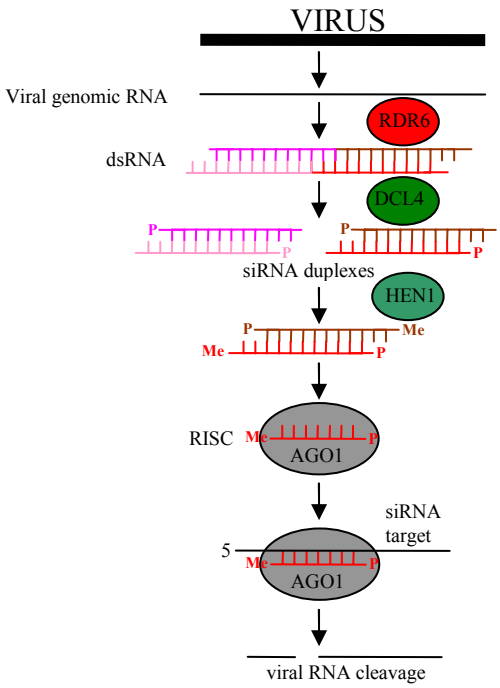
Due to their important role in development and antiviral defense miRNAs and siRNAs are discussed in details.

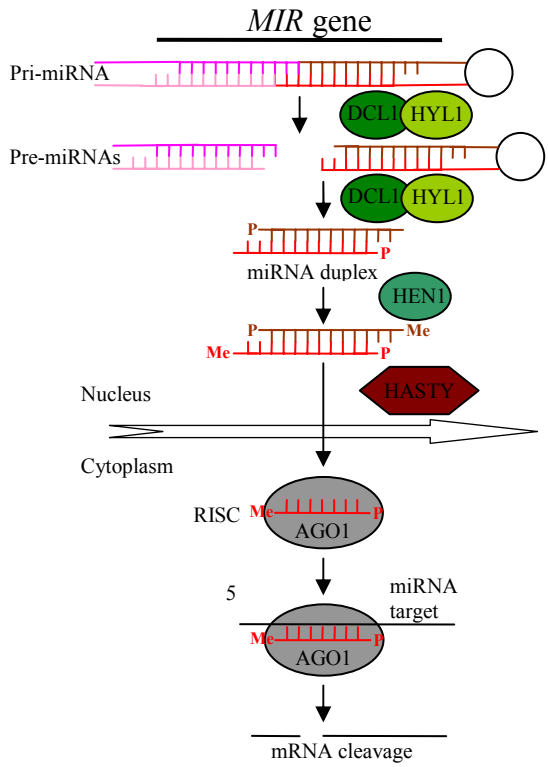
1.1.2.1. MicroRNAs (miRNAs)

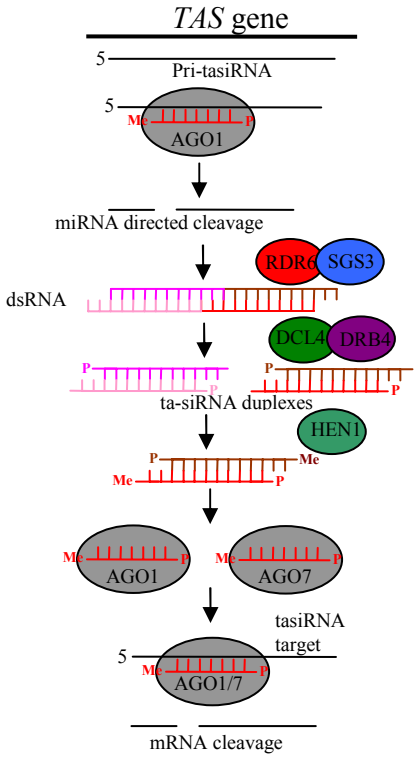
miRNAs mediate either the translational arrest or the cleavage of their target mRNAs, this mode of function is determined by their level of complementerity to the target sequence. Imperfect base pairing between the miRNA, either at the 3' non-translated region or in the coding region of the target mRNA leads to its translational arrest. Alternatively, full complementarity between the miRNA with its target leads to target cleavage by RISC, and subsequent degradation by other cellular nucleases. The former type of interaction occurs more commonly in silencing systems in animals, while the plant miRNAs are usually fully complementary, and like siRNAs, cleave their target RNAs (Bartel & Bartel, 2003; Carrington & Ambros, 2003; Doench *et al.,* 2003; Tang *et al.,* 2003). Several plant miRNAs cleave their target RNAs even with a few nucleotide mismatches in the recognition sites (Ambros, 2003; Kasschau *et al.,* 2003; Lleave *et al.,* 2002b; Palatnik *et al.,* 2003; Jones-Rhoades & Bartel, 2004; Tang *et al.,* 2003). Some plant miRNAs are also known to function through translational arrest (Aukerman & Sakai, 2003; Chen, 2004), and some animal miRNAs via the cleavage of the target mRNAs (Yekta *et al.,* 2004). Thus both the target-homologies, and the regulatory mechanism of animal and plant miRNAs are equally varied (Ambros, 2003; Bartel, 2004).

Many of the silencing-targeted mRNAs, code for transcription factors both in plants and animals. Developmental differentiation of organisms requires accurate temporal and spatial regulation of multiple gene functions, and it has been shown that the regulation of many of these genes happens via RNA silencing, mediated by specific miRNAs which can regulate multiple target genes simultaneously. Correlating with the regulatory role, many of the miRNAs themselves are expressed in specific tissues, and at specific stages of development (Alvarez *et al.,* 2006; Mallory *et al.,* 2002 & 2004; Palatnik *et al.,* 2003; Parizotto *et al.,* 2004; Reinhart *et al.,* 2000; Valoczi *et al.,* 2006). Some of the examples are summarized in Table 1.

Figure 1. Schematic illustration of different small RNA pathways in plants (adapted from Bonnet *et al.,* 2006; Brodersen & Voinnet, 2006; Jones-Rhoades *et al.,* 2006; Vaucheret, 2006).


VIRUS
Viral genomic RNA
RDR6
dsRNA
DCL4
siRNA duplexes
HEN1
RISC
AGO1
siRNA
target
5
AGO1
viral RNA cleavage


MIR gene
Pri-miRNA
DCL1
HYL1
Pre-miRNAs
DCL1
HYL1
miRNA duplex
HEN1
Nucleus
HASTY
Cytoplasm
RISC
AGO1
5
miRNA
target
AGO1
mRNA cleavage


TAS gene
5
Pri-tasiRNA
5
AGO1
miRNA directed cleavage
RDR6
SGS3
dsRNA
DCL4
DRB4
ta-siRNA duplexes
HEN1
AGO1
AGO7
tasiRNA
target
5
AGO1/7
mRNA cleavage

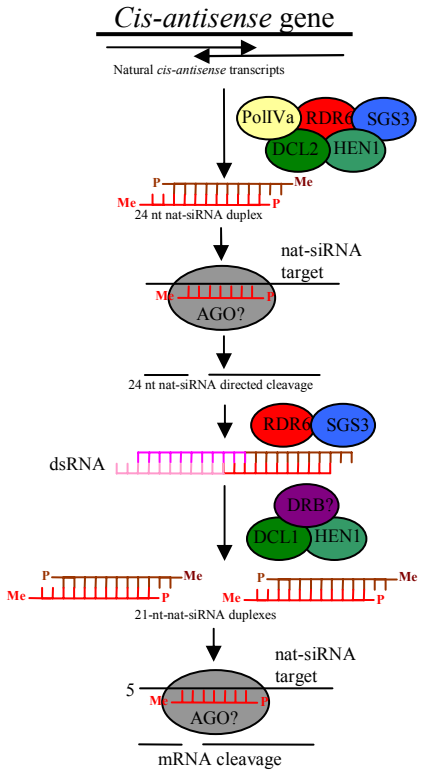

Cis-antisense gene
Natural *cis-antisense* transcripts
PolIVa
RDR6
SGS3
DCL2
HEN1
24 nt nat-siRNA duplex
nat-siRNA
target
AGO?
24 nt nat-siRNA directed cleavage
RDR6
SGS3
dsRNA
DRB?
DCL1
HEN1
21-nt-nat-siRNA duplexes
nat-siRNA
target
5
AGO?
mRNA cleavage

Table 1: Transcription factors and other targets of *A. thaliana*, *Oryza sativa* and *Populus trichocarpa* miRNAs (adapted from Jones-Rhoades *et al.*, 2006)

miRNA family	Target family	Targets
Transcription factors		
miR156	SBP	*SPL2, SPL3, SPL4, SPL10*
miR159/319	MYB	*MYB33, MYB65*
miR159/319	TCP	*TCP2, TCP3, TCP4, TCP10, TCP24*
miR160	ARF	*ARF10, ARF16, ARF17*
miR164	NAC	*CUC1, CUC2, NAC1, At5g07680, At5g61430*
miR166	HD-ZIPIII	*PHB, PHV, REV, ATHB-8, ATHB-15*
miR167	ARF	*ARF6, ARF8*
miR169	HAP2	*At1g17590, At1g72830, At1g54160, At3g05690, At5g06510*
miR171	SCL	*SCL6-III, SCL6-IV*
miR172	AP2	*AP2, TOE1, TOE2, TOE3*
miR393	bZIP	*At1g27340*
miR396	GRF	*GRL1, GRL2, GRL3, GRL7, GRL8, GRL9*
miR444	MADS	*Os02g49840*
Other targets		
miR161	PPR	*At1g06580*
miR162	Dicer	*DCL1*
miR163	SAMT	*At1g66690, At1g66700, At1g66720, At3g44860*
miR168	ARGONAUTE	*AGO1*
miR173	ta-siRNA	*TAS1a, TAS1b, TAS1c, TAS2*
miR390	ta-siRNA	*TAS3*
miR390	Receptor-like kinase	*Os02g10100*
miR393	F-box	*TIR1, ABF1, ABF2, ABF3, At3g23690*
miR394	F-box	*At1g27340*
miR395	APS	*APS1, APS4*
miR395	SO_2 transporter	*AST68*
miR396	Rhodenase	
miR397	Laccase	*At2g29130, At2g38080, At5g60020*
miR398	CSD	*CSD1, CSD2*
miR398	CytC oxidase	*At3g15640*
miR399	PO_4 transporter	
miR399	E2-UBC	*At2g33770*
miR403	ARGONAUTE	*AGO2*
miR408	Laccase	*At2g30210*
miR408	Plantacyanin	*Os03g15340*
miR436	Unknown	*Os12g42390*
miR447	2-PGK	*At5g60760*
miR475	PPR	4 PPR genes
miR476	PPR	1 PPR gene

Well-known mutants of these dominant regulatory genes are due to small nucleotide changes in the miRNA-target sites of their mRNAs, and not due to the altered protein functions (Dugas & Bartel, 2004; Emery *et al.,* 2003; Mallory & Vaucheret, 2004; Mallory *et al.,* 2004; McConnell *et al.,* 2001).

Localization of these miRNAs in actively dividing cells and young organs indicate their crucial role in cell differentiation (Valoczi *et al.,* 2006). A further evidence of the central role of miRNAs in the developmental differentiation comes from several developmental mutants, such as *ago1*, *hen1*, *caf1/dcl1-9* or *hyl1* in *Arabidopsis* (Bouche *et al.,* 2006; Chen *et al.,* 2002; Henderson *et al.,* 2006; Lu & Federoff, 2000; Schauer *et al.,* 2002; Vaucheret *et al.,* 2004). Each of these mutants is related to the gene functions required for the miRNA synthesis or function. e.g. *dcl1* mutants are associated with floral organ morphogenesis defects and altered ovule development, suggesting a critical role of the miRNAs in these developmental processes, also mutations in miRNA genes or miRNA target sequences themselves cause very clean development defects (Allen *et al.,* 2006; Kasschau et al., 2003; Reinhart *et al.,* 2002; Tang *et al.,* 2003).

Like mutants, artificially engineered plants expressing specific miRNAs can also cause developmental disturbances. Transgenic *A. thaliana* expressing miR167 and transgenic *N. benthamiana* expressing miR172, which regulates transcription factor APETALA2 (AP2) in *A. thaliana*, caused severe flower malformation and target transcripts (ARF-miR167, AP2-miR172) were degraded (Mlotshwa *et al.,* 2006; Ru *et al.,* 2006, reviewed in Jones-Rhoades *et al.,* 2006) confirming the central role of miRNAs in regulating different targets by cleaving or translational arrest.

RNA-silencing related developmental mutant phenotypes occur also *in C. elegans* and in other organisms (Bartel & Bartel, 2003; Fagard *et al.,* 2000; Grishok *et al.,* 2001; Zamore, 2002). The first short RNAs, found to be related to developmental disturbances in *C. elegans* were called *lin-4* and *let-7* (Lee *et al.,* 1993; Reinhart *et al.,* 2000). These short RNAs regulate, by translational arrest, the expression level of LIN-14 and LIN-28, the key regulatory factors of early larval developmental transitions (Bartel & Bartel, 2003; Olsen & Ambros, 1999; Pasquinelli *et al.,* 2000; Pasquinelli & Ruvkun, 2002). Homologs of *lin-4* and *let-7* occur also broadly in bilateral animals, regulating developmental transitions (Bartel & Bartel, 2003; Moss & Tang, 2003; Pasquinelli *et al.,* 2000). Other miRNA genes, e.g. *BANTAM* and *mir-14* locuses of *Drosophila*, and *mir-80* locus of *C. elegans* encode for miRNAs, which repress the activation of programmed cell death during early development (Brennecke *et al.,* 2003). These are just a few of the best-known examples of the miRNA genes now known in animals. So far, their number has been estimated to be at least 120 in *C. elegans* (Lim *et al.,* 2003 a & b), and, more recently, more than 1000 in humans (reviewd in Brodersen & Voinnet, 2006), indicating that they compose a major set of regulatory molecules in all organisms. A high portion of these genes is also conserved between different animal species (Bartel & Bartel, 2003; Reinhart *et al.,* 2002). Occurrence of homologous molecular machinery and RNA-silencing processes in plants, animals and fungi indicates that their functions originate from evolutionary stages predating the last common ancestors of plants and animals, and before the onset of multicellular life (Reinhart *et al.,* 2002).

In *Arabidopsis,* 22 distinct short RNAs, of 20-24 nts and with defining features of miRNAs were identified by initial cloning. Subsequent cloning, genetics and bioinformatic work has increased the tally. Now at least 118 potential miRNA genes, grouped in 42 families have been reported in *A. thaliana,* 116 in rice (*Oryza sativa*) and 169 in cotton wood (*Populus trichocarpa*) (reviewed in Jones-Rhoades *et al.,* 2006) and many of them have been verified via *in vitro* or *in vivo* miRNA-guided cleavage assays (Dugas & Bartel, 2004; Kasschau *et al.,* 2003; Lleave *et al.,* 2002 b; Rhoades *et al.,* 2002; Tang *et al.,* 2003; Xie *et al.,* 2003) (Bartel & Bartel, 2003; Dugas & Bartel, 2004; Jones-Rhoades & Bartel, 2004). Out of these, 92 genes, belonging to 21 families have been found conserved in *Arabidopsis*, rice and cotton wood and also their target sites are conserved in all three species (reviewed in Jones-Rhoades *et al.,* 2006). Many of the identified *Arabidopsis* miRNAs are encoded by multiple (2-7) loci in the genome. Many of the miRNA sequences, and their target sites are highly conserved also between other plants species (Alvarez *et al.,* 2006): for instance the miRNA165/166, and its target site in the class-III HD-ZIP transcription factor mRNA is conserved not only in *Arabidopsis*, maize and rice, but also in ferns and bryophytes, which have evolved separately for at least 400 million years (Floyd & Bowman, 2004). In spite of the conserved miRNA genes, the synthesis and degradation processes of miRNAs can be different in different species (Billoud *et al.,* 2005).

The identified target gene families code for 117 (*Arabidopsis*), 178 (rice) and 216 (cotton wood) unique mRNAs, about two-thirds of these (65 from *Arabidopsis*, 66 from rice and 93 from cotton wood) (reviewed in Vaucheret, 2006) code for known or putative transcription factors which regulate developmental differentiation. Some of the miRNAs also target the components of the RNA-silencing pathway, such as DCL1 and AGO1 (see below), indicating that the silencing process it self is targeted by miRNA-mediated feed-back regulation (Bartel & Bartel, 2003; Brodersen & Voinnet, 2006; Jones-Rhoades *et al.,* 2006; Park *et al.,* 2002; Reinhart *et al.,* 2002; Vaucheret *et al.,* 2004; Xie *et al.,* 2003).

1.1.2.2. Short interfering RNAs (siRNAs)

siRNAs differ from miRNAs in the way that they arise from different double-stranded RNAs. They may be cleaved from dsRNAs of external origin, such as replicating viral RNAs, or synthetic dsRNAs or from dsRNA synthesized from aberrant cellular transcripts by RdRP activity (Han & Grierson, 2002; Reinhart *et al.,* 2002). As mentioned earlier, these ssRNA targets include highly expressed transgenes and transposons. Clearly, siRNA production is a defense reaction against different invading nucleic acids (reviewed in Plasterk, 2002; Waterhouse *et al.,* 2001 a; Zamore, 2002).

siRNAs in plants fall into two distinct size classes, i.e. sizes of 21-22, and 24-25 nt (Hamilton *et al.,* 2002; Mallory *et al.,* 2002; Mette *et al.,* 2000; Tang *et al.,* 2003). The smaller siRNAs function in silencing of the target mRNAs by sequence specific cleavage, as described above. The longer siRNAs (24-26 nts) appear to mediate different silencing-related functions, e.g. in histone and DNA methylation to induce transcriptional silencing, and systemic spread of the silencing status throughout the plant (Aufsatz *et al.,* 2002; Hamilton *et al.,* 2002; Mette *et al.,* 2000). Recently, also some 30 nt long RNAs have been

detected, but their role and function in the silencing process is not known (Baulcombe, 2005; Meister & Tuschl, 2004).

The different sizes of siRNAs are apparently produced by different dicer (DCL2 and DCL3) alleles, which can be separately induced in different condition (Hamilton *et al.,* 2002; Tang *et al.,* 2003). The dicers are mostly cytoplasmic enzymes, but at least one of the *Arabidopsis*-encoded dicers functions in the nucleus (Papp *et al.,* 2003). The siRNAs produced in the nucleus could easily be engaged in epigenic RNA-DNA interactions, e.g. in methylation of transposons or heterochromatin DNAs, to repress their transcription (Matzke *et al.,* 2004).

Along with dicers, RNA RDR1 (RNA dependent RNA polymerase, now abbreviated as RDR), RDR2, and RDR6 proteins are also involved in siRNA biogenesis (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000; Peragine *et al.,* 2004; Xie *et al.,* 2004; Yu *et al.,* 2003). For example, DCL3 and RDR2 generate 24 nt siRNAs from retroelements, transposons and from direct and inverted repeats in the heterochromatin (Chan *et al.,* 2004; Xie *et al.,* 2004; Zilberman *et al.,* 2003). RDR6 is involved in posttranscriptional gene silencing (PTGS) of transgenes, as well as in silencing of some viral RNAs and some endogenous mRNAs that are targets of ta-siRNAs (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000; Parizotto *et al.,* 2004; Peragine *et al.,* 2004; Yu *et al.,* 2003).

The silencing process, i.e. degradation of the target RNAs, can be effectively amplified by a chain reaction, where the primary siRNAs, produced from the initial inducer dsRNA, are used to cleave the target mRNAs, and/or to prime mRNAs for copying by RdRP. The produced secondary siRNAs and dsRNA templates are used to continue the process (and spread the silencing status to adjacent cells), as long as suitable templates are available (Tang *et al.,* 2003; Vaistij *et al.,* 2002; Voinnet *et al.,* 1998). This amplification process is described in more detail later.

1.1.3. Enzymatic machinery for production of the mi- and siRNAs

RNA silencing process involves several different pathways and target molecules, and each of these is mediated by specific enzyme catalysis. The main enzymatic steps in silencing process are the conversion of ssRNAs into ds form by RdRP, the cleavage of the dsRNAs by the dicer or dicer-like complexes, degradation of the target RNAs by the RISC, RNA-directed DNA-methylation by RdDM complexes and different histone-modifying and chromatin remodeling activities. According to various mutant analyses, also several accessory proteins are required in reactions mediated by the RNA silencing (Matzke *et al.,* 2004).

1.1.3.1. RNA dependent RNA polymerase (RDR)

As silencing is triggered by dsRNA, conversion of ssRNA sequences to ds form is required for silencing of endogenous transcripts. Total of six RDR-like genes have been identified in *Arabidopsis* (Dalmay *et al.,* 2000). In *Arabidopsis* this RDR function is mediated by proteins encoded by the SILENCING DEFECTIVE1 (SDE1)/ SUPPRESSOR OF GENE

SILENCING2 (SGS2) RDR genes, and the SDE3 RNA helicase gene (Dalmay *et al.,* 2000 & 2001; Mourrain *et al.,* 2000; Tang *et al.,* 2003). According to more recent nomenclature, SDE1/SGS2 is referred as RDR6 (Xie *et al.,* 2004). These gene functions are not needed for virus-induced PTGS (Dalmay *et al.,* 2000; Voinnet, 2001), or for PTGS of transgenes that are transcribed into sense-antisense RNAs, forming dsRNA structures (Beclin *et al.,* 2002; Waterhouse *et al.,* 2001b). RDR activity is neither needed for miRNA biogenesis and function. Accordingly, *SGS2* mutants have no developmental abnormalities (Mourrain *et al.,* 2000; Tang *et al.,* 2003).

Although virus-induced RNA-silencing does not require host-specific RDR activity, a host-encoded and pathogenesis-inducible RDR, identified as NtRdRP1 in tobacco (Xie *et al.,* 2001), and as *AtRdRP1* in *Arabidopsis* (Yu *et al.,* 2003), are known to enhance the silencing-based defense reaction against different RNA viruses. This suggests that the dsRNA accumulation may be the rate-limiting step in the silencing-based defense reaction. Recently, an ortholog of *A. thaliana* RDR6 (*AtRDR6*) in *N. benthamiana* is reported and referred as *NbRDR6*. *NbRDR6* is a key component in initiation of silencing in cells after perceiving the systemic signal and may enhance the signal. However, *NbRDR6* only contributes to siRNA production if infection is not fully established and its activity in antiviral defense is temperature sensitive. In contrast, RDR6 is not required for production of systemic silencing signal in *A. thaliana* indicating that silencing machinery may vary in different species and may also depends on virus-host combination (Qu *et al.,* 2005; Schwach *et al.,* 2005).

One of the silencing pathways leading to DNA methylation is mediated by RNA Polymerase IV (Pol IV), RDR2 and DCL3. In this pathway Pol IV and RDR2 would synthesize dsRNA which is then be cleaved by DCL3 to generate siRNA. In one other pathway, Pol IV acts together with AGO4 and one of the RDR to produce specific siRNAs. These two silencing pathways are independent in the leaves but interdependent in the flowers (Herr *et al.,* 2005).

Synthesis of other small RNAs requires activity of specific RDRs. At least, ta-siRNAs production also depends on RDR6/SGS3 proteins (Allen *et al.,* 2005).

1.1.3.2. Dicer

Dicer is the key enzyme initiating the RNA-silencing process: it is a dsRNA specific Ribonuclease III-like endonuclease which cleaves the target dsRNAs into fragments of 21-24 nts, leaving 3'-hydroxyl and 5'-phosphate ends, and 2 nt 3' overhangs at the termini of the duplex (Bernstein *et al.,* 2001; Lau *et al.,* 2001). The dicer appears to interact with the RISC complex, and after cleavage, the dsRNA fragments disassociate from dicer and become associated with the RISC. Only one strand of the miRNA fragments (the strand complementary to the target sequence), but either strand of the short lived ds-siRNAs are incorporated into the RISC, where these RNAs function as sequence specific tags and target the silencing function to the homologous RNAs (Dunoyer *et al.,* 2004).

Arabidopsis and rice genomes encode at least for four different dicer-like proteins (DCL1 - DCL4) (Schauer *et al.,* 2002). Different dicer-orthologs are involved in the production of distinct short 21-24 nt siRNAs, and in different cellular localisation (cytoplasmic vs. nuclear). (Finnegan *et al.,* 2003; Lee & Ambros, 2001; Tang *et al.,* 2003). DCL1 produces miRNAs (Bartel, 2004; Chen, 2005), DCL3 produces 24 nt long siRNAs involved in DNA methylation and in heterochromatin formation (Xie *et al.,* 2004) and DCL2 cleaves natural antisense transcripts into siRNAs (Deleris *et al.,* 2006). DCL4 generates siRNAs of 21 nt which mediate the viral RNA silencing (Gasciolli *et al.,* 2005; Xie *et al.,* 2005, Dunoyer *et al.,* 2005; Herr, 2005; Qi & Hannon, 2005). If DCL4 is not functional, then DCL2 and DCL3 produce 22 nt and 24 nt long siRNAs, respectively, from viral sequences, but only siRNAs produced either by DCL4 or by DCL2 can mediate antiviral silencing. These dicers can restrict the virus accumulation into inoculated leaves and DCL4 produces the silencing signal which can inhibit the virus spread (Bouche *et al.,* 2006; Deleris *et al.,* 2006; Dunoyer *et al.,* 2007; Fusaro *et al.,* 2006).

The essential role of the dicer enzymes and of the RNA-silencing pathway in the developmental regulation is indicated by the strong morphological disturbances caused by various dicer mutants: well characterized mutants of the DCL1 (*caf /dcl1, sus1 and sin1*) produce a strongly malformed flower phenotype with proliferation of inner floral organs, indicating that the *DCL1* gene is involved in the regulation of organ development and in meristem cell identity and differentiation (Jacobsen *et al.,* 1999; Kasschau *et al.,* 2003; Park *et al.,* 2002; Reinhart *et al.,* 2002; Schauer *et al.,* 2002). These data indicate that the other *DCL* genes (*DCL2-4*) do not compensate for the function of *DCL1*, but function in separate dsRNA cleavage pathways. For instance, the PTGS pathway is operating normally in the *caf /dcl1-9* mutant plants, indicating that this function guided by siRNA is not mediated by the DCL1 enzyme (Finnegan *et al.,* 2003). Still DCL1 may help DCL4 and DCL3 to produce siRNAs from inverted repeats involving it in the other RNA silencing pathways (Dunoyer *et al.,* 2007).

DCL activity in the degradation of dsRNA is dependent on the amount of different inducer sequence. DCL4 is the main producer of siRNAs from viral sequences but beyond a certain accumulation threshold of dsRNA, any DCL (1-3) can substitute for DCL4 activity and slice the dsRNA with the help of AGO1 (Dunoyer et al., 2007). Diaz-Pendon et al. (2007) showed recently that DCL3 can also act upstream of DCL4 to enhance antiviral silencing. Exploring further the functions and involvement of different DCLs in the silencing pathways, Moissiard & Voinnet, (2006) showed that like RNA viruses, DNA viruses also induce DCL-dependent production of siRNAs in the silencing pathway. Both caulimoviral and geminiviral transcripts are processed by the combined effects of DCL2, -3 and -4 producing 22-, 24-, and 21-nt short RNAs, respectively. In these cases, DCL1 does not produce any siRNAs but does facilitate other three DCLs in synthesizing siRNAs. Thus, DCL3 acts differently on RNA and DNA viruses as in RNA virus infections, where DCL3 has no antiviral effects (Deleris *et al.,* 2006) but in the case with DNA viruses, the 24 nt siRNA production by DCL3 exert antiviral effects (Akbergenov *et al.,* 2006; Moissiard & Voinnet, 2006).

1.1.3.3. Effector complexes

RNA-induced effector complexes include RNA-induced transcriptional silencing complex (RITS) guiding methylation of chromatin, siRNA- and miRNA-dependent RNA-induced-silencing complex (siRISC and miRISC, collectively called only RISC) causing cleavage of homologous sequences and translational arrest (reviewed in Li & Ding, 2006). RNA-induced silencing complexes are multi-subunit, large assemblies of 250-500 kDa, associated with the ss fragments of the si- or miRNA (Nykänen *et al.,* 2001; Omarov *et al.,* 2007). They contain an ARGONAUTE (AGO) protein, which in the case of RITS is AGO4 and in the case of RISC is AGO1. AGO1 is a homolog of the translation initiation factor eIF2C (Hall, 2005; Hammond *et al.,* 2000; Song & Joshua-Tor, 2006).

1.1.3.4. **ARGONAUTE** *proteins*

ARGONAUTE proteins are coded by multigene families, which comprise ten members in *Arabidopsis*, four in *Drosophila*, three in *C. elegans*, seven in humans and eight in mouse (Carmell *et al.,* 2002). Based on the analysis of numerous silencing-defective and developmental mutants, ARGONAUTE-like proteins are known to be required for various silencing-related processes. ARGONAUTES are about 100 kDa, highly basic proteins comprising PAZ and PIWI domains. The PAZ domain, which occurs also in the Dicer enzymes, may mediate protein-protein interactions, and facilitate binding with the Dicer complex. PAZ and PIWI domains are responsible for 3' 2 nt overhang recognition and endonucleolytic activities, respectively (Hall, 2005; Hammond *et al.,* 2000; Song & Joshua-Tor, 2006). Due to their basic character the ARGONAUTE proteins bind RNAs (e.g. siRNAs), and guide them to functional complexes (reviewed in Bartel & Bartel, 2003; Dugas & Bartel, 2004; Lecellier & Voinnet, 2004; Matzke *et al.,* 2004; Tang *et al.,* 2003; Vaucheret *et al.,* 2004). It is now well known that AGO1, which has slicer activity, is essential component of RISCs and cleaves the target mRNAs which are homologous to the miRNA or siRNA sequences in the complex (Baumberger & Baulcombe, 2005; Qi *et al.,* 2005; Ronemus *et al.,* 2006; Vaucheret *et al.,* 2004) and AGO4 is essential for DNA and histone methylation in *Arabidopsis* (Fagard *et al.,* 2000; Irvine *et al.,* 2006; Morel *et al.,* 2002; Zilberman *et al.,* 2003). ARGONAUTE protein PINHEAD/ZWILLE has a role in maintenance of undifferentiated stem cells in the shoot apical meristem, but is not required for PTGS (Morel *et al.,* 2002). Also in other species, specific argonaute proteins are required for RNA-silencing, for transcriptional gene silencing via methylation, and for cellular differentiation and in virus resistance; e.g. in humans, although hAGO3 (full) and hAGO1 (partial) have catalytic components but only hAGO2 posses the cleavage activity (Matzke *et al.,* 2004; Morel *et al.,* 2002, reviewed in Parker & Barford, 2006).

1.1.3.5. Other required proteins

HEN1, a dsRNA methylase (Boutet *et al.,* 2003; Park *et al.,* 2002; Xie *et al.,* 2004), is required for miRNA accumulation in *Arabidopsis* and for the methylation of miRNA duplexes (Yu *et al.,* 2005). HEN1 can also methylate the 3'-OH of siRNAs with less efficiency when they are in duplexes with 2 nt overhang, and preferably of 23 nt. HEN1 is also involved in siRNAs production, at least in the case of begomovirus silencing (Akbergenov *et al.,* 2006; Yang *et al.,* 2006).

HYL1, a dsRNA binding protein, affects miRNA, but not siRNA accumulation (Anantharaman *et al.,* 2002; Han *et al.,* 2004; Vazquez *et al.,* 2004; Wu *et al.,* 2007). HYL1 function is also required for hormonal (auxin, ABA, and cytokinin) responces (Lu & Federoff, 2000). *hyl1* and *hen1* single mutants are fertile, but double mutants are infertile, suggesting that they function synergistically (Boutet *et al.,* 2003).

Mutations in genes coding for HEN1, HYL1 and the proteins of the ARGONAUTE family cause developmental defects very similar to those caused by the *dcl1* mutants, i.e. delay in the switch to floral development and over-proliferation of the floral meristem, disrupted shoot meristem formation, and leaf deformation (Park *et al.,* 2002; Vaucheret *et al.,* 2004; Vazquez *et al.,* 2004). These phenotypes appear to be related to miRNA processing defects: processing of AGO1 mRNA by miR168 is disturbed in *ago1* mutants (Vaucheret *et al.,* 2004). Mutants *dcl1, hen1 and hyl1* also lead to reduction of different miRNAs, and/or to increase of their target mRNAs (Grishok *et al.,* 2001; Kasschau *et al.,* 2003; Reinhart *et al.,* 2000; Vaucheret *et al.,* 2004; Vazquez *et al.,* 2004; Xie *et al.,* 2004).

It is possible that other cellular proteins are also affecting the silencing processes. For instance enhanced pectin methylesterase (PME) can enhance the degradation of the viral RNAs and the RNA silencing mechanism (Dorokhov *et al.,* 2006).

Still some other proteins, e.g. dsRNA binding proteins (Kurihara *et al.,* 2006; Han *et al.,* 2004; Vazquez *et al.,* 2004), SDE3 (Dalmay *et al.,* 2001), and SGS3 (Mourrain *et al.,* 2000) may operate with different DCL proteins in different RNA silencing pathways (Herr *et al.,* 2006).

1.2. Other activities associated with silencing

1.2.1. Methylation

RNA silencing is often associated with sequence-specific methylation of homologous DNA sequences, caused via interaction between DNA and inducing dsRNA. The *de novo* initiation of DNA methylation by the traditional DNA-methyltransferase (DMTases, DRM1 and DRM2 in plants) are cued, or triggered, by the small RNAs, and termed RNA-directed DNA methylation (RdDM) (Aufsatz *et al.,* 2002; Mette *et al.,* 2000; Wassenegger *et al.,* 1994). In plants, the DNA-methylation is initiated by production of 24 nt siRNAs by DCL3 and may or may not lead to methylation, depending on the pre-methylation status of the siRNA-producing locus or on some other unknown factors (Chan *et al.,* 2006). RdDM is strictly sequence specific: if the silencing is triggered by mRNA coding sequence, the methylation is directed to the corresponding DNA sequence, and if it is triggered by a dsRNA transcripts corresponding to a promoter sequence, methylation is directed to the homologous promoter and leads to transcriptional silencing (Jones *et al.,* 1999; Matzke *et al.,* 2004; Sijen *et al.,* 2001b). RNA silencing and DNA methylation pathways co-exist in the same species, i.e. in plants, vertebrates, protozoa, *Drosophila, Neurospora* fungi and in *S. pombe*, while *S. cerevisiae* lacks the machineries both for the RNAi and for DNA methylation (reviewed in Matzke *et al.,* 2004).

Also chromatin structure modifications and establishment of heterochromatin are mediated via RNA-silencing related pathways. At least in *S. pombe* yeast, the Dicer, AGO and RDR proteins, and short RNAs (siRNAs) are needed in conjunction with histone methyltransferase Clr4 to establish heterochromatin at centromeres, and at and around regions of retrotransposons with long terminal repeats (LTRs) (Hall *et al.,* 2003; Schramke & Allshire, 2003; Sijen *et al.,* 2001a). AGO4 is also required in *Arabidopsis* for accumulation of heterochromatic siRNAs (Zilberman *et al.,* 2003), and distinct miRNAs and endogenous siRNAs appear to function in heterochromatin formation in *Arabidopsis*, *C. elegans* and *Drosophila* (reviewed in Matzke *et al.,* 2004; Zamore, 2002). Differing from the DNA methylation, the histone methylation and heterochromatin formation is not confined to the locations which are homologous to the inducing RNA, but can spread several kilobases from the initial target site. These RNAi-mediated heterochromatin assembly mechanisms may, or may not be related with DNA-methylation activity. It appears to be derived from ancient defense mechanism against retrotransposons, but has now a general role in gene silencing, chromosome structure and segregation (Chicas *et al.,* 2004; Matzke *et al.,* 2004; Schramke & Allshire, 2003). The mechanism still functions also in the repression of the transposons, since loss of RNAi function leads to activation of multiple transposable elements (Novina & Sharp, 2004; Plasterk, 2002).

Three nuclear proteins, RDR2, DCL3 and AGO4 are known to be key palyers in RdDM. RDR2 produces dsRNA from ss templates, DCL3, which cleaves dsRNA synthesized by RDR2 into 24 nt (heterochromatin) hc-siRNAs. AGO4 can either, induce RdDM by cleaving trarget RNA and thus producing small RNAs or by engaging methylation machinary, independent of its catalytic activity. Subsequent work has described that some other plant specific proteined are essential for RdDM. e.g. DRD (*defective in RNA-directed DNA* methylation) proteins. DRD1, which is a plant specific chromatin remodeling protein, while DRD2 and DRD3, which are subunits of plant specific RNA polymerase Pol IV, mediates RdDM (reviewed in Huettel *et al.,* 2007). Also AGO4 can affect RdDM in two different ways. In addition to its role in the RdDM activity, it may mediate methylation also by producing longer siRNAs which are then converted into dsRNA by RDR2 and processed into siRNAs by DCL3 (Qi *et al.,* 2006). In *Arabidopsis*, HEN1 causes the methylation of siRNA at the 2' or the 3' OH group of ribose so it seems that methylation may be a general modification of siRNAs in *Arabidopsis* (Akbergenov *et al.,* 2006).

While in plants, RdDM is regulated by the RNAi pathway, in mammalian cells the assembly and maintenance of the transgene induced heterochromatic assembly and maintenance can be RNAi independent (Wang *et al.,* 2006).

1.2.2. Amplification, and local and systemic spread of silencing

Once silencing is induced, it can be relay-amplified by synthesis of (multiple rounds of) dsRNAs on the primary cleavage product templates, and by their cleavage into secondary siRNAs. This amplification leads to the transitory nature of the silencing reaction. Transitory nature means that the reaction may spread along the mRNA, although it is initiated by a locally targeted single siRNA (Klahre *et al.,* 2002; Sijen *et al.,* 2001a). In *C. elegans* the reaction spreads only into the 5' direction from the initial cleavage site, while

in plants it spreads into both the 5' and into the 3' direction (Tang *et al.,* 2003; Vaistij *et al.,* 2002; Voinnet *et al.,* 1998). The bi-directional transition may be explained by a process where both the 5' and 3' cleavage products of the initial target RNA act as aberrant mRNAs to trigger dsRNA synthesis (Herr *et al.,* 2006), and induce secondary silencing reactions. The transitory spread appears to apply only to silencing process induced by exogenous dsRNA or by transgenes, but not to miRNA-mediated silencing - maybe because the miRNA target RNAs are not adequately abundant to be copied by RDR into dsRNA forms (Tang *et al.,* 2003). The transitory silencing is neither induced against endogenous mRNAs, such as mRNAs of small subunit of Rubisco (RBS) or phytoene desaturase (PDS), indicating that these endogenous mRNAs are not targets for RDR (Himber *et al.,* 2003; Vaistij *et al.,* 2002). Endogenes are also not targets for RNA-directed DNA methylation, which appears to be related to the amplification, and to transitory RNA-silencing (Jones *et al.,* 1999). The silencing process is also enhanced by the enzymatic activity of the RISC complex, mediating multiple turnover reactions (Hutvagner & Zamore, 2002; Tang *et al.,* 2003).

Furthermore, production of the secondary siRNAs leads to enhancement of silencing via its spread from the first activated cell to neighboring cells, and systemically through the organism (Himber *et al.,* 2003; Palauqui *et al.,* 1997). The cell-to-cell spread can be mediated as passive spread of the small RNAs via plasmodesmata or by the silencing signal complex which is between 27 and 54 kDa (Himber *et al.,* 2003; Kobayashi & Zambryski, 2007; Voinnet *et al.,* 1998 & 2005 b). It can also involve relay-amplification of the RNA cleavage in each subsequent cell; the amplification is initiated by the 21 nt siRNAs, and requires the presence of a target transcript, SDE/SGS (RDR6) function and the activation of transitory silencing (Dunoyer *et al.,* 2005; Himber *et al.,* 2003; Voinnet *et al.,* 1998, 2000 & 2005 b).

The systemic spread in phloem is mediated by the 24 nt siRNAs (Hamilton *et al.,* 2002; Himber *et al.,* 2003), unloading of the systemic signal appears to be mediated via plasmodesmata, since it does not spread into meristematic cells (Voinnet *et al.,* 1998 & 2005 b). The discovery of RNA-binding protein (PSRP1) in the phloem and its ability to bind 25 nt ssRNA species add further to the argument that siRNAs (24-26 nt) are the key components for systemic silencing signal. Exclusion of meristems from virus infection may also be due to systemic silencing signal, spreading ahead of the virus infection (reviewed in Xie & Guo, 2006).

Also miRNA-mediated mRNA processing can spread locally and systemically in the plant, indicating that also some miRNAs can be mobile regulatory signals (Juarez *et al.,* 2004). The extent of cell-to-cell movement is dependent on the levels of siRNAs produced at the site of silencing initiation, but is independent of the presence of siRNA target transcripts in either source or recipient cells (Himber *et al.,* 2003, reviewed in Li & Ding, 2006; Voinnet, 2005b).

1.3. RNA-silencing as a defense mechanism against plant RNA viruses

1.3.1. Silencing defense

One of the main biological roles of RNA-silencing in plants is now known as the viral defense mechanism (Covey *et al.,* 1997; Vance & Vaucheret, 2001; Voinnet, 2001; Voinnet, 2005 a; Waterhouse *et al.,* 2001 b). Recovery of plants from initial viral symptoms and cross-protection between related viruses are manifestations of inducible, RNA-silencing mediated plant defense reactions against viruses (Ratcliff *et al.,* 1999). In these reactions the dsRNA replicative intermediates, or internal dsRNA structures of viral genomic RNAs (Voinnet *et al.,* 2005 a) of RNA viruses are the inducers, and their ss genomic RNAs are the targets of the silencing function. RNA-silencing works as a viral defense also in insects (Keene *et al.,* 2004; Li *et al.,* 2002). In plants, antiviral defense is guided prefereably by RISC mediated cleavage of the viral target RNA than by inhibiting its translation (Pantaleo *et al.,* 2007).

Benefits of the RNA-silencing based defense system are, for instance, its versatility and flexibility. The defense-specificity is not pre-formed, but is determined by the invading RNA sequence, and therefore the defense can be directed against any invading virus. In this sense the system resembles the immune system of vertebrates (Voinnet, 2001). Another benefit of the silencing system is that it is effectively amplified by the very presence of it's trigger, i.e. the replicative RNAs, as described above. It is also transmitted from its initial induction site to the neighboring cells, and systemically within the whole plant. This pre-conditioning of remote tissues may significantly enhance the plants' defense-potential against the spreading viral infection.

The mere fact that viruses can effectively infect their specific host plants indicates that they can successfully combat or counteract the host defenses. Viruses have various means to protect their genomes against the silencing process: they often replicate inside of membranous vesicles, or in proteinaceous inclusions, which exclude the intracellular enzymes. Viruses with dsRNA genomes sequester their entire dsRNA stage inside of capsid particles, and thus avoid the exposure of the dsRNA (the inducing agent) to the cytoplasmic surveillance system. Moreover, all viruses encapsidate their ssRNA genomes into ribonucleoprotein, and usually capsid structures to protect them from degradation (Ahlquist, 2002, reviewed in Li & Ding, 2006; Voinnet *et al.,* 2005 a). Some plant viruses can escape the silencing defense for instance by mutation of the target sequences, making them unavailable for suitable siRNA pairing and subsequent cleaving. However, most viruses combat silencing by coding for efficient silencing suppressors that inhibit silencing activity (Simon-Mateo & Garcia, 2006).

1.3.2. Silencing suppressors

Many plant viruses, and also some insect viruses (Li *et al.,* 2002) have developed specific counter-defense system, mediated by viral-encoded silencing suppressors. Silencing suppressor factors have been identified for numerous viruses and are now assumed to be produced by most, while maybe not by all viruses. The silencing suppressors known so far

are listed in Table 2. The suppression of the host's defense may be very critical in determining whether a given virus can multiply and spread successfully in a given host. The importance of this function is reflected by the fact that many of the viral silencing-suppressor proteins have been previously identified as pathogenicity factors, and as essential for infectivity in indicated hosts (Brigneti *et al.,* 1998; Voinnet *et al.,* 1999).

Several (but not all) of the identified suppressor proteins have also been identified as viral cell-to-cell or long distance movement proteins. One characteristic feature of these proteins is that they are able to move independently, either together or ahead of the spreading viral infection, to the recipient cells. This function may thus be essential for reducing the hosts' induced (silencing) defense against viral spread (Dunoyer *et al.,* 2004). Viral silencing suppressors also show efficient adaptation and specificity to their susceptible host species, i.e. closely related viruses may show different silencing suppression activity in different hosts (Voinnet, 2001; Voinnet *et al.,* 1999). This indicates specific interactions of the suppressor molecules with their targets, and strong selective co-evolution between the virus and it´s host.

A surprising feature of silencing suppressor proteins, encoded by unrelated RNA and DNA viruses, is that they bear no similarity to each other either in coding sequence or protein structure, indicating multiple and separate origins. Different suppressor proteins also show very varied functional mechanisms, and they interfere with different steps of the silencing process. They can, for instance, inhibit the initiation or the maintenance stage, or the systemic spread of the silencing status, and they may, or may not interfere with the miRNA-mediated silencing pathway or the silencing-associated DNA methylation. Due to these various modes of action the viral silencing suppressors have turned out to be very useful tools to elucidate the different steps of the silencing process (Dunoyer *et al.,* 2004; Lecellier & Voinnet, 2004; Roth *et al.,* 2004).

The best characterized silencing suppressor proteins are the HcPro proteins of potyviruses, 2b proteins of cucumoviruses, P19 of tombusviruses, and P25 of potexviruses (Roth *et al.,* 2004). Some of their functional features are listed in Table 2.

Table 2: Plant viral suppressors of RNA silencing

Genus	Virus	Suppressor and its other function(s)	Reference
Aureusvirus	*Pothos latent virus* (PoLV)	P14 (dsRNA binding)	Merai *et al.,* 2005
Carmovirus	*Turnip crinkle virus* (TCV)	P38 (Coat Protein)	Dunoyer *et al.,* 2004; Qu *et al.,* 2003; Thomas *et al.,* 2003
	Hibiscus chlorotic ringspot virus (HCRSV)	P38 (Coat Protein, symptom determinant)	Meng *et al.,* 2006
Closterovirus	*Beet yellows virus* (BYV)	P21 (Replication enhancer, dsRNA binding)	Reed *et al.,* 2003
	Beet yellow stunt virus (BYSV)	P22	
	Citrus tristeza virus (CTV)	P20 (Replication enhancer) P23 (Nucleic acid binding) Coat Protein	Lu *et al.,* 2004
Comovirus	*Cowpea mosaic virus* (CPMV)	S protein (Small coat protein)	Liu *et al.,* 2004
Crinivirus	*Sweet potato chlorotic stunt virus* (SPCSV)	P22 RNaseIII	Kreuze *et al.,* 2005
Cucumovirus	*Cucumber mosaic virus* (CMV)	2b (Movement Protein, dsRNA binding)	Brigneti *et al.,* 1998; Guo & Ding, 2002;, Li *et al.,* 2002
	Tomato aspermy virus (TAV)	2b	
Dianthovirus	*Red clover necrotic mosaic virus* (RCNMV)	P27 (Replicase) P38 (Replicase)	Takeda *et al.,* 2005
Furovirus	*Beet necrotic yellow vein virus* (BNYVV)	P14 (RNA2 & Coat protein accumulation)	Dunoyer *et al.,* 2002
	Soilborne Wheat mosaic virus (SBWMV)	19K (cysteine rich protein)	Te *et al.,* 2005
Geminivirus	*African cassava mosaic virus* (ACMV) and *Srilankan cassava mosaic virus* (SLCMV)	AC2 (Transcriptional activator protein-TrAP, dsRNA binding) AC4 (miRNA binding)	Chellappan *et al.,* 2005; Dong *et al.,* 2003; Vanitharani *et al.,* 2004; Van Wezel *et al.,* 2002; Voinnet *et al.,* 1999
	Bhendi yellow vein mosaic virus (BYVMY)	C2 C4 βC1 (satellite-encoded protein)	Gopal *et al.,* 2007

	Mungbean yellow mosaic virus (MYMV)	AC2	Trinks *et al.,* 2005
	Tomato yellow leaf curl virus-China (TYLCV- C)	C2	Bisaro 2006; Dong *et al.,* 2003; Van Wezel *et al.,* 2002
	Tomato yellow leaf curl virus-China (TYLCCNV- Y10)	βC1 (satellite-encoded protein)	Cui *et al.,* 2005
	Tomato yellow leaf curl virus-Israel (TYLCV-Is)	V2	Zrachya *et al.,* 2007
Hordeivirus	*Barley stripe mosaic virus* (BSMV)	γb (Replication enhancer, Movement Protein, cysteine rich protein, pathogenicity determinant)	Yelina *et al.,* 2002
	Poa semilatent virus (PSLV)		
Pecluvirus	*Peanut clump virus* (PCV)	P15 (Movement Protein)	Dunoyer *et al.,* 2002
Phytoreovirus	*Rice dwarf virus* (RDV)	Pns10	Cao *et al.,* 2005
Polerovirus	*Beet western yellows virus* (BWYV)	P0 (pathogenicity determinant)	Pfeffer *et al.,* 2002
	Cucurbit aphid- borne yellows virus (CABYV)	P0	
Potexvirus	*Potato virus X* (PVX)	P25 (Movement Protein)	Voinnet *et al.,* 2000
	White clover mosaic virus (WCMV)		Foster *et al.,* 2002
Potyvirus	*Potato virus Y* (PVY)	HcPro P1/HC-Pro (Movement Protein, polyprotein processing, aphid transmission, pathogenicity determinant)	Dunoyer *et al.,* 2004; Kasschau & Carrington, 1998
	Turnip mosaic virus (TuMV)		
	Tobacco etch virus (TEV)		
Sobemovirus	*Rice yellow mottle virus* (RYMV)	P1 (Movement Protein, pathogenicity determinant)	Voinnet *et al.,* 1999
	Cooksfoot mottle virus (CfMV)	P1	Sarmiento *et al.,* 2007
Tenuivirus	*Rice hoja blanca virus* (RHBV)	NS3	Bucher *et al.,* 2003
Tobamovirus	*Tomato mosaic virus* (ToMV)	130K (Replicase)	Kubota *et al.,* 2003
	Tobacco mosaic virus (TMV)		
Tobravirus	Tobacco rattle virus (TRV)	16K (Cysteine rich protein)	Liu *et al.,* 2002
Tombusvirus	*Tomato bushy stunt virus* (TBSV)	P19 (Movement Protein, pathogenicity determinant)	Qu & Morris, 2002; Takeda *et al.,* 2002; Voinnet *et al.,* 2003
	Cymbidium ringspot virus (CymRSV)		

Tospovirus	*Tomato spotted wilt virus* (TSWV)	NS (Pathogenicity determinant)	Bucher *et al.,* 2003; Takeda *et al.,* 2002
Trichovirus	*Apple chlorotic leaf spot virus* (ACLSV)	P50 (Movement Protein)	Yaegashi *et al.,* 2007
Tymovirus	*Turnip yellow mosaic virus* (TYMV)	P69 (Movement Protein, pathogenicity determinant)	Chen *et al.,* 2004
Vitiviruses	*Grapevine virus A* (GVA)	P10	

1.3.3. Comparison of different suppressors

Dunoyer *et al.* (2004) and Chapman *et al.* (2004), in two independent studies, investigated and compared the mode of function of five different silencing suppressors, encoded by five unrelated RNA-viruses, by expressing them as transgenes in *Arabidopsis*. The tested silencing suppressors were the P1/HcPro (Helper component protease) of *Turnip mosaic potyvirus* (TuMV), P38 of *Turnip crinkle carmovirus* (TCV), P19 of *Tomato bushy stunt tombusvirus* (TBSV), P25 of *PVX potexvirus*, and P15 of *Peanut clump pecluvirus* (PCV) (Dunoyer *et al.,* 2004), P1/HcPro of TuMV, P38 of TCV, P19 of TBSV, P21 of *Beet yellows virus* (BYV) and 2b of *Cucumber mosaic virus* (CMV) (Chapman *et al.,* 2004). Of these viruses, TuMV, TCV infect *Arabidopsis* naturally, while TBSV, BYV and CMV do not. These suppressors cause developmental abnormalities in the transgenics and disturb miRNA cleavage of endogenous target genes.

Among these and other suppressors, mentioned above (Table 2) the HcPro, P19 and 2b have been studied more extensively.

1.3.3.1. HcPro from Potyviruseses

HcPro of potyviruses is a strong suppressor which inhibits effectively initiation of silencing, and reverses already established silencing. However, but it does not prevent the systemic spread of silencing (Brigneti *et al.,* 1998; Lleave *et al.,* 2000). It appears to interfere with RNA-silencing in different ways, depending on how the silencing is initiated, and whether it is mediated by the miRNA or siRNAs. HcPro sequesters specifically siRNA duplexes by binding small RNAs (< 24) and blocking their assembly into RISC (Lakatos *et al.,* 2006; Merai *et al.,* 2006). HcPro-mediated suppression of transgene silencing did not eliminate the transgene methylation, or the systemic signaling, indicating that these are mediated by the longer siRNAs. In some experiments, HcPro has actually enhanced systemic spread of silencing (Mallory *et al.,* 2001, 2002 & 2003). Accumulation of specific miRNAs was enhanced in the HcPro-expressing plants, but the function of miRNAs appeared to be repressed, because the un-processed target RNAs also accumulated in these plants (Kasschau *et al.,* 2003; Mallory *et al.,* 2002). HcPro suppressor, when over-expressed in transgenic *Arabidopsis*, also causes developmental defects similar to the *caf/dcl1-9, dcl-1, ago-1* and *hen-1* mutants, i.e. disturbance of organ differentiation and polarity. These developmental disturbances appear to be related to the HcPro interference with the miRNA accumulation, and/or with their reduced function (Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Kasschau *et al.,* 2003; reviewed in Syller,

2006). This type of interference with the RNA-regulatory pathways may also be related to the viral symptoms, manifested as various organ malformations and reduction of growth.

1.3.3.2. P19 from Tombusviruses

To date, the best understood viral suppressor is P19 from tombusvirus. The function of the P19 has varied in different assays of silencing suppressors: When applied on established silencing, it can reverse the silencing, but this occurs only in the region of veins (Voinnet *et al.,* 1999). However, when applied simultaneously with the silencing inducer (in a transient silencing suppression assay) it effectively blocks both the local and systemic silencing (Hamilton *et al.,* 2002; Silhavy *et al.,* 2002; Voinnet *et al.,* 2003). It functions by binding specifically the RNaseIII-cleaved short RNA duplexes, i.e. to the siRNAs, and also to the double-stranded primary cleavage products of miRNA precursors (Dunoyer *et al.,* 2004; Lakatos *et al.,* 2004; Silhavy *et al.,* 2002; Vargason *et al.,* 2003; Ye *et al.,* 2003). Due to this specific siRNA and miRNA binding function, P19 suppressors of different tombusviruses can function as silencing suppressors in a wide range of organisms, and even in mammalian cells (Dunoyer *et al.,* 2004). P19 is the only suppressor whose structure has been solved so far. X-ray crystal structure showed that dimers of P19 bind one molecule of siRNA (Ye *et al.,* 2003) and like HcPro, sequester duplex siRNAs and block the antiviral silencing (Omarov *et al.,* 2007).

The ALY proteins in *A. thaliana* can be either nuclear or cytoplasmic. Nuclear ALY proteins can drag P19 into the nucleus and by doing so, inhibit the P19 function (Canto *et al.,* 2006). If RISC becomes activated due to absence or malfunctioning of P19, the process cannot be reversed. Silencing suppression activity by P19 depends on its high expression levels and its quick accumulation after the TBSV infection. The selective binding of P19 with 21 nt and 22 nt siRNAs describes a unique pathway of RNA silencing suppression by sequestration of siRNA. In addition the binding of the protein with 21 nt siRNAs, its presence in phloem affects the systemic movement of the silencing signal (Omarov *et al.,* 2006; reviewed in Li & Ding, 2006; Scholthof, 2006).

1.3.3.3. 2b from Cucumoviruses

In earlier work, 2b was described as a weak silencing inhibitor, which can suppress the initiation, but not the maintenance of established silencing. It does not effect (much) the local silencing reaction, but blocks the systemic movement of the silencing signal (Brigneti *et al.,* 1998; Guo & Ding, 2002). Now it's believed that 2b proteins coded by some strains of CMVare strong suppressors (Lewsey *et al.,* 2007). The effects of 2b are very strain-specific and depending on the CMV strain, it may or may not bind to ss-siRNA or siRNA or miRNA duplexes (Merai et al., 2006; Zhang et al., 2006; Goto et al., 2007). E.g. 2b from subgroup I CMV can suppress both local and systemic silencing. It binds viral siRNAs of all three classes (21-, 22- and 24 nt) effectively but 21- and 22 nt siRNAs are the main targets (Diaz-Pendon *et al.,* 2007). The 2b proteins from mild strains may bind miRNA duplexes very poorly, and that could be the reason why they do not cause much (or any) phenotypic effects when expressed in transgenic plants (Lewsey *et al.* 2007). The 2b can even bind small stretches of dsRNA that are longer than siRNAs. It is not clear how

2b functions, but its localization in the nucleus (in contrast to the cytoplasmic localization of the other known virus suppressors) suggests that it does not function by inhibiting directly the degradation of viral RNAs, but rather indirectly, by effecting the host responses (Goto *et al.,* 2007). A special feature of the 2b of CMV is that its function requires SDE/SGS, indicating that it suppresses conversion of the ssRNAs into ds form, which is initiated by aberrant, or abundant viral RNAs (Goto *et al.,* 2007; Lucy *et al.,* 2000; Voinnet, 2001). Thus, 2b suppresses RNA silencing by a mechanism different from that used by P19 and P1/HcPro. The severe developmental abnormalities caused by 2b (CMV-Fny strain) in transgenic plants indicate that 2b interferes with endogenous silencing mediated by miRNA pathway. At least the plants accumulate more miR164 and also its target, NAC1 mRNA and the developmental defects depend on the accumulation level of intact 2b protein (Lewsey *et al.,* 2007; Ruiz-Ferrer & Voinnet, 2007 and references therein; Zhang *et al.,* 2006).

1.3.3.4. P25 from Potexvirus

P25, the cell-to-cell movement protein of PVX, prevents the systemic spread of the silencing signal (Hamilton *et al.,* 2002; Voinnet *et al.,* 2000). However, corresponding P25 protein of the closely related *White clover mosaic virus* (WCMV) does not function as silencing suppressor in *Arabidopsis*. P25 of WCMV may function as such in *N. benthamiana*, a systemic host of this virus, because it causes severely altered leaf morphology in this species (Foster *et al.,* 2002). P25 might be a very weak suppressor as it neither blocks siRNA biogenesis nor has effects on endogenous miRNAs. P25 may block the silencing pathway by interfering with silencing effector complexes (Bayne *et al.,* 2005).

1.3.3.5. Other investigated suppressors

Different suppressors appear to affect very differently the silencing (siRNA) pathway and the miRNA metabolism. P50 of *Apple chlorotic leaf spot virus* (ACLSV), P1 of *Rice yellow mosaid virus* (RYMV) and coat protein (CP) of *Citrus tristeza virus* (CTV) (Himber *et al.,* 2003; Lu *et al.,* 2004) all suppress systemic silencing, induced by both ss and dsRNA, but do not have any effect on local silencing (Yaegashi *et al.,* 2007). P50 of ACLSV and CP of CTV do not effect the accumulation of either short (20-23 nt) or long (24-26 nt) small RNAs (Yaegashi *et al.,* 2007), while P1 of RYMV reduces the accumulation of long species of siRNA (Hamilton *et al.,* 2002; Himber *et al.,* 2003). The P21 suppressor of the BYV, like P19 and HcPro, inhibit the RISC assembly and target cleavage, but preassembled RISC is not affected by either P19 or P21 silencing suppressors. HcPro and P21 also do not prevent siRNA biogenesis from long dsRNA precursor (Chapman *et al.,* 2004; Lakatos *et al.,* 2006; Yu *et al.,* 2006). Many viral suppressors, e.g. HcPro, P19, P21, P15 of PCV and γB of *Barley stripe mosaic virus* inhibit silencing pathways by binding siRNA- and miRNA duplexes, preferably bind 21 nt siRNA duplexes. They containing 2 nt 3' overhangs but fail to bind long dsRNA (Lakatos *et al.,* 2006; Merai *et al.,* 2005 & 2006), while CP of TCV and P14 of *Pothos latent virus* bind dsRNA without obvious size selection and do not require 3' overhangs (Merai *et al.,* 2005 & 2006). *Hibiscus chlorotic ringspot virus*-CP can suppress sense RNA-induced

PTGS but does not effect dsRNA-induced local and systemic PTGS (Meng *et al.,* 2006). Geminivirus (DNA virus) suppressor protein AC4 binds with ssRNAs including miRNAs (Chellappan *et al.,* 2005). The same transgene and/or virus can have different effects in different hosts. AC2 of *African cassava mosaic virus* (ACMV) in transgenic *N. benthamiana* cleaves 21 nt siRNAs, whereas ACMV infection in *N. benthamiana* generates 22- and 24 nt siRNAs. However, in cassava, both the transgene and virus are cleaved into also 23 nt siRNA (Akbergenov *et al.,* 2006). It seems that binding with siRNA and/or miRNA is a general functional strategy of different viral silencing suppressors (Chapman *et al.,* 2004; Chellappan *et al.,* 2005; Lakatos *et al.,* 2006; Merai *et al.,* 2005 & 2006; reviewed in Bisaro, 2006; Li & Ding, 2006; Ruiz-Ferrer & Voinnet, 2007). In some cases, plant viruses have more than one suppressor protein. e.g. CTV has three suppressors (Lu *et al.,* 2004). *Red clover necrotic mosaic virus* suppresses silencing pathway and inhibits miRNA biogenesis by using multiple (P27 and/or P38-replicase proteins) viral components required for viral RNA replication (Takeda *et al.,* 2005).

The varied responses indicate that all the tested suppressor proteins affect the siRNA and miRNA-mediated pathways in distinct ways, and that the different forms of the short RNAs are differently regulated. The results from separate, independent work also indicate the complexity and diversity of the silencing-related pathways and interactions, which may be related to the diversity of the Dicer and ARGONAUTE families in *Arabidopsis* and other plant species. Moreover, the data obtained from other species, mainly *N. benthamiana* indicates that the effects of different viral silencing suppressors may be different in different species. However, the experimental work so far has focused mostly in *Arabidopsis*, and therefore, the specific features of different suppressors, or of specific suppressor-host interactions are not well known. All together, the race between the viral RNA replication and spread, the RNA-silencing-mediated local and systemic defense of the host, and the virus-encoded silencing suppression determine the outcome of the disease (Ahlquist, 2002). The optimal balance in this multi-factorial interaction may be very sensitive: it is a selective advantage for the virus to replicate and spread efficiently, and to suppress the host RNA-silencing, but it must not be too effective in these functions to avoid excessive harm or lethality to it´s host.

2 AIMS

In this work, I have investigated the symptom causing mechanisms of TMV, focusing particularly on the roles of viral CP, and of the plant silencing defense mechanisms in the outcome of TMV infection. In addition, I have studied the effects of different viral silencing suppressors on the plants and on infections by different viruses. Using transgenic plants, I have specifically aimed to elucidate the following:

1. The role of TMV CP in induction of disease symptoms.

2. The role of PTGS in induction of TMV symptoms.

3. Developmental abnormalities caused by various viral silencing suppressors in closely related plant species.

4. Interference of the various viral silencing suppressors with the recovery of plants from nepovirus infections, and with severity of different viral infections.

3 MATERIALS AND METHODS

3.1. TMV transgenic plants

Transformation constructs for the full length, infectious cDNA in pBGC89 vector of wt TMV and of the CP-deleted mutant of TMV, were obtained from Dr. T. Turpen (Turpen *et al.,* 1993, Paper I)). In these constructs, the 35S promoter is fused to the 5' end of TMV cDNA and a ribozyme sequence to the 3' end of the viral cDNA in such a way that infectious, viral transcripts are produced of the transgene in plant cells. These constructs were first transferred to a binary vector, and then transformed into tobacco plant via *Agrobacterium* mediated transformation, to produce R0 progeny of wt-TMV and ΔCP-TMV transgenic plants. Transgenic plants were propagated in a greenhouse at 25 ^{o}C under a 16 h photoperiod. Three R2 independent lines were used for further analysis. For TMV inoculations, the sap was derived from the tobacco plants infected with the same cDNA clone that had been used for the plant transformations, i.e. pTMV304, a T7-promoter-controlled derivative of the original infectious clone pTMV204 (Dawson *et al*., 1986).

3.2. Viral silencing suppressor transgenic plants

Viral silencing suppressors, i.e., P19 of *Tobacco bushy stunt virus* (TBSV, tombusvirus), P25 of *Potato virus X* (PVX, potexvirus), HcPro of *Potato virus Y* (strain N) (PVY, potyvirus), 2b of *Cucumber mosaic virus* (strain Kin) (CMV, cucumovirus), AC2 of *African cassava mosaic virus* (ACMV, geminivirus) and P1 of *Rice yellow mottle virus* (RYMV, sobemovirus) in pBin61 vector, obtained from the laboratory of Prof. Dr. Baulcombe (through Plant Bioscience Ltd.) and P1 of *Cocksfoot mottle virus* (CfMV, sobemovirus) in pBin61 vector (Sarmiento *et al.,* 2007) were used for transformation (Paper II). These constructs and empty vector were introduced into *Agrobacterium tumefaciens* through electroporation, and transformed into leaf discs of *N. benthamiana* and *N. tabacum* cv Xanthi (nn) L. by standard procedures. The transformants were regenerated on Murashige-Skoog (MS) medium using kanamycin (Km) selection (Km 100 μg/ml, cefotaxime 250 μg/ml and vancomycin 100 μg/ml). Rooted plantlets were transferred to pots and grown to maturity in the greenhouse at 25 °C, with a 16 h photoperiod. A total of 10 lines with each silencing suppressor construct in both *Nicotiana* species, except P1-RYMV lines, were regenerated with observation of phenotypes and collection of seeds. In case of P1-RYMV, only 3 of the regenerated *N. benthamiana* plants survived. All lines produced adequate amount of seeds for propagation.

3.3. Selection of homozygous lines

Seeds of each of the five selected R0 transgenic lines, except P1-RYMV (only 3 lines) were germinated on Km-containing MS-medium, transferred to soil, and grown in the

greenhouse, as mentioned above, with observation of the phenotypes and collection of seeds. For each line, seeds of the ten separate R1 plants were germinated on Km-containing MS medium, to test the rate of Km-resistance. For each transgene, two or three independent R2 lines, showing 100 % Km-resistant germination, indicating homozygote transgene status, were selected for further analysis. For any further experiments the seeds were germinated in soil, and the plants propagated in controlled greenhouse conditions as described above.

3.4. Transgene detection

DNA was extracted by Genomic DNA from Plant (Macherey-Nagel & Co. KG) kit according to the manufacturer's instructions. Transgene sequences were amplified from the corresponding plant DNA samples by PCR, using either primers annealing in the 35S promoter and terminator sequences (for P1-RYMV gene), or specific primers annealing in the coding regions of the other transgenes, plus *Taq* polymerase (See paper II). Amplification products were run in 1 % agarose gels.

3.5. RNA extractions, Northern blot and siRNA analysis

Total RNA was extracted according to Sijen *et al.* (1996). Briefly, 0.5 g of leaves were ground in liquid nitrogen and the powdered leaves were extracted in hot phenol and RNA extraction buffer (100 mM Tris-HCl: pH 8.0, 100 mM LiCl, 10 mM EDTA, 1 % SDS) (1:1) followed by extraction with one volume of chloroform. An equal amount of 4 M LiCl was added to the supernatant and RNA was separated from DNA by incubating on ice overnight at 4 ^{o}C, followed by centrifugation (13,000 rpm for 20 min at 4 ^{o}C). Pellets were resuspended in double distilled water and RNA was recovered by ethanol precipitation. Five µg RNA was separated on a 1 % agarose denaturing gel, running in MOPS buffer, and transferred to Hybond-N (GE Healthcare, Munich, Germany) membrane via capillary blotting by standard methods (Sambrook and Russell, 2001) and fixed by baking at 80 ^{o}C for 2 hours. Probes for the different silencing suppressor genes, as well as for the corresponding viruses were generated by PCR amplification using DIG labelled UTP nucleotide, from original plant transformation constructs using forward primer annealing in the 35S promoter and reverse primer annealing in the 35S terminator region. The probe for TMV sequences was produced by PCR from the infectious TMV cDNA clone, pTMV304, and using specific primers. Probes for the PVY-N strain and for the TRSV calico strain were produced by PCR from virus-specific cDNA clones, which were produced from the total RNA extracted from infected plants as described in manuscripts IV and III, respectively. Analysis of TMV and TRSV specific small interfering RNAs (siRNAs) was performed according to Sarmiento *et al*. (2006) using 30 µg of total RNA. The radioactive probe was a ^{32}P-labeled *in vitro* transcript corresponding to the anti-sense strand of the viral RNA covering movement protein (MP), CP and 3' UTR sequences for TMV and 3' UTR sequence for TRSV specific siRNAs. Radioactive signals were detected with Personal Molecular Imager FX (BioRad, Hercules, CA, USA).

3.6. Protein extraction and Western immunoblotting

TMV 126 kDa replicase protein, MP and CP were extracted from leaf tissues, separated by SDS-PAGE and analyzed by Western blotting using chemiluminescence for detection, as described earlier (Lehto *et al*., 2003). The 126 kDa replicase and MP antisera were kind gifts of Drs. Y. Dorokhov and T. Ahola, respectively (Ahola and Kääriäinen, 1995; Zvereva *et al*., 2004).

3.7. Methylation analysis

The methylation of the transgenes corresponding to TMV coding and promoter regions was analyzed as described by Mallory *et al*., (2001), and Rodman *et al*., (2002). DNA samples were extracted from the wt-TMV and ΔCP-TMV transgenic plants, both before (4-weeks old) and after (8-weeks old) the resistance breakage, using CTAB-extraction buffer (2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.3 % β-mercaptoethanol) and equal volume of chloroform, with incubation at 65 ^{o}C for 30 min, followed by repeated phenol-chloroform-isoamyl alcohol extractions and ethanol precipitation. Aliquots of the plant genomic DNAs were digested with the methylation-sensitive enzyme *Sau*96I. Fragments of the viral genomic sequences, as well as of the 35S promoter sequence were amplified by PCR both from the *Sau*96I-digested and undigested samples, using reverse primers annealing in TMV or 35S-promoter sequences upstream of the *Sau*96I restriction sites, and forward primers annealing downstream of the restriction sites, to promote amplification over the restriction sites. The amplified products were analyzed by gel electrophoresis.

3.8. Microscopy

For microscopic analysis, samples were collected from the first fully expanded leaves of young *N. benthamiana* and *N. tabacum* plants and immediately fixed with 3 % glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.0, and post fixed in 1 % osmium tetroxide in the same buffer. After dehydration in an alcohol series, the samples were embedded in Epon. Thin sections were cut with Reichert ultramicrotome and examined with a Reichert zetopan microscope, mounted with Canon EOS 20D digital camera.

3.9. *Agrobacterium* infiltration

Fresh over-night cultures of *A. tumefaciens* cells, carrying 35S-controlled infectious clone of the crTMV cDNA with a GFP-gene replacing the coat protein gene (a kind gift of Prof. Y. Dorokhov), adjusted to OD_{600}= 1.0 as final density, were induced with acetosyringone as described by Hamilton *et al.* (2002). Equal volumes (approx. 100 μl) of the cell suspension were infiltrated to the two uppermost, fully expanded leaves of three plants of each transgenic *N. benthamiana* line. Plants were incubated for one to two weeks for GFP

analysis**.** The infiltrated plants were maintained in the greenhouse under the conditions mentioned above. The GPF was visualized by using a hand held 366 nm wave length UV lamp (BLAK RAY, UVL-21, Ultra-violet Products Inc, California, USA). Photographs were taken at 15 days post infiltration with Canon EOS 20D digital SLR camera. The total brightness of the GFP spots was measured with an aperture photometry technique, in which the background level brightness was automatically subtracted. From repeated measurements of the same sample the accuracy of the surface area was estimated as ± 5% and the integrated brightness within ± 5%, giving an estimated error of ± 7% for the surface brightness.

3.10. Testing of the silencing suppression effect on different viral infections

Two plants of each of selected homozygous transgenic lines both in *N. benthamiana* and *N. tabacum* in repeated experiments were inoculated with four different viruses including, TRSV, TMV-U1, PVY-N and PVX to see the effects of homologous and heterologous viruses on different transgenic lines (Papers III & IV). The used TMV inoculum was derived from infectious clone pTMV 304 (obtained from Prof. W. O. Dawson, University of Florida, USA), PVY-N, was obtained from Prof. A. Kurppa (Agricultural Research Centre, Jokioinen, Finland), TRSV-calico strain inoculum was obtained from Dr. Stephan Winter (DSMZ Plant Virus Division, Braunschweig, Germany), and PVX inoculum was obtained from Dr. David Baulcombe, Sainsbury Lab, UK. Infected leaves were ground in 50 mM phosphate buffer (pH 7.0), and the sap was rubbed onto Carborundum–dusted leaves of transgenic and wt plants. Two plants of each of selected homozygous transgenic lines in *N. benthamiana,* in three independent experiments were also inoculated with TMV-*ChlH* construct pTb549a. *In vitro* transcription and inoculation was done as described by Hiriart *et al.,* (2002).

4 RESULTS AND DISCUSSION

In this work I have investigated the effects of either wild type, or mutated viral genomes or of isolated viral genes on their hosts. These viral elements have been expressed in transgenic plants under the regulation of the 35S promoter, so that they are produced in all the cells, and through all different developmental stages of the plants.

The aim of the work has been to study different interactions between the viral genomes or gene products and the host plants. In the first part of the studies, the work was done with transgenic plants expressing either wild type or mutated TMV genomes. Using these plants I investigated, the role of the viral CP in viral replication and in induction of disease symptoms, and the plant reactions to TMV infections during different developmental stages (Paper I). The other part of the work focused on the effects of different viral silencing suppressors on the host phenotypes (Paper II) and on their effects on the infections of different homologous and heterologous viruses (Papers III & IV).

4.1. Resistance of young plants against endogenously expressed TMV RNAs

Of special interest in this study was the role of the TMV-CP in the virus accumulation and symptom production in TMV-infected plants. So far, it has been thought that the main function of CP is in virus encapsidation and possibly, in cell to cell movement; although role in replication has also been suggested recently (Asurmendi *et al.,* 2004; Kawakami *et al.,* 2004). However, CP strongly effects symptoms, as different deletions of the CP gene may change the symptoms from very mild to very severe (Dawson *et al.,* 1988; Lindbeck *et al.,* 1991), and alteration of just one or two amino acids, which affect the folding of the CP, can change the symptoms to very severe (Banerjee *et al.,* 1995; Jockusck *et al.*, 2001). It is thus possible that CP affects the infection outcome in several different ways, i.e. by protecting the genome from silencing mediated degradation, and by participating in replication and the viral movement in the host tissue.

Transgenic plants were used to study the plant responses to endogenously expressed viral genomic RNAs. In this study I particularly wanted to study the balance between viral replication and host defenses during different developmental stages of the plant, and the role of the CP in these processes.

Upon the propagation of the transgenic plants through R1 and R2 generations, it was found that seedlings of either the wt-TMV or ΔCP-TMV-expressing transgenic plants did not show any obvious mosaic symptoms during the first 5-7 weeks post germination. However, they exhibited very stunted growth, wt-TMV expressing plants being stunted by about 50 %, and ΔCP plant by about 30 % as compared to control plants (Fig. 1B, Paper I). During this growth period viral RNA accumulated at a very low level in the wt-TMV plants, and not at all in the ΔCP-TMV plants (Fig. 2 A & B, Paper I). The presence and

infectivity of transcripts in the wt-TMV expressing plants was confirmed by inoculating their sap onto leaves of a local lesion host, *N. tabacum* L. 'Xanthi' NN, where production of very few necrotic spots (1-6) confirmed the low amounts of viral RNA. Also, TMV-specific siRNAs (if any) were below detection level during this time (Fig. 3, Paper I). When these transgenic plants were inoculated with wt-TMV during these early weeks, they developed disease symptoms, but the symptoms were much milder and developed much slower than in the control plants (Fig. 4 A & B, Paper I). However, viral RNA accumulated in all inoculated transgenics and control plants to similar levels (Fig. 4 C & D, Paper I). These results indicated that the young plants expressed strong resistance against the endogenously expressed viral transcripts, and also strong tolerance against the induction of viral symptoms, but they were not resistant against the replication and accumulation of exogenously applied viral inoculum. Lack of disease symptoms and the very low to no accumulation of viral RNA in wt-TMV and ΔCP-TMV expressing plants resembled silencing mediated antiviral defense. However, lack of detection of TMV specific siRNAs (Fig 3, Paper I), which is hallmark of such defense phenomenon, indicated that silencing was not activated in these plants. Thus, some other defense strategy against initial, endogenous inoculum was responsible for the observed resistance in the young transgenic plants.

Between 7-8 weeks post germination, resistance was broken in both types of transgenic plants. After this, wt-TMV plants developed typical systemic TMV symptoms (Fig. 1C, Paper I), and genomic length TMV RNA accumulated in the leaves to high levels similar to normal wt TMV infections (Fig. 2A, Paper I). This result is slightly different from the results reported by Yamaya *et al*. (1988) who also expressed full-length TMV genome in tobacco plants, and in that case plants started to show symptoms within 4 weeks after germination.

ΔCP-TMV transgenics also displayed, occasionally, chlorotic symptoms, but these were very mild (Fig. 1D, Paper I), and plants accumulated low levels of the truncated viral RNA (Fig. 2 A & B, Paper I). These data indicated that the CP is necessary both for efficient virus replication, and consequently, also for symptom production (Asurmendi *et al*., 2004; Dawson *et al*., 1988; Kawakami *et al*., 2004). Interestingly, a TMV-specific RNA of about 210 nt, corresponding to the size of truncated 3'-terminal subgenomic mRNA, accumulated to high levels in these plants (Fig. 2 A & B, Paper I).

Surprisingly, when growth of the side shoots was induced in ΔCP-TMV plants by removing apex of the main shoot, these plants displayed stronger symptoms than the intact shoots, although still much milder than the wt-TMV plants (Fig. 1 C, E, Paper I) suggesting that the plant response to viral infection was dependent on the physiological status of the tissue. TMV-specific siRNAs were below (if any) detection in these plants (Fig. 3, Paper I). After the resistance break, the TMV-specific 21-25 nt siRNAs were also detected in both types of transgenic plants, being more abundant in the wt-TMV plants, corresponding to the accumulation of viral RNAs (Fig. 3, Paper I). Detection of siRNAs indicated that RNA silencing became active at this stage. Simultaneous breaking of resistance, virus accumulation and activation of silencing implies again that this form of

defense was not active during the early resistance and that it cannot even maintain resistance against wt-TMV when active replication is initiated.

4.1.1. Accumulation of the virus-specific proteins

After resistance breakage, when viral RNAs accumulated at higher levels, the viral 126 kDa replicase protein and MP were also detected from both types of transgenic plants. The 126 kDa replicase protein was detectable at a very low level from the ΔCP-TMV plants, but interestingly, the MP from same plants was detected at a much higher level than in the wt-TMV plants (Fig. 5, Paper I). The increase in MP in ΔCP-TMV plants together with the increased level of the truncated subgenomic transcript indicated that the transcriptional and translational regulation of the internal genes was disturbed by the deletion of the CP coding region. The increase of the MP expression in ΔCP virus constructs has been reported before (Culver *et al*., 1993).

4.1.2. DNA methylation

To check weather the resistance in young transgenics was correlated with methylation status of the transgene promoter or coding regions, the purified DNAs from both types of transgenic plants, before and after the resistance break, were cleaved with methylation-sensitive *Sau*96I restriction enzyme. To check for the positive cleavage, samples were PCR-amplified over the restriction site. Samples extracted from the transgenic plants before resistance break, after *Sau*96I-digestion, produced a very faint PCR amplification of the coding region and no amplification at all from 35S promoter region, indicating that the transgenes or their promoter region were not methylated. However, after the resistance breakage, clear amplification from both the coding and promoter regions was obtained from the *Sau*96I-digested samples (Fig. 6A & B, Paper I) indicating that these sequences became methylated as soon as the sequence-specific silencing was activated in the plants. No or very low DNA methylation either at the transgene coding region or at the promoter region in the young resistant plants further confirmed that resistance in these the plants was not due to RNA silencing or transcriptional gene silencing.

Simultaneous resistance breakage in both types of transgenic plants, and stronger symptoms observed in the side shoots of the ΔCP-TMV plants indicated that breaking of the resistance was associated with some specific physiological stage of the plants. The mode of this resistance is not known.

4.2. Expression of different viral silencing suppressors in two *Nicotiana* species: their effects on plant phenotypes and on the infections of other viruses

The silencing suppressors, encoded by different and unrelated RNA and DNA viruses,not only inhibit the siRNA mediated RNA silencing pathway but may also interfere with the miRNA mediated pathways which can lead to developmental disturbances in the host (Chapman *et al.,* 2004; Dunoyer *et al.,* 2004). In recent years the interactions of different

suppressors with the RNA silencing pathways have been intensively studied (Deleris *et al.,* 2006; Lakatos *et al.,* 2006; Lewsey *et al.,* 2007; Merai *et al.,* 2006; Xie & Guo, 2006; Yaegashi *et al.,* 2007; Zhang *et al.,* 2006). The available data indicates that these interactions are very complex, and vary between different suppressor-host combinations. Most of the experimental work has been done using *A. thaliana* as host and in some cases *N. benthamiana* and therefore, the degree of host specificity of these interactions are not known.

In this study, we wanted to investigate and compare the effects of various viral silencing suppressors in two closely related species; *N. benthamiana* and *N. tabacum.* The plants were transformed with silencing suppressor genes derived from six different viral genera (see Paper II). To avoid any possible tissue culture effects and to have stable lines, two or three independent homozygous R2 generation lines for each of the transgenes were selected based on their 100 % germination on Kanamycin-containing medium.

4.2.1. Silencing suppressor–associated phenotypes of the two *Nicotiana* species

Five transgenic lines were propagated through the R1 generation, with observation of phenotypes. From these seeds, three R2 generation homozygous lines were selected. In the R2 generation, three homozygous lines of each transgene in both *Nicotiana* species were initially selected and for the final analyses, single lines with more pronounced phenotypes were used for each transgene in both species. The transgenes caused specific effects in the two tobacco species through the three generations of propagation. *N. benthamiana* displayed more disturbed phenotypes than *N. tabacum* through different generations (Tables 3 & 4).

In general, separate lines of all transgenes showed different degrees of phenotype effects. In some of the *N. tabacum* R0 regenerants (Table 4), the HcPro, AC2 and 2b caused clearly disturbed flowers and reduced seed set, while most of the transgenes caused reduced seed set in *N. benthamiana* R0 regenerants (Table 3).

4.2.1.1. P1-RYMV and P1-CfMV

The P1-RYMV transgene caused high lethality in *N. benthamiana* in the R0 generation, the surviving regenerants grew very poorly and their seed set was much reduced. The progeny plants were stunted also in the R1 generation, and produced occasionally cup-shaped leaves. These stunted growth and cup-shaped leaves persisted in the R2 generation in the selected line. The same line also produced sterile flowers with malformed petals and bent flower stalks (Fig. 2, Paper II). On the other hand, P1-CfMV did not produce any specific malformation in *N. benthamiana* except in the R2 generation, where the flowers had bent stalks.

In *N. tabacum* the P1-RYMV transgene did not cause any (observable) adverse effects, except that the flowering was delayed in the selected line. Most of the P1-CfMV lines in *N. tabacum* produced malformed flowers in the R1 generation, but the R2 generation plants did not exhibit any specific abnormalities.

4.2.1.2. PVY HcPro

PVY HcPro transgenes caused the most striking phenotypes in both *N. benthamiana* and *N. tabacum* plants throughout the three generations. In the R0 generation, many *N. benthamiana* lines grew bushy, had creeping stems and produced much less seeds than wt plants. In the R1 generation, the lines developed mild to very sever rolling of leaves and bending of stems. The line selected for further analysis had leaves fully curled and very soft and creepy stems, while another line (Line B) was more erect, with thick leaves which were rolled down only at the margins. The line A produced leaves without petioles and with altered vein pattern. Also flowers were emerging directly from the stem, with no stalk, and were strongly malformed and sterile (Fig. 2, Paper II).

Several HcPro-expressing *N. tabacum* lines produced malformed flowers with reduced seed set during all three generations, although these malformations were more severe during the R1 generation. Plants grew normal during the R0 generation, but in the R1 and R2 generations produced stunted plants with large, thick, hairy and dark leaves. Transgenic *N. tabacum* cv Havana 425 plants also produced some developmental abnormalities when transformed with TEV-HcPro transgene (Anandalakshmi *et al.,* 2000; Pruss *et al.,* 2004).

4.2.1.3. ACMV AC2

Seed production was strongly reduced throughout all three generations in plants of both species, expressing the ACMV AC2 gene. Some plants in both species also showed funnel- or cup shaped leaf forms in the R1 generation, and also in the R2 generation of *N. benthamiana* of the selected line. Leaves also displayed mild blistering in this line.

AC2 expressing *N. tabacum* plants produced strongly malformed flowers during all three generations, although malformation was more pronounced during the R1 generation than in the R2 generation. Plants of selected lines (R2) were also very stunted, and their leaves were thick and hairy with short internodes, and flowering was very late. The phenotypes caused by the AC2 and HcPro genes occurred more strongly in the R1 generation than in the the R2 generation. Disturbed leaf phenotypes in plants expressing either HcPro or AC2 suggested disturbance of the differentiation of the leaf abaxial/adaxial morphology.

4.2.1.4. CMV 2b

N. benthamiana plants with CMV 2b transgenes grew normally in both the R0 and R1 generations, but produced less seeds, and had mild mosaic appearance (chlorotic spotting) on the leaves. In both the R1 and R2 generations the plants were slightly stunted, with a bushy appearance in some lines.

In *N. tabacum*, one line produced heavily malformed flowers in the R0 generation, while in the R1 generation, most of the plants produced malformed flowers, and plants of one line exhibited mosaic patterns. No phenotypes were observed in the R2 generation.

4.2.1.5. TBSV P19

The TBSV P19 expressing *N. benthamiana* transgenic plants grew normally in both the R0 and R1 generations, but their seed set was reduced. In the R2 generation, one of the lines showed mild flower malformations with slightly larger, rounded petals and bent flower bases. The other (selected) line showed altered leaf phenotype with mildly blistered leaf surfaces, and more serrated and hairy leaves.

In P19 expressing *N. tabacum* lines, no specific phenotypic alterations were observed during any of the three generations of these transgenes, except for some flower malformations in the selected line (Fig. 3, Paper II)

4.2.1.6. PVX P25

Of the PVX P25 expressing transgenic *N. benthamiana* lines, only plants of the selected line in the R2 generation were slightly stunted, with distinctly small, un-opening flowers and reduced seed set, and very early senescence of the plants.

In *N. tabacum*, plants of one of the lines were taller than the wt control plants during the R1 generation. Due to lack of detection of transgene mRNA and any pronounced phenotypes, the expression of the transgene was not confirmed.

It has been shown that viral suppressors interfere with miRNA biosynthesis in *Arabidopsis* and inhibit the cleavage of target genes by specific miRNA in the plant developmental pathway (Alvarez *et al.,* 2006; Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Jacobsen *et al.,* 1999; Kasschau *et al.,* 2003; Llave *et al.,* 2002 b; Mallory *et al.,* 2002, 2004; Millar and Gubler, 2005; Park *et al.,* 2002; Ray *et al.,* 1996; Vazquez *et al.,* 2004). The disturbed phenotypes in the silencing suppressor-expressing transgenic plants, observed in this study, could thus be due to the interference of these suppressors with the endogenous RNA silencing pathways.

As the HcPro or the AC2 genes caused the most severe leaf malformations in both *N. benthamiana* and *N. tabacum* transgenic plants, thin sections from the leaves of these transgenic plants were prepared to observe the alteration in tissue structures. Microscopic analysis showed that in both *Nicotiana* species, HcPro transgene caused a significant increase in the numbers of mesophyll cells (Fig 5 B, E, H, K, Paper II), and disturbed abaxial/adaxial differentiation of the leaves, as guard cells were present on the upper epidermis (Fig. 5 E, Paper II). On the other hand, the AC2 transgene in both *Nicotiana* species did not cause an increase in the number, but rather, produced larger mesophyll cells as compared to wild type plants. Cells were also distorted or wrinkled with disturbed and clumped chloroplast distribution, indicating weak cell wall structures (Fig. 4 I, L, Paper II). Thus, each transgene caused similar malformation in the two *Nicotiana* species, although their effects were different effects at the cellular level, i.e. hyperplasia in the HcPro plants, and hypoplasia in the AC2 plants.

Table 3: Phenotypes observed in transgenic *N. benthamiana.* Selected lines are marked in bold.

Transgene	R0	R1	R2
P1-RYMV	Very lethal, only 3 of 10 regenerants survive	Stunted growth, occasionally funnel shaped leaves, reduced seed set	**Line 1**: very stunted, funnel shaped leaves, few flowers with malformed petals and bent stalks, sterile Line 2: normal
P1-CfMV	Normal	Normal	Line 1: normal **Line 2**: flowers with bent stalks
PVY HcPro	Strongly reduced seed set, most plants were bushy and creepy	Stems soft and bending, reduced seed set, occasionally malformed leaves	**Line 1**: very strong rolling of leaves, change vein pattern, no petioles, flowers with small petals and protruding carpels, sterile Line 2: leaf margins strongly rolled, smaller flowers, strongly reduced seed set Hyperplasia of mesophyll
ACMV AC2	Very reduced seed set	Occasionally stunted growth, funnel shaped leaves, reduced seed set	Line 1: normal **Line 2**: mild blistering on leaves, sterile Hyperplasia of mesophyll
CMV 2b	Occasionally plants with mild mosaic pattern and reduced seed set	Early flowering, occasionally reduced seed set	**Line 1**: normal Line 2: normal
TBSV **P19**	Reduced seed set	Reduced seed set	Line 1: flowers larger and round with bent stalks and reduced seed set **Line 2**: normal
PVX P25	Normal	Normal	Line 1: normal **Line 2**: early senescence, small and partially opened flowers with reduced seed set

Table 4: Phenotypes observed in transgenic *N. tabacum*. Selected lines marked in bold.

Transgene	R0	R1	R2
P1-RYMV	Occasional malformed flowers	Normal	**Line 1**: late flowering Line 2: normal
P1-CfMV	Normal	Occasional malformed flowers	Line 1: normal **Line 2**: reduced seed set
PVY HcPro	Mostly malformed flowers with reduced seed set	Thick, big and dark green leaves, malformed flowers with reduced seed set	Both lines similar: thick, dark green leaves with hair, short internodes, stunted growth, late flowering, strongly reduced seed set Hyperplasia of mesophyll
ACMV AC2	Mostly malformed flowers with reduced seed set	Occasionally stunted growth, heavily malformed flowers	**Line 1**: leaves thick and hairy, short internodes, stunted growth, very late flowering, malformed flowers with reduced seed set Line 2: heavily malformed flowers, transformation of petals into stamens, reduced seed set Hyperplasia of mesophyll
CMV 2b	Normal	Heavily malformed flowers, occasional mosaic patterns on the leaves	**Line 1**: late flowering Line 2: normal
TBSV P19	Normal	Occasional malformed flowers	**Line 1**: late flowering, flowers heavily malformed Line 2: normal
PVX P25	Normal	Some plants very tall, occasional malformed flowers	Line 1: normal **Line 2**: normal

4.2.2. Confirmation of transgene expression

To verify the transgene expression northern analysis was performed to detect the mRNAs levels. The presence of the transgene, especially from those plants where mRNA detection was below detection, was also confirmed by amplifying the trangene sequences from the corresponding plant DNA samples by PCR (See paper II).

4.2.2.1. TMV-ChlH

To verify the silencing suppressor functionality in the transgenic plants, I wanted to show that a heterologous, weak viral pathogen is enhanced in these plants. For this purpose I used a chimeric TMV-30b vector expressing ChlH gene, a key enzyme for the chlorophyll biosynthesis pathway, which causes the VIGS-mediated silencing on this pathway, and functions thus as an observable genetic tag on the virus. This construct can spread easily in inoculated *N. benthamiana,* reduces the chlorophyll biosynthesis by silencing *ChlH* gene through VIGS, and can be observed by specific (yellow/white) symptoms in the systemic leaves (Hiriart *et al.,* 2002). *N. benthamiana* transgenic plants were inoculated with the TMV-ChlH construct. The observed symptoms indicated that the wt and all vector-transformed control plants were more yellow and stunted than the transgenic plants, indicating that the silencing activity was reduced in the transgenic plants. The symptoms, with special focus on the visually estimated levels of the silencing of the chlorophyll biosynthesis, and viral RNAs levels quantified by Northern blotting, are described in Table 5.

Table 5: Descriptions of the phenotypes and virus accumulation of TMV-ChlH infected *N. benthamiana* transgenic plants. Visually estimated level of silencing is indicated in relation to the silencing level of wt plants, with following ratings.
Very strong: 100 % silencing, Strong: 70-90 % silencing, Moderate: 50-60 % silencing, Mild: 20-40 % silencing, Weak: less than 20 % silencing.
Selected lines are marked in bold.

Transgene	**Symptoms, with silencing indicated in relationship to wt plants**	**Virus titer**
Wt-*N. benthamiana*	severely stunted, stems mostly white, **ChlH silencing: very strong**	(++++)
pBin61		
1	heavily infected, stunted, curled, green**, ChlH silencing: weak**	(++++)
2	stems yellow/recovered, one plant strongly stunted, other tall, **ChlH silencing: moderate**	(++++)
3	stems and veins yellow, small leaves, strongly yellow flakes, **ChlH silencing: moderate**	(++++)
P1-RYMV		
1	both plants green, heavily infected,**ChlH silencing (local): weak**	(++)
2	both plants very green, bushy, **ChlH silencing (local): weak**	(++)
3	both plants very green, bushy, one plant tall, other plant smaller, **ChlH silencing (local): weak**	(++++)

P1-CfMV		
1	both plants heavily infected, bushy. **ChlH silencing (local): weak**	(+++)
2	both plants heavily infected, bushy, **ChlH silencing (local): weak**	(+++)
3	one plant small and silenced, one plant tall, infected, **ChlH silencing (local): weak**	(+++)
TBSV P19		
1	both plants green, heavily infected, one plant with **ChlH silencing: moderate**, other plant with **ChlH silencing: strong**	(+)
2	both plants green, heavily infected, distortion of leaves**, ChlH silencing: weak**	(++++)
3	one plant tall, **ChlH silencing: moderate**, other plant smaller with **ChlH silencing: mild**	(++)
PVX P25		
1	both plants tall, green, heavily infected, **ChlH silencing: mild**	(++++)
2	both plants stunted, heavily infected, **ChlH silencing: mild**	(++)
3	both plants tall and green, heavily infected, **ChlH silencing (local): moderate**	(+++)
PVY HcPro		
1	heavily infected, upper leaves very narrow, **ChlH silencing: no**	(+++++)
2	stunted, heavily infected, very green, apical leaves very small, **ChlH silencing: no**	(++++)
3	stunted, heavily infected, very green, apical leaves very small**, ChlH silencing: no**	(++)
ACMV AC2		
1	both plants heavily infected, taller than wt-*N. benthamiana*, **ChlH silencing (local): mild**	(++)
2	one plant stunted, **ChlH silencing (local): mild**, other plant tall, bushy, leaves strongly curled, distorted, **ChlH silencing (local): moderate**	(++)
CMV 2b		
1	both plants very bushy, heavily infected, distorted leaves, **ChlH silencing (local): mild**	(++++)
2	both plants very bushy, heavily infected, distorted leaves, **ChlH silencing: mild**	(++++)
3	both plants very bushy, heavily infected, distorted leaves, **ChlH silencing (local): mild**	(+++++)

The TMV-ChlH infection responses varied between sibling plants of the different transgenic lines, but in most of the plants some level of silencing suppression was observed. Especially, in the P1-RYMV and P19 lines, where the transgene mRNA remained below detection level, the positive silencing suppression response was evident. Generally, all the selected lines except HcPro, which is known as strong viral suppressor, reduced the infection level of the TMV-ChlH, as compared to control plants. Interestingly, 2b siblings, which were highly mRNA positive, had no effect on the virus accumulation level, although the silencing of the ChlH-endogene was reduced also in these plants. It was not possible to quantify the degree of silencing, or silencing suppression, as these features

appeared to vary in the course of the infection. This type to variation in TMV-ChlH infections has been reported also earlier (Hiriart *et al.,* 2003). The plants displayed severe symptoms soon after inoculation, but with the passage of time, started to show first mild symptoms, then totally recovered phenotypes, and then re-activation of infection. The timing of the phenotypes varied and fluctuated over time, according to the changing balance between virus replication and silencing. This appears to be the reason of the silencing suppression variations observed also in these studies. Different tissues from different leaves were displaying different symptoms, and also accumulating different levels of virus. Thus it was not possible to quantify the silencing levels, but qualitative effect of ChlH-silencing and silencing suppression was observed in most of the cases.

4.2.2.2. crTMV-GFP

As the quantification of TMV-ChlH silencing or silencing suppression was not possible, I wanted to test the suppression activity with a different virus construct that does not so heavily interfere with the silencing dynamics. The crTMV-GFP construct is very suitable to observe silencing suppression effects as, according to Kurihara and Watanabe (2004), crTMV itself does not suppress silencing, at least in *Arabidopsis* (which is a natural host of crTMV). A major benefit is also that the level of its accumulation (replication vs silencing) can be quantified from the size and brightness of the GFP-lesions. As this construct infects *N. benthamiana* locally but is barely infectious in *N. tabacum,* analysis was carried out only in *N. benthamiana*.

In a total of three experiments, the crTMV-GFP inoculum was agro-infiltrated into small lesions in two leaves of 2-4 *N. benthamiana* plants (see Materials and Methods), and the development of GFP-expressing viral lesions was observed for the course of 2-3 weeks. Again, the strength of the silencing suppressor effects on crTMV-GFP infections varied to some extent between sibling plants, but still it was clear that the different silencing suppressors affected the crTMV-GFP accumulation in specific ways. The spread and the intensity of the GFP lesions was enhanced, indicating that the RNA silencing was positively suppressed in P1-RYMV, P19, AC2 and 2b expressing transgenic lines. The P19 transgene even enhanced the spread of infection into new infection foci and to the upper leaves of the plants (Fig. 2), indicating that the plants expressed the active silencing suppressor, although the transgene mRNA remained below detection level in these plants. In the P1-CfMV expressing lines the transgenic mRNAs were positively detected, but they did not show any difference in crTMV-GFP spread, as compared to the control plants. Surprisingly, the HcPro and P25 expressing lines, showing high transgene mRNA accumulation and specific transgenic phenotypes, did not enhance, but rather reduced the crTMV-GFP proliferation (Fig. 4, Paper II).

These varied reactions indicate that the tested suppressors affected differently the accumulation of this virus in cells, and also its spread in plant tissues. In particular, HcPro, known to be a strong silencing suppressor, was expected to enhance viral infections, but the observations were the opposite. However, these results confirm previous observations that HcPro of TEV, expressed in transgenic tobacco plants enhances their resistance against TMV, and against *Tobacco black ring virus* (Pruss *et al.,* 2004).

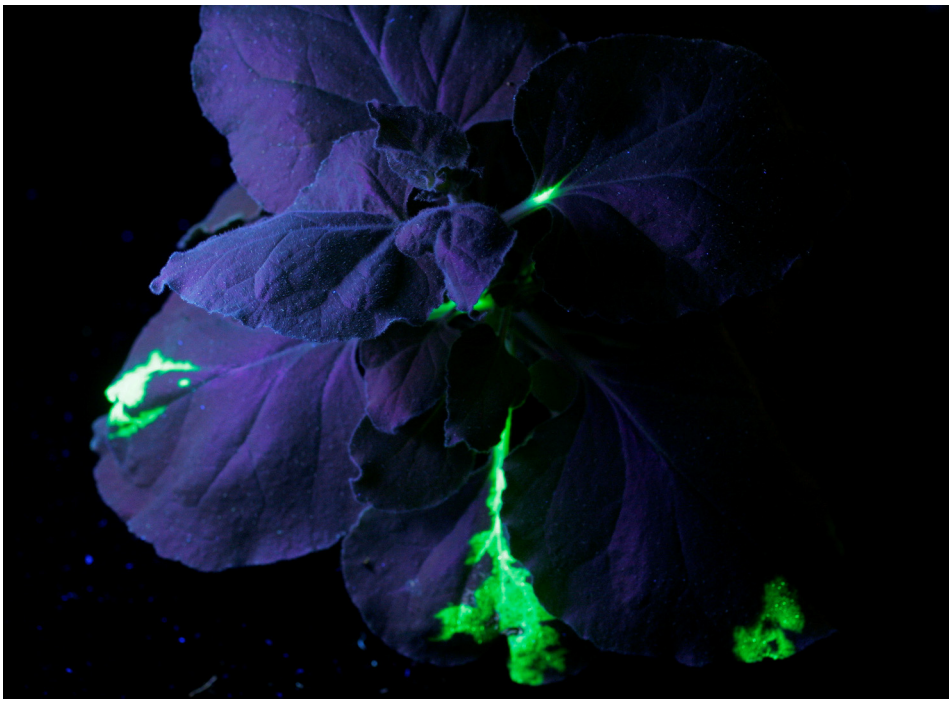


Figure 2. Spread of crTMV infection to upper leaves in P19 transgenic plant

4.2.3. Silencing suppressor effects on infections of different viruses

To see if the viral suppressors used in this study have any effect on infection, accumulation rate, symptom development and disease severity of different viruses, plants from selected lines were challenged with the same virus and with different heterologous viruses.

4.2.3.1. Responses against TRSV

The calico strain of the nepovirus TRSV produces very clear initial ringspot symptoms, with obvious later recovery in *N. benthamiana* hosts. The reaction of *N. tabacum* plants to this virus is strongly dependent on the growth conditions, and the plants may or may be susceptible, depending on the growth conditions. To see, whether the silencing suppressors could block the recovery of *N. benthamiana* plants from infection by TRSV *calico*, and whether the susceptibility and symptom production are enhanced by these silencing suppressors in *N. tabacum*, selected lines of both *Nicotiana* species were inoculated with this virus.

In *N. benthamiana,* all transgenic and wt plants exhibited typical ringspot symptoms within a week after inoculation (Fig. 1 A, Paper III). High virus accumulation was confirmed by northern blot analysis from these symptomatic leaves (Fig. 2, Paper III). Inoculated wt and vector-transformed control plants, as well as P1, P19, AC2 or 2b expressing plants recovered from early infection (Fig. 1 B, Paper III). At 5 weeks pi the virus levels were strongly reduced in the upper leaves of all these plants, although not as much in the transgenic plants as in the wt or in the pBin61 transformed control plants (Fig. 2, Paper III)

suggesting that the silencing was partly suppressed in these transgenic plants. Only very low levels of virus-specific siRNAs were detected in the systemically infected leaves of these plants at 7 and 40 dpi (Fig. 3, Paper III). In contrast, the infected P25 and HcPro expressing plants continued to show strong symptoms until the end of the experiment (40 dpi), and the northern analysis from systemically infected leaves indicated that these plants were not recovering from the infection (Fig. 2, Paper III). In addition, TRSV-specific siRNAs were detected from systemically infected leaves of these plants both at 7 and at 40 dpi, correlating with the high level of viral RNA (Fig. 3, Paper III).

In non-transformed *N. tabacum*, TRSV infection was strongly dependent on the growth conditions. Under low temperature (18 ^{o}C) and normal light conditions (200 μ mol m^{-2} s^{-1}) for 16 hours a day, the plants were susceptible to the virus, and developed infections with strong virus accumulation and systemic spread, and with severe ringspot symptoms. Northern analysis showed that in these conditions, the virus RNA accumulated to high levels in the systemically infected leaves at 10 and 40 dpi (Fig. 4 B, Paper III). Also at 25 ^{o}C temperature, with higher illumination during the mid-day hours, all the plant became systemically infected and accumulated high level of the viral RNA. Under these conditions the transgenic plants, expressing the different viral silencing suppressor genes accumulated the viral RNA at similar levels as did the wt and pBin61 transformed *N. tabacum* plants. In spite of the equal virus RNAs accumulation in the wt and transgenic plants, the specific oak-leaf pattern symptoms we observed only in some of transgenic plants, and particularly in plants expressing the P1 and P19 genes.

On the other hand, when plants were grown under conditions slightly above 26 ^{o}C, or under high temperatures (33 ^{o}C), with 200 μ mol m^{-2} s^{-1} illumination for 16 hours a day, the wt plants were resistance against the virus, and no virus symptoms and viral RNA was detected upon inoculation. In these preventative conditions, at a temperature of about 26 ^{o}C, prominent RNA accumulation was detected in the inoculated leaves of the all the transgenic plants, except for the P19 expressing plants, where the detection of the viral RNA varied between different experiments. (Fig. 5, Paper III). Virus RNA accumulated also in the systemically infected leaves of HcPro and AC2 expressing transgenic plants to levels equal to the inoculated leaves, and to somewhat variable levels in the systemic leaves of P1-RYMV or 2b expressing plants (Fig. 5, Paper III). It is possible that the lack of TRSV accumulation in the P19 or P25 expressing *N. tabacum* lines was due to the low expression level of these transgenes in the selected lines (Paper II). The transgenic plants infected under these conditions did not show any symptoms, except for some very mild ringspots occasionally occurring in the HcPro transgenic plants. No siRNAs were detected either from locally or systemically infected leaves of any of the plants.

Silencing suppressors are assumed to increase the plant susceptibility to other invading viruses. However, the interactions of different silencing suppressors with heterologous viruses are not always so straight forward and even the same suppressor can produce different effects under different conditions (Pruss *et al.,* 1997 & 2004). In these experiments we observed that the susceptibility of the *N. tabacum* plants to TRSV depended strongly on the growth conditions, but in the limiting conditions, the silencing suppressors significantly assisted in the establishment of the systemic infections.

The varied response of TRSV calico strain in the different transgenic *N. benthamiana* lines was perhaps due to the different modes of action of different viral suppressors. The data suggest that except for P25 and HcPro transgenes, all other silencing suppressors could not block the systemic silencing, and the recovery was induced in these plants. The lack of the siRNAs apparently correlated with the absence of the genomic viral RNAs in these tissues. The high virus-specific siRNAs accumulation in the leaves of the P25 and HcPro expressing plants, at 7 dpi, indicated active silencing. However, these plants did not recover from the virus symptoms, and at 40 dpi, both the virus RNA, and virus-specific siRNA accumulation in these plants was as high as early in the infection. Thus, the silencing continued in the presence of the silencing suppressors in these systemically infected plants, apparently, in balance with the viral replication and silencing suppression as it was still not able to induce recovery of the plants.

The data suggest that the wt *N. tabacum* plants were susceptible to TRSV in principle, but under restrictive conditions, the infection appeared to be strongly repressed by the silencing-mediated defense reaction. As in *N. benthamiana*, HcPro was an adequately strong suppressor able to stop the local silencing reaction also under the restrictive growth conditions, at elevated temperatures, to allow the initial infection and to prevent recovery. Also the AC2 silencing suppressor allowed the initial infection and the systemic spread of the virus, and prevented recovery in this host. As in *N. benthamiana,* 2b, P1 and P19 were able to suppress the local silencing process in this host, but did not allow the systemic spread under the restrictive conditions, suggesting that they failed to stop the induction of systemic silencing and the recovery of plants in these conditions.

4.2.3.2. TMV infection

TMV is one of the most studied plant viruses. In *N. benthamiana* it causes rapid systemic necrosis and kills the plants, while in *N. tabacum* it causes severe systemic mosaic symptoms. The synergistic effects of TMV were first studied in 1963 by Murakishi and Honma who showed that TMV enhanced the PVX infection in tomatoes. To see how different viral suppressor proteins affect TMV infections, selected lines were inoculated with wt TMV. All *N. benthamiana* transgenic and control plants inoculated with TMV developed symptoms 3 days post inoculations (dpi), and died one week later. Symptoms displayed by the control plants and by the transgenic plants were similar and viral RNA levels were equal in all the tested plants (Fig. 2 A, Paper IV).

N. tabacum transgenic plants, inoculated with wt-TMV, developed mosaic symptoms 5 dpi, at the same time with the non-transgenic control plants. All symptoms were initially very similar, but after four weeks of inoculation, very specific symptoms appeared in some of the transgenic lines. Transgenic plants expressing the 2b gene induced classical filiformism (shoe-string symptoms), which are typical in mixed infections of TMV and CMV. P1-RYMV transgenic plants also showed similar, although not as severe symptoms as the 2b transgenic plants, and P1-CfMV transgenic plants exhibited somewhat different leaf malformation, as the leaves in these plants were bifurcated rather than shoe-string like (Fig. 2 B, Paper IV). In spite of these different symptoms the virus titers were homogenous in all the inoculated plants (Fig. 2 C, Paper IV). These results are analogous with those obtained by

Shams-Bakhsh *et al.* (2007), showing that HcPro from PVY did not have any effect on TMV accumulation. My results showed that the heterologous silencing suppressors do not have any effect on TMV replication and accumulation, and indicate that the TMV on its own has very efficient means to escape from silencing. The special synergistic effects of 2b and P1 proteins on TMV symptoms, i.e. disturbance of the leaf blade differentiation, indicate that these factors allowed the TMV infection to invade the plant meristem.

3.2.3.3. PVX infection

PVX is the most studied virus in synergy experiments, and its infection was shown to be enhanced significantly when it was co-infecting plants with one of the potyviruses (Damirdagh & Frank, 1967; Vance *et al.,* 1995; Yang & Ravelonandro, 2002).

Five days after PVX, virus RNA accumulation and symptoms were enhanced in all the *N. benthamiana* transgenic plants as compared to wt controls. At the second sampling time, at 20 dpi, virus RNA levels remained higher in all the transgenic lines as compared to wt control plants. Virus RNA levels remained the same in P19 and P1-CfMV expressing plants and were increased in HcPro expressing plants during the infection (Fig. 3 A, Paper IV). At this time, HcPro transgenic plants showed very severe symptoms (Fig. 3 B, Paper IV). In the rest of the transgenic plants, virus RNA levels were reduced as compared to the early infection, indicating that the plants were recovering to some extent from the infection (Fig. 3 A, Paper IV).

In *N. tabacum,* all the transgenes enhanced the accumulation of viral RNA at the early sampling time, at 5 dpi as compared to control plants. Throughout infection, HcPro, AC2 and 2b transgenic plants showed more severe symptoms than the wt and pBin61 control plants. No differences were observed in the symptom severity of P19 or P1 expressing plants. Interestingly, the P25 expressing plants showed even milder symptoms than the control plants, suggesting that the virus and the transgene may have been co-silenced to some extent. Northern blot analysis at this time (20 dpi) from systemic leaves indicated that the transgenic plants appeared to have recovered from the early infection to some extent, except for the HcPro, AC2 and 2b expressing plants, where the viral RNA levels remained similar as were in the early samples. Also in wt plants, RNA levels remained at similar low levels. In the other plants, the levels were reduced to the same levels as in the wt control plants. (Fig. 3 C, Paper IV). Earlier, it was shown that PVX infection could be enhanced by synergistic co-infection only if it was either co-infected with PVY potyvirus, or when PVY infection was established before PVX inoculation (Damirdagh & Frank, 1967). Our data confirm that constitutive expression of HcPro transgene in all plants cells effectively prevents silencing of PVX, indicating that the responses in PVX infection are very dynamic and strongly affect the outcome of the disease symptoms.

4.2.3.4. PVY infection

The PVY isolate used in this study causes severe mosaic and rolling in *N. benthamiana* leaves, and rolling of leaves and necrotic lesions on the inoculated leaves of *N. tabacum*, but no systemic necrosis in either of these tobacco species.

N. benthamiana transgenic plants infected with this PVY strain started to show moderate systemic symptoms at 5 dpi. Through the early infection the symptoms in HcPro expressing plants remained milder than in the wt plants. Symptoms were observed until 20 dpi and at this time all plants showed very severe symptoms. Northern analysis showed that all the transgenes, except for P1-RYMV and HcPro, strongly enhanced the virus RNA accumulation in the early stage of the infection. The HcPro transgene totally silenced the virus in the beginning, but in all plants the virus eventually accumulated to the same high level (Fig. 4 A, Paper IV).

In *N. tabacum* all plants started showing necrotic lesions in the inoculated leaves after 5 days of PVY inoculation. Through the next week of infection, P1-RYMV reduced, but all other transgenes enhanced the symptoms as compared to wt plants. However, at 20 dpi, after establishing systemic infections, most of the plants had recovered from PVY symptoms to some extent, although HcPro, AC2, 2b and P1-CfMV expressing plants were still showing mild systemic symptoms (Fig. 4 B, Paper IV). Northern blot results of the early samples (5 dpi) indicated that the initial infection in AC2 and P1-CfMV expressing transgenic plants was low and remained at the same level throughout the later sampling time (20 dpi). The rest of the plants had high initial virus levels, but the levels were significantly reduced during the course of infection (i.e. plants recovered from early infection) except for pBin61 control and HcPro expressing plants, which retained fairly high virus levels throughout the infection (Fig. 4 C, Paper IV). The observed interference of HcPro with PVY infection is in accordance with earlier results where HcPro from cowpea aphid-borne mosaic virus enhanced the infection of the parental virus in transgenic plants early in infection, and the severe initial symptoms were followed by brief recovery and subsequent re-establishment of infection (Mlotshwa *et al.,* 2002). This effect may be related to the mode of action of HcPro. As a locally functioning suppressor, it can not prevent systemic silencing, when expressed from the replicating viral genome. Thus, the systemic silencing can activate the systemic defence, which leads to efficient restriction of viral movement, and recovery of the plants. However, when this local suppressor is expressed in all plant tissues, it prevents the establishment of this systemic silencing stage.

Among the tested silencing suppressor transgenes, only HcPro was able to enhance the systemic infection of TRSV, PVX and PVY, and to maintain the infection at high levels throughout the late infection times in both *Nicotiana* hosts. Also P1-CfMV and P19 genes, in *N. benthamiana* and the AC2 and 2b genes in *N. tabacum* could prevent, to some extent, the reduction of PVX RNAs during systemic infection. In addition, the P25 transgene in *N. benthamiana* plants, and the AC2 transgene in *N. tabacum* plants prevented the recovery of plants from the TRSV infections. These host-virus and silencing suppressor interactions may have been affected, to some extent, by the different expression levels of the transgenes in different plants. Nevertheless, our results suggest that there is considerable variation between the different silencing suppressors in the two *Nicotiana* hosts, except for the HcPro, which effectively prevented silencing mediated defences in both species.

5 CONCLUSIONS

The defenses mechanism operating in the plants against invading viruses, and the plant-viurs interactions in infection establishment are very complex phenomenon. The functions of viral genomes and individual viral genes can be effectively studied by using transgenic plants, which express either the intact, or differently modified virus genomes, or individual viral genes. Such transgenic plants allow expression of different combinations of viral proteins and RNA, either independently from the virus spread or encapsidation, or in combination with external viral infections. Suppressor proteins, encoded by different viruses can easily suppress the host defenses and affect the host phenotype and virus symptom in the host. Based on my results obtained, using different transgenic plants, I have concluded that;

Young plants can exert more resistance to viral infection and symptoms development against endogenously expressed viral transcripts.

Plants possess more than one defense mechanisms to protect themselves against different pathogenic attacks. Some of these defense mechanisms are still unknown.

Viral suppressors cause developmental disturbances in transgenic plants. The effects of viral suppressors are dependent on the specific suppressor-host combination.

The balance between the plant defense mechanism and viral replication potential, with the aid of silencing suppressors, is very fine-tuned.

Different suppressors affect differently the silencing functions targeted against the virus accumulation or cell-to-cell and systemic apread of silencing signal, and establishment of the systemically silenced stage and recovery of the plants.

N. benthamiana are more sensitive and susceptible to both endogenously expressed suppressors and against exogenous virus infections, compared to *N. tabacum* and infection establishment and symptoms development in *N. tabacum* can be affected by temperature.

6 FUTURE PERSPECTIVES

In the present study, I have tried to answer some key questions, but still some more important questions needed to be addressed.

TMV infection is very lethal to *N. benthamiana* plants and kills them quickly after the infection. It would be of great importance to transform *N. benthamiana* wt-TMV and mutated versions (either with the Replicase-, Movement-, or Coat Protein genes deleted) to see if these plants can show some resistance to this aggressive virus. Also by using different mutated forms of wt-TMV, the hypothesis "TMV might have more than one viral suppressor" can be answered.

The transgenic plants with different silencing suppressors can be used to elucidate the functions of these viral suppressors. I would like to study the questions such as: How they cause developmental disturbances, and do all transgenes have some effects on histology or developmental differentiations? Do the silencing suppressors affect the entry of silencing signals into the plant meristems? At what stage do different suppressors act in silencing pathways? How do they help viruses to overcome systemic silencing? If two transgenic plants, carrying different viral suppressor are crossed, how would these two transgenes interfere for altering plant functions or susceptibility?

ACKNOWLEDGEMENTS

First of all, I am grateful to The Almighty Allah, The infinitely Compassionate and Merciful, for all of His countless blessings. He is the One Who has the power to do anything, and Who encompasses all things with His Knowledge.

I would like to thank the Head of the Department, Prof. Eevi Rintamäki, for solving my academic problems, and I am indebted to Prof. Eva-Mari Aro for her support during my work.

I would like to express my sincere thanks to my supervisor, Docent Kirsi Lehto, for providing me with the possibility to work on this project and for her support during these years. Surely, without her kind help, it would have been very hard for a plant pathologist to become a molecular biologist.

I am grateful to Profs. Paula Elomaa and Peter Palukaitis for their critical reading of the thesis and their valuable comments.

My gratitude also goes to Dr. Saif Khalid, who introduced me to the world of plants viruses and laid down the foundation.

Elina, Kurt, Maisa and Mika, I do not have the words to thank you all for your endless and patient help with non-scientific matters just as important as the scientific ones.

I warmly thank all current and previous colleagues at the University Hill and in BioCity, especially Alexey, the only other full-time member in our group.

I am very grateful to all my friends, especially to my little Asian community here in Turku, for all the respect and help, for all the parties and fun moments. Shabash, Phate chuck k rakho. This work would have much more difficult without your warm support. Bohat bohat shukria.

I cordially thank my Pakistani and all my Finnish families (the Lehtos, the Orpanas, the Suorantas) for their unconditional love and support and for inspiring my interest in natural sciences.

I dedicate this thesis to my mother; without your prayers, I could have never done this.

This work was carried out in the Laboratory of Plant Physiology and Molecular Biology, University of Turku and was supported financially by the Academy of Finland and the Finnish Cultural Foundation.

Shahid Aslam Siddiqui
Turku, December, 2007

Nobody is perfect and I am NOBODY

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