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Suppressors of RNA silencing in plants

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Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination.

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CECILIA SARMIENTO

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INTRODUCTION

During the past fifteen years, our view of eukaryotic gene regulation has changed in a remarkable way, due to discoveries in plants and animals that revealed a novel mechanism of RNA-mediated gene silencing. RNA silencing collectively refers to the suppression of gene expression through sequence-specific interactions that are mediated by RNA. This mechanism is involved in the control of endogenous genes' expression during development and growth, maintenance of genome stability, as well as antiviral response in both animals and plants.

Viruses and their hosts have co-evolved and this is reflected by the diverse range of viral proteins coded to counteract the RNA silencing mechanism. These proteins are known as viral suppressors of RNA silencing. During the last ten years, many different viral suppressors have been described, particularly for plant viruses. The study of these proteins has provided important knowledge about the RNA silencing mechanism itself. Therefore, the research on viral suppressors is not only meant to develop tools for controlling the viral infections but it is also a suitable way to gain an understanding about a mechanism which can be exploited as a powerful biotechnology instrument with countless promising applications.

The fact that RNA silencing is involved in many different processes implies that it is finely tuned to act when, where and how it is needed. As a consequence, there are also negative regulators coded by the host itself, known as endogenous suppressors of RNA silencing. Up to now, few such suppressors have been described in both plants and animals.

Taking into account how useful the study of suppressors is for knowing more about RNA silencing, the goal of the present study was to identify in plants new viral as well as endogenous suppressors. The aim also included the analysis of the effects of transgenically expressed suppressors on plant phenotype and viral infection.

ABBREVIATIONS

abRNA	aberrant RNA
ADP	adenosine diphosphate
AGO	Argonaute protein
ATP	adenosine triphosphate
cDNA	complementary DNA
DCL	Dicer-like protein (*Arabidopsis*)
DIG	digoxigenin
DNA	deoxyribonucleic acid
dpi	days post-infiltration
ds	double-stranded
EST	expressed sequence tag
GFP	green fluorescent protein
GST	glutathione *S*-transferase
His-tag	hexa histidine-tag
HR	hypersensitive response
miRNA	microRNA
mRNA	messenger RNA
nat-siRNA	natural antisense transcript-derived siRNA
NBD	nucleotide-binding domain
nt	nucleotide
NUS	NusA protein from *Escherichia coli*
OD	optical density
ORF	open reading frame
^{32}P	phosphorus 32 radioactive isotope
PCR	polymerase chain reaction
PKR	protein kinase dsRNA-activated
Pol	polymerase
rasiRNA	repeat-associated siRNA
RdDM	RNA-dependent DNA methylation
RDR	RNA-dependent RNA polymerase (*Arabidopsis*)
RdRp	RNA-dependent RNA polymerase (viral)
RdRP	RNA-dependent RNA polymerase (cellular)
RISC	RNA-induced silencing complex
RLI	RNase L inhibitor
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RPA	RNase protection assay
RT-PCR	reverse transcriptase PCR
SA	salicylic acid
siRNA	small or short interfering RNA
sRNA	small RNA

ss	single-stranded
tasiRNA	trans-acting siRNA
T-DNA	transfer-DNA (*Agrobacterium tumefaciens*)
TGS	transcriptional gene silencing
UV	ultra violet
wt	wild-type

ORIGINAL PUBLICATIONS

The present dissertation is based on the following publications, which will be referred to in the text by their Roman numbers.

I **Sarmiento, C., Nigul, L., Kazantseva, J., Buschmann, M., Truve E.** (2006) AtRLI2 is an endogenous suppressor of RNA silencing. *Plant Molecular Biology* **61**, 153-163

II **Sarmiento, C., Gomez, E., Meier, M., Kavanagh, T. A., Truve, E.** (2007) *Cocksfoot mottle virus* P1 suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum*. *Virus Research* **123,** 95-99

III **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*. *Molecular Plant-Microbe Interactions* **21**, 178-187

1. REVIEW OF THE LITERATURE

1.1. RNA silencing

RNA silencing was first described in plants following attempts to overexpress key enzymes in the anthocyanin biosynthesis pathway in transgenic petunia (Napoli *et al.,* 1990; van der Krol *et al.,* 1990). Contrary to expectation, the flowers' pigmentation was not enhanced, but reduced and so was the mRNA level of the endogenous gene. The phenomenon was termed "co-suppression", because both the transgene and the endogenous gene were suppressed. In addition, in fungi, a transient inactivation of gene expression was observed when *Neurospora crassa* was transformed with homologous sequences and this mechanism was called "quelling" (Romano and Macino, 1992). Later, the fact that transgenic expression of viral sequences protected plants from viral infection was also found to be based on RNA silencing (Dougherty *et al.,* 1994; Angell and Baulcombe, 1997). As the silencing in virus-infected plants operates at the RNA level, the effect was termed "post-transcriptional gene silencing". The observation by Fire and colleagues (1998) that double-stranded (ds) RNA was a potent trigger for RNA silencing in the nematode *Caenorhabditis elegans* suggested a simple approach for efficient induction of RNA silencing in *C. elegans* and other organisms. This kind of RNA silencing was named "RNA interference" (RNAi) and in 2006 the Nobel Prize in medicine or physiology was awarded to A. Fire and C. Mello for the discovery of this mechanism. Nowadays, RNAi is a widely used research tool in reverse genetics with promising perspectives at the therapeutic level.

In 1999, Hamilton and Baulcombe reported that RNA silencing in plants was accompanied by the appearance of 25 nt short or small interfering RNAs (siRNAs). Thereafter, Dicer, the ribonuclease-III (RNase III) type enzyme that processes long dsRNA into siRNAs during the initiation of RNA silencing, was identified (Bernstein *et al.,* 2001). At about the same time, a target-specific nuclease complex called RISC (RNA-induced silencing complex) was purified from *Drosophila melanogaster* cells and found to be associated with siRNAs, which confer the specificity to the enzyme through homology to the substrate mRNA (Hammond *et al.,* 2000). Thus, RNA silencing was already understood as a mechanism blocking gene expression through sequence-specific interactions triggered by dsRNA and targeted to single-stranded (ss) RNA.

RNA silencing can be considered an evolutionarily conserved process that operates through diverse pathways. It is an important defense mechanism against viruses at least in plants and invertebrates. Furthermore, it also regulates endogenous gene expression, transposon taming and heterochromatin formation (Brodersen and Voinnet, 2006). Following, I will describe the most important components of the RNA silencing machinery.

1.1.1. Dicer and Dicer-like enzymes

Drosophila Dicer and its homologs cut dsRNA, the RNA silencing inducer, into 21-26 nt long sRNA (small RNAs) molecules characterized by a double-stranded body with 5'-phosphate and 3'-hydroxyl ends and a 2 nt 3' overhang on each strand of the duplex (Fig. 1a). Dicer enzymes are multidomain proteins that contain putative RNA helicase, PAZ (Piwi/Argonaute/Zwille), two tandem RNAse III, and one or two dsRNA-binding domains (Bernstein *et al.*, 2001; Hutvagner *et al.,* 2001; Tang *et al.,* 2003; Fig. 1b). There are at least four proteins homologous to *D. melanogaster* Dicer in *Arabidopsis thaliana*, one in *C. elegans* and one in mammals (Bernstein *et al.*, 2001; Provost *et al.*, 2002; Tang *et al.*, 2003). *Drosophila* itself has two Dicer paralogs Dcr1 and Dcr2. The first produces micro RNAs (miRNAs) while Dcr2 products are siRNAs (Hammond, 2005). For human Dicer, it has been shown that the enzyme processes siRNAs preferentially starting from the ends of the dsRNAs (Zhang *et al.*, 2002). According to the model of its activity, both RNase III domains form one single dsRNA cleavage center. Dicer then cuts the dsRNA from its terminus in ~20 nt long fragments, measuring this length through recognition of 3' overhang by the PAZ domain (Zhang *et al.*, 2004).

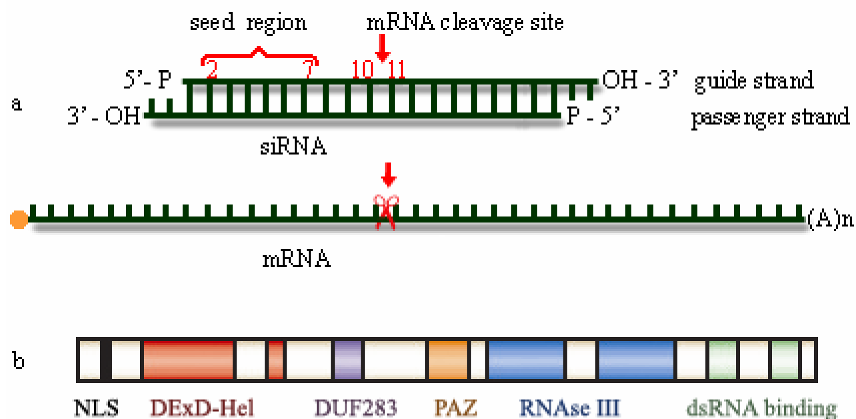


Fig. 1. Dicer and its product (siRNA)
a) siRNA duplex schematically represented. b) *Arabidopsis thaliana* DCL1with its domains (adapted from Chapman and Carrington, 2007).

In the case of *A. thaliana*, the diverse Dicer homologs act in different RNA silencing pathways but a clear hierarchical redundancy has been observed in their functions (Gasciolli *et al.*, 2005; Deleris *et al.*, 2006). Dicer-like protein 1 (DCL1) primarily synthesizes miRNAs (Bartel, 2004), which are normally 21 nt in length, but can be also longer, up to 24 nt (Kurihara and Watanabe, 2004). This protein was previously called Carpel Factory (CAF) or Short Integuments 1 (SIN1) or Suspensor 1 (SUS1), a protein needed for normal flower and ovule morphogenesis, for flowering time control and for embryogenesis (Golden *et al.*, 2002). DCL2, DCL3 and DCL4 produce siRNAs of 22, 24 and 21 nt, respectively. DCL3 acts in the nucleus and is involved in RNA-dependent DNA methylation (RdDM) with the production of 24 nt long siRNAs guiding chromatin silencing (Xie *et al.*, 2004; Matzke and Birchler, 2005). DCL4 is involved in endogenous *trans*-acting siRNAs' (tasiRNAs) production (Gasciolli *et al.*, 2005; Yoshikawa *et al.*, 2005). Moreover, this DCL protein produces the 21 nt long siRNAs that are part of the plant cell-to-cell silencing signal and are also involved in the amplification of the systemic silencing signal (Dunoyer *et al.*, 2005). DCL2 and DCL1 generate natural antisense transcript-derived siRNA (nat-siRNA) of 24 and 21 nt in lenght, respectively (Borsani *et al*., 2005). Usually, the products of DCL2 cut are 22 nt long, but the length of nat-siRNAs is an exception. Another role for DCL2 is to act together with DCL4 in transitivity, downstream from RDR6, one RNA-dependent RNA polymerase (RdRP; Moissiard *et al.*, 2007). The difference in size between DCLs' products may arise from intrinsic structural characteristics of the enzymes or from needed factors that associate with the different DCLs (Qi and Hannon, 2005).

The redundancy of different DCL functions is evident in the case of *Arabidopsis* mutants lacking one or more DCL paralogs. Thus, DCL1 is able to produce 21 nt tasiRNAs in the absence of DCL2, DCL3 and DCL4 (Bouche *et al.*, 2006). DCL4 can be replaced also by DCL2 or by DCL3 in the production of tasiRNAs, which turn to be 22-24 nt long, instead of 21 nt in lenght (Gasciolli *et al.*, 2005; Xie *et al.*, 2005; Bouche *et al.*, 2006). Interestingly, an antagonist function has been reported for DCL2, which usually acts compensating other DCLs. In this case, DCL2 partially inhibits DCL1 miRNA and tasiRNA production (Bouche *et al.*, 2006). DCL1 has not been reported to be replaced by another DCL and null-mutations in *DCL1* are embryo-lethal (Ray *et al.*, 1996).

DCL enzymes not only produce endogenous sRNAs but they also participate in defense mechanisms against invading nucleic acids such as transgenes or viruses. siRNAs from inverted-repeat transgenes are produced by DCL4 (Dunoyer *et al.*, 2005) and recently, DCL1 and DCL3 were also found to be needed. It seems that DCL1 cuts the hairpin produced by the transcript derived from the inverted-repeat and this facilitates the siRNA production by DCL3 and DCL4 (Dunoyer *et al.*, 2007). Virus-derived siRNAs are produced by different DCLs. DCL2 and DCL4 are the major and minor producers of *Turnip crinkle virus* siRNAs (Bouche *et al.*, 2006). In the case of *Cabbage leaf curl virus*, a DNA virus, DCL2, DCL3 and DCL4 generate 22, 24 and 21 nt long siRNAs, respectively (Blevins *et al.*, 2006). Besides all other DCL proteins, DCL1 is involved in the production of 21 nt long siRNAs from the 35S leader region of the DNA virus *Cauliflower mosaic virus* (CaMV). Apparently, DCL1 is required for the accumulation of DCL3- and DCL4-

dependent siRNAs generated from the 35S leader, because it excises hairpins from that region, facilitating the further access by the other DCLs (Blevins *et al.*, 2006; Moissiard and Voinnet, 2006). The primacy of DCL4 in the production of viral siRNAs has been reported for *Oilseed rape mosaic virus*, *Cucumber mosaic virus* (CMV) and *Tobacco rattle virus*, all RNA viruses (Blevins *et al.*, 2006; Bouche *et al.*, 2006; Deleris *et al.*, 2006). DCL enzymes tend to compensate their functions also in the case of virus-derived siRNAs. It is worth mentioning that in the case of DNA viruses, the accumulation of 24 nt long siRNAs, produced by DCL3, is stronger than in the case of RNA viruses, where it is only observed in the case of mutants lacking DCL4 and DCL2 (Blevins *et al.*, 2006; Bouche *et al.*, 2006; Deleris *et al.*, 2006).

Interestingly, it has been reported that poplar encodes five DCLs and rice six (Margis *et al.*, 2006).

1.1.2. RISC complex

Once Dicer has produced the siRNAs or miRNAs, these molecules enter a protein complex in order to guide it to complementary mRNAs, the target to be silenced. The RISC complex contains Argonaute proteins (AGO), which are composed of four domains: the N-terminal, PAZ, Middle and PIWI domains. PAZ are small domains (~ 140 residues) found in AGO and Dicer enzymes, whereas PIWI domains are present only in AGO proteins. Purification of minimized RISC with cleavage activity suggests that AGO proteins are the major component of the complex (Martinez *et al.,* 2002). The crystal structure of PfAgo, AGO from the archae *Pyrococcus furiosus*, revealed that the PIWI domain is structurally related to the RNase H family of ribonucleases (Song *et al.,* 2004). Crystal structures of AfPiwi (Piwi of *Archaeoglobus fulgidus*) in complex with siRNA-like duplexes have provided structural perspective on the importance of the 5' end of the siRNA strand which confers the sequence-specificity to the complex, the so-called "guide" strand (Ma *et al*., 2005; Parker *et al*., 2005). The 5' nt of the guide strand is unpaired and bound in a basic pocket where the first base forms a stacking interaction with a conserved aromatic position in AGO proteins. The 5′ phosphate group is bound directly to this pocket by side chains of four conserved residues and by a divalent cation. This phosphate group is important for the stability of the complex and in addition, it may be important for the fidelity of the position of mRNA cleavage (Rivas *et al.,* 2005). The PIWI domain recognizes phosphate groups of the first to the fourth or fifth bases of the guide strand. No contacts are made to the additional RNA backbone or bases. The contact with the mRNA target strand is minimal. The mRNA target is always cleaved at the phosphodiester bond between the bases complementary to the 10th and 11th positions of the guide siRNA (Elbashir *et al,* 2001a, 2001b; Haley and Zamore, 2004; Martinez and Tuschl, 2004). This cleavage site is one helical turn displaced from the end of the siRNA molecule (Elbashir *et al,* 2001a). The target cleavage step itself is ATP independent and leaves the siRNA intact, allowing RISC to function as a multiple-turnover enzyme. Although ATP is not essential for cleavage, the turn-over of RISC is faster in the presence of ATP, which indicates that ATP can drive product release,

promote a conformational step that restores RISC to a productive ground state, or both (Haley and Zamore, 2004).

The PAZ domain is a modified oligonucleotide/oligosaccharide binding fold and recognizes the 2 nt 3' overhangs of siRNAs through a binding pocket (Lingel *et al.,* 2004; Ma *et al.,* 2004). Although PAZ domains share little sequence homology, the binding cleft for the 3' end of the oligonucleotide is hydrophobic and is mediated by conserved aromatic residues (Rashid *et al.*, 2007). Tomari and Zamore (2005) proposed a model for the target recognition by AGO: the 5' end of the guide siRNA binds to the target mRNA while the 3' end remains bound to the PAZ domain. After that, the 3' end of the siRNA is released from the PAZ domain to pair to the target mRNA. The siRNA-mRNA pairing surface is probably provided by a positively charged cleft that extends from the 3' terminal RNA-binding site of the PAZ domain into the PIWI domain (Lingel *et al.,* 2004; Ma *et al.,* 2004).

It has been shown that the second RNase III domain of Dicer interacts with the PIWI domain (Tahbaz *et al.*, 2004; MacRae *et al.*, 2006). Recently, the AGO of the bacterium *Aquifex aeolicus* was crystallized in the presence of ss siRNA (Rashid *et al.*, 2007). The model based on this study proposes that the siRNA just cleaved by Dicer, enters AGO through the S-groove located between the PIWI and the N-terminal domains, which is positively charged. After that, the passenger strand is cleaved and the guide strand is orientated with the 5' end anchored in the PIWI domain and the 3' end fixed to the PAZ domain. The orientation of the guide strand is facilitated by a conserved residue in the PAZ domain (Arg195, in the case of *A. aeolicus*). Interestingly, a tendency for the PAZ domain, and to a lesser extent the N-terminal domain, to change conformations, leading to a widening and narrowing of the M groove has been observed. The shift in the case of the PAZ domain is of 24°. Free AGO is flexible and it is plausible that after formation of the RISC complex, the flexibility of the PAZ domain is restricted. The rigid status of PAZ may allow for recognition of the incoming target mRNA as well as discrimination between perfect and imperfect base pairing in the seed region of the guide strand. The target mRNA may enter the AGO through the M or the S groove; this remains to be tested (Rashid *et al.*, 2007, Fig. 2).

RISC cleavage ("slicing") results in products having ends similar to those of RNase H-type enzymes' products, this means, a 5' product carrying a 3' hydroxyl and a 3' product with a 5' phosphate. To perform these reactions, RISC uses a conserved Asp-Asp-His motif located in the PIWI domain, for divalent metal ion (Mg^{2+}) binding and catalysis (Schwarz *et al.*, 2004; Rivas *et al.*, 2005). This motif is moderately degenerated and therefore it could be considered as Asp-Asp-Asp/Glu/His/Lys (Tolia and Joshua-Tor, 2007). Argonautes can be divided into three clades: AGO-like, PIWI-like and group III also called WAGOs. AGO-like and PIWI-like proteins division is based on the similarity to AtAgo1 (AGO1 from *A. thaliana*) and DmPiwi (Piwi from *D. melanogaster*), respectively (Carmell *et al.*, 2002). WAGOs are worm-specific and predominantly contain Argonautes that do not have the Asp-Asp-His motif (Yigit *et al.*, 2006). Of the eight human Argonaute family members, four (HsAgo1, HsAgo2, HsAgo3 and HsAgo4) have been tested for activity and only HsAgo2 is a Slicer, that means an endonuclease responsible for the cleavage of the target mRNA (Liu *et al.*, 2004; Meister *et al.*,

2004). Both HsAgo1 and HsAgo2 are involved in RNA silencing and in transcriptional gene silencing (TGS; Janowski *et al.*, 2006).

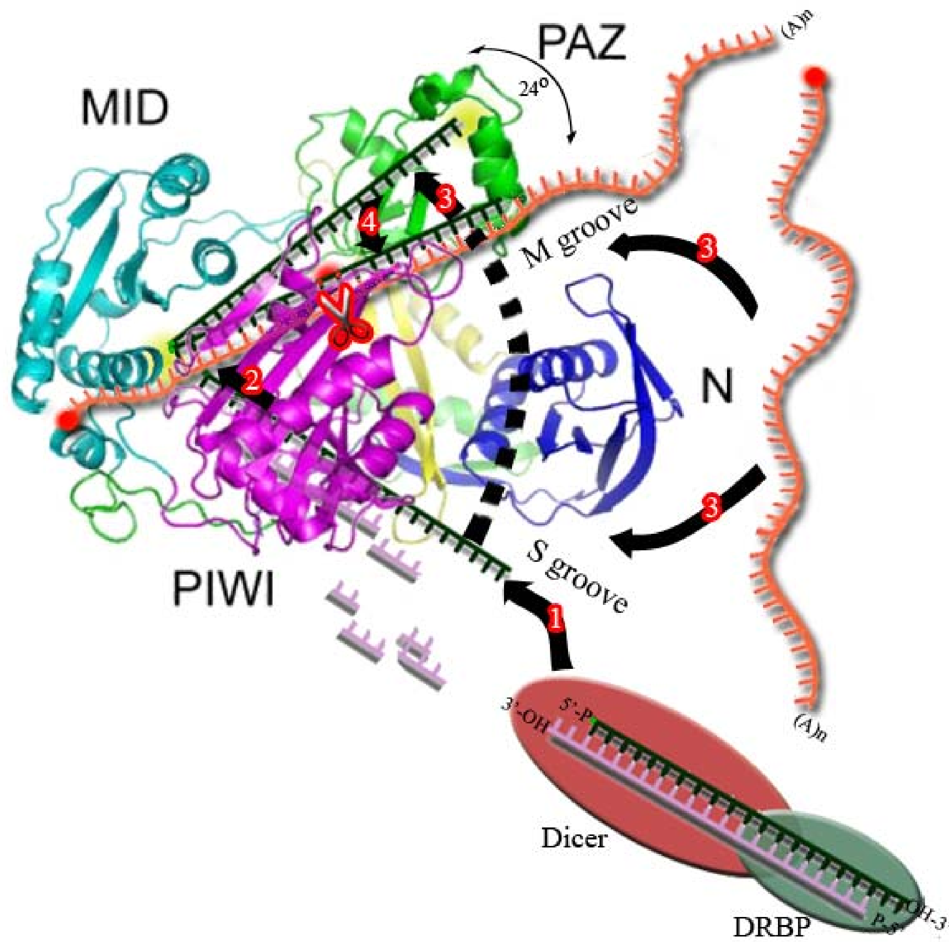


Fig. 2. Hypothetical catalytic activity of *Aquifex aeolicus* AGO
AGO crystal structure is represented as in Rashid *et al.* (2007). The other proteins and RNA molecules are schematically represented. Steps are more simultaneous than sequential:
1) siRNA enters AGO in complex with Dicer and dsRNA-binding protein (DRBP). 2) 5' end of the guide strand of the siRNA (black) is orientated to the binding pocket of the PIWI domain (yellow spot). Passenger strand (violet) is cleaved. 3) 3' end of the guide strand is orientated to the binding pocket of the PAZ domain (yellow spot). Target mRNA (orange) enters AGO. 4) 5' end of the guide strand pairs to the target mRNA. Both RNA molecules (guide strand and mRNA) move to the active site in the PIWI domain (red spot). RNA-RNA pairing is complete. AGO stays rigid. Cleavage of the target mRNA takes place between 10th and 11th nt of the guide strand (small red points, scissors point to the cleavage site).

The 27 *C. elegans* AGOs can be partially divided in primary and secondary AGOs, according to the siRNAs they are binding to (primary or secondary). RDE-1 is the primary AGO, with the Slicer activity. Secondary AGOs, with redundant functions are SAGO-1, SAGO-2 and PPW-1 (Yigit *et al.*, 2006). ALG-1 and ALG-2 are needed for miRNA processing (Grishok *et al.*, 2001). An emerging theme is the importance of AGO proteins for germline maintenance and function. In *C. elegans* four distinct groups of AGO genes are required for fertility: *csr-1*, *prg-1*/*prg-2*, *alg-1*/*alg-2* and the multiple AGO mutant strain (MAGO) that includes *sago-1* and *sago-2* (Yigit *et al.*, 2006). PRG-1 and PRG-2 are clustered in the PIWI-like group and although PIWI-interacting RNAs (piRNAs) have been described only in *Drosophila*, zebrafish and mammals (Aravin *et al.*, 2007), it is tempting to relate these AGOs with those sRNAs.

Drosophila has five AGOs: DmAgo1, DmAgo2, DmAgo3, DmPiwi and DmAub. The first two are from the AGO-like proteins and the others from the PIWI-like proteins. Slicer activity has been demonstrated *in vitro* for all these proteins (Rand *et al.*, 2004; Miyoshi *et al.*, 2005; Saito *et al.*, 2006; Gunawardane *et al.*, 2007). AGO1 functions in miRNA processing and AGO2 in the siRNA pathway (Okamura *et al.*, 2004; Saito *et al.*, 2005). DmPiwi and DmAub (Aubergine) bind repeat-associated siRNAs (rasiRNAs) and therefore here rasiRNAs represent a subset of piRNAs (Saito *et al.*, 2006; Vagin *et al.*, 2006).

Schizosaccharomyces pombe has only one AGO which is not only a component of RISC but also of RNA-induced transcriptional silencing complex (Sigova *et al.*, 2004).

Notably, all 10 *Arabidopsis* Argonautes belong to the AGO-like group, having either a complete Asp-Asp-His motif or an Asp-Asp-Asp motif. Only two *Arabidopsis* AGOs have been tested and both (AtAgo1 and AtAgo4) possess Slicer activity (Baumberger and Baulcombe, 2005; Qi *et al.*, 2006). AGO7 (ZIPPY) is involved in the production of tasiRNA (Peragine *et al.*, 2004) as well as in the transition from juvenile to adult phase (Hunter *et al.*, 2003). AGO10 (PINHEAD/ZWILLE) has a role in meristem maintenance (Moussian, *et al.*, 1998; Lynn *et al.*, 1999). AGO1 associates with miRNAs in RISC and probably AGO10 is able to replace it (Vaucheret *et al.*, 2004). Interestingly, *AGO1* mRNA is subjected to negative feedback regulation through the action of miR168 (Vaucheret *et al.*, 2004), resembling the regulation of *DCL1* mRNA through miR162 (Xie *et al.*, 2003). The minimal *Arabidopsis* RISC contains AGO1 and an associated sRNA. AGO1 interacts selectively with sRNAs. It binds miRNAs, transgene-specific siRNAs, some virus-specific siRNAs and some tasiRNAs, whereas it does not associate with 24 nt long siRNAs involved in chromatin silencing. This selection could be dictated by the sRNA biogenesis, i. e. by the DCL producing one or another sRNA (Baumberger and Baulcombe, 2005). AGO4 binds siRNAs originated from transposable and repetitive elements. This AGO is involved in RdDM (Zilberman *et al.*, 2003; Chan *et al.*, 2004) and is localized to the nucleolus-associated Cajal bodies, which are centers for the processing of many ribonucleoparticles (Cioce and Lamond, 2005; Li *et al.*, 2006). AGO4 forms a ribonucleoprotein complex together with siRNA and NRPD1b, a subunit from RNA polymerase IV (Pol IV). NRPD1b interacts with AGO4 through its C-terminal domain (Li *et al.*, 2006). It seems that AGO4 has two distinct functions in

RdDM. On the one hand, this protein directs chromatin remodeling factors to target loci, probably through interactions between siRNAs and nascent transcripts. This process does not require the catalytic activity of AGO4. On the other hand, the catalysis is required for secondary siRNA production; i. e. cleavage of the target RNA by AGO4 may trigger RDR2 to synthesize a dsRNA that will be cut by DCL3 to produce secondary siRNAs (Qi *et al.*, 2006). AGO6 functions redundantly with AGO4 because both AGOs induce DNA methylation at transgene and endogenous promoters, causing TGS and accumulation of chromatin-related siRNAs. For some target loci the effect of AGO4 is stronger than the one of AGO6. AGO6 is important for cytosine methylation at all possible sequence contexts for some loci, but is only required for methylation at non-CpG sites for other loci (Zheng *et al.*, 2007). Although there is only one example, it is worth mentioning that in the case of one specific locus (*AtMu1*), AGO1 is involved in RdDM (Lippman *et al.*, 2003). This could be a further hint of redundant functions between different AGOs.

As already explained, Dicer interacts directly with AGO proteins. This has been demonstrated for *Drosophila*, *C. elegans* and human cells (Hammond *et al.,* 2001; Tabara *et al.,* 2002; Tahbaz *et al.,* 2004). A further interaction was recognized with the characterization of R2D2, a dsRNA-binding protein of *Drosophila* (Liu *et al.,* 2003). R2D2 heterodimerizes with Dcr2, the Dicer generating siRNAs in *Drosophila*, and only siRNAs binding to this heterodimer are channeled into RISC. Both Dcr2 and R2D2 directly contact siRNAs and form, probably with other factors, a precursor from RISC called RLC (RISC-loading complex) (Pham *et al.,* 2004; Tomari *et al.,* 2004a, 2004b; Liu *et al.*, 2006). All this shows that Dicer has a role downstream of dsRNA processing, namely in RISC assembly.

When the mRNA target is cleaved, only one strand of the siRNA incorporated into RISC is bound to AGO. This implies that the siRNA duplex must be unwinded before. Several putative helicases have been implicated in RNAi but none has been unambiguously linked to a discrete siRNA unwiding step. In *Drosophila* cells, AGO2 cleaves not only the target mRNA but also the "passenger" strand (anti-guide strand) of the siRNA duplex (Matranga *et al.,* 2005; Rand *et al.,* 2005). Thus, siRNAs are loaded into RISC as a duplex, not as a single strand. The RLC (at least R2D2 and Dicer) determines which 5' end of the siRNA duplex will be directed to the phosphate binding pocket of the AGO2 Piwi domain (Tomari *et al.,* 2004b) and once bound, AGO2 will cleave the passenger strand, triggering its dissociation from the complex. AGO2 receives the siRNA duplex first from Dicer, with whom it makes a protein-protein contact, and then from R2D2. Release of the passenger strand cleavage products may be facilitated by an ATP-dependent cofactor, much as release of the products of target cleavage facilitated by ATP (Haley and Zamore, 2004). Passenger strand cleavage by AGO2 is not obligatory but is the normal mechanism. This cleavage follows rapidly after AGO2 binds the siRNA duplex, but when this cleavage is blocked, a slower bypass pathway for RISC activation dissociates and destroys the passenger strand (Matranga *et al.*, 2005).

Concerning the composition of RISC complex, some forms of active RISC have been sufficiently purified to allow direct subunit identification by mass spectrometry. In other cases, components have been identified by Western blotting. Proteins that have been found to be in physical contact with active RISC are for

example: Gemin3 (putative human helicase; Hutvagner and Zamore, 2002; Mourelatos *et al.*, 2002), Dmp68 (putative *Drosophila* helicase; Ishizuka *et al.*, 2002), Tsn (Tudor and SNase domains in *Drosophila*; Pham *et al.*, 2004), human MOV10, a homolog of *Drosophila* Armitage (essential for assembling of miRNA-containing RISC; Cook *et al.*, 2004; Tomari *et al.*, 2004a; Meister *et al.*, 2005), human PRMT5 (arginine methyl-transferase; Meister *et al.*, 2005), etc. Moreover, large ribosomal subunit components have been found in *Drosophila* AGO2-containing complexes (Ishizuka *et al.*, 2002) and in the case of humans, a suggestive fraction of Dicer has precipitated at ~ 80S (Zhang *et al.*, 2004). Ribosome association might facilitate mRNA-target scanning by RISC *in vivo*, by exploiting the ability of ribosomes to disrupt secondary structures. Many of the proteins related to RISC localize to the same intracellular compartment where AGO2 is concentrated, namely the P-bodies (Sen and Blau, 2005).

1.1.3. RNA-dependent RNA polymerases

Although RNA silencing core reactions are mediated by Dicer and RISC, there are other key components needed for the different RNA silencing pathways. One of them are RdRPs. RdRPs are important for RNA silencing in several organisms, including plants, *C. elegans*, *S. pombe*, *N. crassa* and *Dictyostelium discoideum* (Tijsterman *et al.*, 2002). It seams that insects and vertebrates do not possess these enzymes (Wassenegger and Krczal, 2006). However, the rasiRNAs found in *Drosophila* have characteristics of so-called secondary siRNAs synthesized by RdRP in *C. elegans* (Vagin *et al.*, 2006; Baulcombe, 2007). The first RdRP isolated in plants was the one found in tomato by Schiebel and co-workers (1993a, 1993b, 1998).

Arabidopsis encodes six different RdRPs: RDR1, RDR2, RDR3a, RDR3b, RDR3c and RDR6 (Yu *et al.*, 2003; Wassenegger and Krczal, 2006). No established function has been attributed yet to RDR3s. RDR1 is induced as part of the virus defense response (Xie *et al.*, 2001; Yu *et al.*, 2003). RDR2 is implicated in the methylation of histones and silencing of certain repetitive DNAs such as SINE-like retroelement *AtSN1*. It is expressed in inflorescences and appears to have a role in the timing of flowering (Chan *et al.*, 2004; Matzke and Birchler, 2005). RDR6 (SDE1/SGS2) is necessary for sense transgene-mediated silencing (Beclin *et al.*, 2002) and is also important in antiviral silencing-based defense (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). This RDR is able to initiate RNA silencing when it perceives aberrant RNAs (abRNAs) spuriously produced by sense transgenes (Dalmay *et al.,* 2000), transposons or viruses (Mourrain *et al.,* 2000), and transforms them *de novo* into dsRNA. The absence of 5’ cap in a transgene mRNA has been shown to be recognized by RDR6 as abRNA (Gazzani *et al.,* 2004). Other missing features in an mRNA could render it aberrant, like for example the absence of poly(A) tail. Indeed, aborted viral transcription products frequently lack 5’ cap or poly(A) tail (Brodersen and Voinnet, 2006). In addition, identification of mutations in genes affecting splicing or 3’ end formation, enhance RNA silencing, providing more abRNAs (Herr *et al.*, 2006). Another important function of RDR6 is its involvement in the amplification of RNA silencing and in systemic silencing, which will be explained later. Furthermore, this RDR is

required for production of tasiRNAs (Peragine *et al.*, 2004; Vazquez *et al.*, 2004a) and for the amplification of siRNAs from many endogenous loci that form transcripts targeted by one or more miRNAs (Chen *et al.*, 2007; Howell *et al.*, 2007). The existence of six RDR paralogs in *Arabidopsis* suggests possible redundancy and specialization, as is the case for DCLs (Ding and Voinnet, 2007).

Three of the four *C. elegans* RdRPs have been studied until now (Grishok, 2005). EGO-1 is needed for germline-specific RNAi while RRF-1 is indispensable for somatic RNAi (Smardon et al., 2000; Sijen et al., 2001). RRF-3 competes with EGO-1 and RRF-1 for intermediates or components of the RNAi pathway (Grishok, 2005). RRF-1 is required for the accumulation of secondary siRNAs during amplification of RNA silencing. Recently, it has been demonstrated that these secondary siRNAs do not have features characteristic for Dicer products and therefore it has been suggested that RRF-1 itself is responsible for the production of these siRNAs (Pak and Fire, 2007; Sijen *et al.*, 2007).

1.1.4. RNA polymerase IV

Eukaryotes have three DNA-dependent RNA polymerases. Interestingly, *Arabidopsis* and rice have a fourth nuclear polymerase, Pol IV (*Arabidopsis* Genome Initiative, 2000) that is involved in RdDM. This polymerase exists as Pol IVa and Pol IVb complexes, which differ in the largest subunits NRPD1a or NRPD1b, respectively. Both have as second subunit NRPD2a. The C-terminal domain (CTD) of NRPD1b is about 500 amino acid residues longer than the one of NRPD1a (Matzke *et al.*, 2006). The extended CTD in NRPD1b contains a WG/GW-rich region that is necessary for its binding to AGO4 (Li *et al.*, 2006; El-Shami *et al.*, 2007). Pol IVa was shown to be implicated in the silencing of transposable elements and other repeated elements via siRNAs (Herr *et al.*, 2005; Onodera *et al.*, 2005). It is required for siRNA accumulation and therefore it is thought to be transcriptionally active, but the substrate is still uncertain. One possibility is that Pol IVa transcribes methylated DNA (Herr *et al.*, 2005). Another possibility is that this protein transcribes nascent RNA at the target locus (Pontes *et al.*, 2006). It is also not known if Pol IVb transcribes anything or if it only opens chromatin at the siRNA-targeted sites to expose them to methyltransferases (Kanno *et al.*, 2005). Each Pol IV isoform is associated with a SNF2-like factor, CLSY1 works together with Pol IVa and DRD1 acts with Pol IVb (Kanno *et al.*, 2005; Smith *et al.*, 2007). Pol IVa together with DCL3 and RDR2 are thought to carry out siRNA biogenesis, whereas Pol IVb and AGO4 are supposed to act downstream of Pol IVa triggering *de novo* DNA methylation at the siRNA-targeted site. The concerted action of both isoforms is needed for efficient silencing of transposons and highly repeated sequences (Pontier *et al.*, 2005; Li *et al.*, 2006; Pontes *et al.*, 2006). It is worth mentioning that NRPD1a, RDR2 and CLSY1 are required for the spread of the silencing signal between cells (Smith *et al.*, 2007).

1.1.5. Double-stranded RNA binding proteins

Proteins that specifically bind dsRNA are characterized by a conserved dsRNA-binding motif, which consists of ca 70 amino acid residues that form an α-β-β-β-α fold, whose two α-helices interface interacts with the dsRNA (Fierro-Monti and Mathews, 2000; Saunders and Barber, 2003).

Different Dicers interact specifically with dsRNA-binding proteins. In *C. elegans*, the dsRNA-binding protein RDE-4 binds long dsRNA with high affinity and interacts with DCR-1 and RDE-1. According to Tabara *et al.* (2002), RDE-4 and RDE-1 function together to detect and retain foreign dsRNA and to present it to DCR-1. RDE-4 dimerization is important for the assembly of active RDE-1/DCR-1, thus it is necessary for cleavage of dsRNA to siRNA (Parker *et al.*, 2006). *Drosophila* dsRNA-binding protein R2D2, as already explained, binds to Dcr2 to channel siRNA into RISC. In a similar way, Dcr1 interacts with Loquacious to process miRNAs (Förstemann *et al.*, 2005; Saito *et al.*, 2005). TRBP [*Human immunodeficiency virus-1* (HIV-1) transactivating response RNA-binding protein] is the human homolog of R2D2 (Chendrimada *et al.*, 2005; Haase *et al.*, 2005). Before discovering its role in RNA silencing, TRBP was known as a protein that inhibits protein kinase dsRNA-activated (PKR; Daher *et al.,* 2001) and modulates HIV-1 gene expression (Dorin *et al.,* 2003). The dsRNA-binding proteins seem to have a role in antiviral immunity (Ding and Voinnet, 2007). Mutations in *r2d2* enhance the accumulation of flock-house virus, cricket paralysis virus and *Drosophila* X virus (Wang X. H. *et al.*, 2006; Zambon *et al.*, 2006). *rde-4* mutations have the same effect in the case of vesicular stomatitis virus (Schott *et al.*, 2005).

A. thaliana has five dsRNA-binding proteins (DRB1/HYL1, DRB2-DRB5) that associate specifically to the different DCLs in order to optimize their activities. It has been suggested that the dsRNA-binding motif of DCL C-terminus plays a role in the interaction with other dsRNA-binding proteins but not with dsRNA directly (Hiraguri *et al.*, 2005). All DRBs have two dsRNA-binding domains in their N-terminal region and no additional catalytic domains (Hiraguri *et al.*, 2005). The dsRNA-binding activity of HYL1 has been biochemically identified and it is known as a nuclear protein regulating plant response to multiple hormones such as cytokinin, auxin and abscisic acid (Lu and Fedoroff, 2000). HYL1 also controls leaf morphology by regulating cell division, cell elongation and polarity, therefore the name “HYPONASTIC LEAVES 1” (Lu and Fedoroff, 2000). In RNA silencing HYL1 role is to bind DCL1 to catalyze the release of miRNAs (M. H. Han *et al.*, 2004; Vazquez *et al.*, 2004b). Apparently, HYL1 binding to DCL1 is mandatory for the processing of pre-miRNA to mature miRNA whereas it is not fully necessary for the production of pre-miRNA from pri-miRNA (Kurihara *et al.*, 2006; Dunoyer *et al.*, 2007; Wu *et al.*, 2007). DRB4 specifically binds to DCL4 for the biogenesis of tasiRNAs (Nakazawa *et al.*, 2007). DRB2 and DRB5 interacted *in vitro* with DCL3 (Hiraguri *et al.*, 2005). Probably, *Arabidopsis* requires five

DRB proteins to regulate different individual functions of the four DCLs (Nakazawa *et al.*, 2007).

1.1.6. Other components of RNA silencing pathways in plants

- **HEN1:** *Arabidopsis HEN1* (*Hua Enhancer 1*) was identified as a gene that plays a role in stamen and carpel identities during flower development (Chen *et al.*, 2002). Afterwards, its function in RNA silencing was discovered. Namely, this protein methylates the 2' hydroxyl groups at the 3' termini of miRNA and siRNA duplexes as well as tasiRNAs, protecting them from exonucleases' attack (Yu *et al.*, 2005; Li *et al.*, 2005). In the *hen1* mutant, miRNAs are reduced in abundance and when detectable are uridylated, a hallmark of miRNA destabilization (Li *et al.*, 2005). Plant viral siRNA are also methylated (Blevins *et al.*, 2006).

HEN1 contains a dsRNA-binding domain at the N-terminus and a catalytic domain at the C-terminus that transfers methyl groups from S-adenosyl methionine (SAM) to the ribose of the last nt of the sRNAs (Yu *et al.*, 2005). HEN1 homologs are found in bacterial, fungal and metazoan genomes (Park *et al.*, 2002). Recently, it has been shown that HEN1 homologs in mouse and rat methylate piRNAs at the 3' end (Kirino and Mourelatos, 2007; Ohara *et al.*, 2007). The *Drosophila* homolog of HEN1 does not contain the dsRNA-binding domain; nevertheless it methylates piRNAs and ss siRNAs (Horwich *et al.*, 2007).

- **SERRATE:** *SERRATE* regulates meristem activity and leaf axial patterning in *Arabidopsis* (Grigg *et al.*, 2005). It encodes a C_2H_2 zinc finger protein critical for the accumulation of multiple miRNAs and tasiRNAs (Lobbes *et al.*, 2006; Yang *et al.*, 2006). SERRATE localizes in the nucleus and interacts physically with HYL1 (Yang *et al.*, 2006). Fang and Spector (2007) have recently described the *in vivo* interaction of SERRATE, DCL1 and HYL1, all proteins related to miRNAs, which colocalize in discrete nuclear bodies, called D-bodies.

- **HASTY (HST):** it is the *Arabidopsis* homolog of mammalian Exportin 5, the nuclear export receptor that transports pre-miRNAs as well as tRNAs from the nucleus to the cytoplasm (Lund *et al.*, 2004). HASTY is required for the biogenesis or stability of some miRNAs in different plant tissues but there is no evidence for its role in miRNA export (Park *et al.*, 2005). This protein has been also found to be important in tasiRNA biogenesis (Peragine *et al.*, 2004).

- **SGS3/SDE2:** the function of this *Arabidopsis* coiled-coil protein is unknown. However, it has a clear role, together with RDR6, in the tasiRNA and nat-siRNA pathways as well as in the RNA silencing of sense transgenes and in transitivity (Mourrain *et al.*, 2000; Vazquez *et al.*, 2004a). Loss-of-function mutations in *SGS3* or in *RDR6* have a similar phenotype to *AGO7* mutants and together with *AGO7* and *HASTY*, these genes regulate vegetative phase change and floral development in *Arabidopsis* (Peragine *et al.*, 2004).

- **SDE3:** *SDE3* is one of the silencing defective loci encoding proteins required for sense-transgene RNA silencing (Dalmay *et al.*, 2001). SDE3 is an RNA helicase-like protein similar but at the same time clearly distinct from the SMG-2 RNA helicase, involved in RNA silencing in *C. elegans* (Domeier *et al.*, 2000). It is likely that this protein is required, together with RDR6, for the production of dsRNA because both RDR6 and SDE3 are needed for transitivity (Dalmay *et al.*, 2001, Himber *et al.*, 2003). However, unlike RDR6, SDE3 is dispensable for transgene RNA silencing, acting probably as an accessory factor to resolve secondary structures found in some RDR templates (Dalmay *et al.*, 2001). Hypothetically, SDE3 could act at other RNA silencing steps, because the *Drosophila* homologous protein Armitage is required for RISC assembly and flies lack RDRs (Tomari *et al.*, 2004a).

- **SDE5:** This protein has similarity to the human mRNA export factor TAP, which binds to nucleoporin complexes (Kang and Cullen, 1999; Schmitt and Gerace, 2001; Hernandez-Pinzon *et al.*, 2007). Another feature of this protein is the region of similarity to the PAM2 motif, characteristic of poly(A) binding proteins (Albrecht and Lengauer, 2004; Hernandez-Pinzon *et al.*, 2007). Because of these similarities, it is tempting to imagine SDE5 targeted to nucleoporins or RNA species in order to present them to RDR6 as substrate. Together with RDR6, SDE5 is required for sense-transgene RNA silencing and for the tasiRNA pathway (Hernandez-Pinzon *et al.*, 2007).

- **WEX: "**Werner syndrome-like exonuclease" is a 3'-5' exoribonuclease, related to the exonuclease domain of MUT-7 that is needed for RNAi and transposon silencing in *C. elegans* (Ketting *et al.*, 1999; Glazov *et al.*, 2003). WEX is indispensable for RNA silencing but not for TGS. It is possible that WEX is required to remove the endonuclease products from the RISC complex or it could act upstream from RISC (Glazov *et al.*, 2003).

- In the case of transcriptional silencing, there is a wide set of proteins involved. These are for example: cytosine DNA methyl-transferases CMT3, DRM1/2 and MET1 (Chan *et al.*, 2005); putative histone deacetylase HDA6 (Aufsatz *et al.*, 2002); histone 3 (lysine 9) methyl-transferases KYP and SUVH2 (Jackson *et al.*, 2002; Naumann *et al.*, 2005); chromatin remodeling factor DDM1 (Mittelsten-Scheid *et al.*, 2002; Brzeski and Jerzmanowski, 2003); factor for maintenance of intermediate heterochromatin MOM1 (Amedeo *et al.*, 2000; Habu *et al.*, 2006).

1.2. Different small RNA molecules and their silencing pathways

Although RNA silencing operates through different pathways generating diverse sRNAs, the core reactions carried out by Dicer/DCL and RISC are invariably present and the trigger is always dsRNA. The dsRNA can have different origins: it can be produced from an inverted-repeat transgene, from viral sequences or endogenous sequences which give rise to hairpins, from the product of an RdRP on an aberrant RNA coming from a sense-transgene, etc. (Brodersen and Voinnet, 2006). During the replication of RNA viruses, the RdRp produces replicative forms which are dsRNA (Voinnet, 2005a). Nevertheless, in the case of DNA viruses, the dsRNA originates via Pol II-driven transcription on the circular viral genome that could produce overlapping sense/antisense transcripts (Blevins et al., 2006). Indeed, the bidirectional Pol II promoters of geminiviruses generate converging transcripts (Hanley-Bowdoin et al., 1999; Shivaprasad et al., 2005). For many positive-strand RNA viruses, the dsRNA source is directly part of the highly structured single-stranded viral RNA (Molnar et al., 2005) and strikingly, the RISC-mediated cleavages occur on hot spots along the viral genome (Pantaleo et al., 2007).

The diversity of RNA silencing pathways reflects the wide spectrum of biological functions covered by this mechanism (Ding and Voinnet, 2007; Chapman and Carrington, 2007). The pathways known until now to exist in plants are summarized in Fig. 3 and the proteins known to be related to each pathway are presented in Table 1.

Figure 3. Different sRNA pathways in *Arabidopsis*
The scheme represents two cells connected through a plasmodesma (Pl.). The principal cell shows part of its nucleus (N) containing the plant DNA with different genes (*MIR*, *TAS*, *a*, *b*, *c*, *d* and transgenes). The proteins involved in TGS (in the light green shadow) are not shown, but histone 3 (H3) is represented with its methylated (-CH3) lysine 9 (K9). Two viruses are indicated in red, one in the nucleus and the other in the cytoplasm (C). Dark green arrows represent promoters. Red points and (A)n at the termini of the mRNAs represent cap and poly(A) tail, respectively. P-body is still hypothetical in plants. The relay amplification shown in cell 2 is in principle the same for primary siRNAs derived from viral RNA. The different pathways are depicted on different backgrounds. As the pathways share common proteins, they are supposed to be more interconnected than shown in this scheme. DRBP: dsRNA-binding protein; i: intron.

Table 1. Proteins involved in the different sRNA pathways in *Arabidopsis*

Proteins	Different sRNA pathways								
	viral siRNA	**siRNA from IR-transgene**	**siRNA from sense-transgene**	**secondary siRNA**	**miRNA**	**tasiRNA**	**nat-siRNA**	**lsiRNA**	**siRNA in TGS**
DCL	DCL1 DCL2 DLC3 DCL4	DCL1 DCL2 DCL3 DCL4	DCL3? DCL4?	DCL2 DCL4	DCL1 (DCL4)	DCL4	DCL1 DCL2	DCL1 DCL4	DCL3
DRBP	HYL1? DRB4?	DRB4	DRB4?	DRB4?	HYL1	DRB4	HYL1	HYL1 DRB4?	ND
AGO	AGO1	AGO1	AGO1	AGO1	AGO1	AGO1 AGO7	AGO1? AGO7?	AGO7 AGO1?	AGO4 AGO6 (AGO1)
RdRP	RDR6 (RDR1)	RDR2 (c-t-c)	RDR6	RDR6 RDR2 (c-t-c)	---	RDR6	RDR6	RDR6	RDR2
helicase	SDE3?	---	(SDE3)	SDE3	---	SDE3?	SDE3?	SDE3	---
methylase	HEN1	HEN1	HEN1	HEN1	HEN1	HEN1	HEN1	HEN1	HEN1
transporter	ND	ND	ND	---	HASTY	HASTY	HASTY?	HASTY	---
other	SGS3	NRPD1a (c-t-c) CLSY1 (c-t-c)	SGS3 SDE5	SGS3 SDE5 NRPD1a (c-t-c) CLSY1 (c-t-c)	SERRATE	SGS3 SDE5 SERRATE	SGS3 NRPD1a	Pol IVa Pol IVb XRN4	CMT3 MOM1 DRM1/2 FCA MET1 FPA HDA6 PolIVb KYP DRD1 SUVH2 PolIVa DDM1 CLSY1

DRBP: dsRNA-binding protein, IR: inverted-repeat, ND: non-determined, c-t-c: cell-to-cell movement, ( ) sometimes required,
--- non-required, ? possibly required

1.2.1. siRNAs

Already in 1999, Hamilton and Baulcombe identified siRNAs as the hallmark of RNA silencing. These sRNAs are directly derived from different dsRNA sources through the aforementioned action of Dicer/DCL enzymes. The siRNAs can be of endogenous, transgenic or viral origin.

In the case of plants and of *C. elegans* they can be classified in primary and secondary siRNAs, depending on their specific biogenesis during the transitivity process (Himber *et al.*, 2003). The transitivity refers to the way the target of silencing spreads within a single strand of RNA. First of all, the primary siRNAs are produced by Dicer from the trigger dsRNA. When these primary siRNAs have been incorporated into RISC and have guided the cleavage of the mRNA target, the secondary siRNAs are formed. In the case these secondary siRNAs correspond to regions adjacent to the target sites of the primary siRNAs, the silencing transits, i.e. transitivity occurs (Baulcombe, 2007). In such way, transitivity increases the initial pool of siRNAs and therefore amplification of silencing takes place, i.e. the silencing is maintained even without the presence of the original dsRNA inducer through secondary synthesis of dsRNA by RdRPs, using the siRNA-complementary target RNA as template. Remarkably, recent studies show that the amplification process differs in plants and *C. elegans* (Baulcombe, 2007). Namely, in the case of plants, secondary siRNAs production is optimal when the target RNA is cleaved by RISC at two target sites (Axtell *et al.*, 2006) and after this cleavage, the RdRP is recruited for the synthesis of dsRNA from which the secondary siRNAs are diced. In the case of *C. elegans*, however, the primary siRNA associated to RISC targets one site of the ssRNA and recruits the RdRP RRF-1 that transcribes directly the 22-23 nt long secondary siRNAs, which have a 5' triphosphate instead of the classical 5' monophosphate, hallmark of Dicer cleavage (Ruby *et al.*, 2006; Pak and Fire, 2007; Sijen *et al.*, 2007). Transitivity in *Arabidopsis* requires RDR6, DCL4 and DCL2, SGS3, SDE3 and SDE5 (Voinnet, 2005b; Ding and Voinnet, 2007; Hernandez-Pinzon *et al.*, 2007; Moissiard *et al.*, 2007). It is noteworthy that in plants and in *C. elegans*, amplification has been found sometimes related to epigenetic effects at the DNA or chromatin level (Voinnet *et al.*, 1998; Vastenhouw *et al.*, 2006).

The RNA silencing triggered by a virus can be a natural process occurring during viral infections or it can be a response to a viral vector used to artificially target the silencing of a gene whose sequence has been inserted into that vector. In both cases, silencing is a consequence of the defense of the infected host against the pathogen and therefore viruses are at the same time triggers and targets of RNA silencing, which give rise to virus-derived siRNAs. The accumulation of secondary virus-derived siRNAs in plants has been verified recently (Diaz-Pendon *et al.*, 2007). Observations from long ago, like recovery and cross-protection can be explained, at least to certain extent, by this same mechanism (Voinnet, 2005a; Hohn *et al.*, 2007). Plants are able to silence RNAs systemically, i.e. in a non-cell autonomous way, and one reason is the necessity to have an efficient defense

throughout the host. RdRPs influence how silencing moves through plasmodesmata and through the phloem. In the case of cell-to-cell movement of the RNA silencing signal through plasmodesmata, it is possible to distinguish between a short-range and a long-range movement (Himber *et al.*, 2003). The short-range signaling (10-15 cells in adult leaves and up to 35 cells in embryonic hypocotyls) means that the 21 nt long siRNAs produced by DCL4 move and for this movement, RDR6 and SDE3 are not needed (Himber *et al.*, 2003; Dunoyer *et al.*, 2005; Kobayashi and Zambryski, 2007). The long-range spreading results from reiterated short distance movement and in this case SDE3 and RDR6 are needed, as it implies synthesis of secondary siRNAs, i.e. relay-amplification (Himber *et al.*, 2003). RDR6-mediated amplification of the long distance silencing excludes viruses from meristems (Schwach *et al.*, 2005). For the short-range movement of the 21 nt long siRNAs, three "silencing movement deficient" genes (*smd1*, *smd2*, *smd3*) are required (Dunoyer *et al.*, 2005) and recently, *smd1* and *smd2* were found to be allelic to *RDR2* and *NRPD1a*. Probably, these two genes promote physical silencing spread between cells or facilitate its detection in recipient cells (Dunoyer *et al.*, 2007). *CLSY1* is also required for this cell-to-cell signaling (Smith *et al.*, 2007) and its action could be coupled to *RDR2*. The 21 nt long siRNA which are part of the cell-to-cell silencing signal are normally produced by DCL4. Nevertheless, in the case of an extreme dosage of dsRNA (e.g. in an RNAi experiment) the other DCLs can also produce siRNAs which will be incorporated into AGO1 and will then be transported to other cells (Dunoyer *et al.*, 2007). Yoo *et al.* (2004) showed that the PSRP1 protein, purified from cucurbit phloem sap, binds and facilitates the transport of ~25 nt long ssRNAs. The significance of this finding for the movement of RNA silencing signal is unclear (Voinnet, 2005b), but it is possible, that one strand of the siRNA duplex is selected by PSRP1 for transport, in an analogous way to the selection of one strand from the duplex for the binding to AGO (Xie and Guo, 2006).

The way how RISC determines which strand from the siRNA duplex will be bound to the specific AGO, and as a consequence, which mRNA will be targeted, is dictated by thermodynamic rules. In nature, small RNAs (siRNAs and miRNAs, explained later) tend to be thermodynamically asymmetric, that means, that the internal stability at both 5' termini of the duplex is different (Khvorova *et al.,* 2003; Schwarz *et al.,* 2003). In the case of *Drosophila*, R2D2 binds the 5' end of the strand with the greatest stability (passenger strand) while Dicer binds the 5' end of the strand that is less stable (guide strand). siRNA asymmetry is sensed by the Dicer/R2D2 heterodimer (Tomari *et al.,* 2004b). Tuschl and co-workers have shown that when Dicer generates a siRNA from one specific end of a longer dsRNA, only the strand with its 3' terminus at the processed end enters RISC (Elbashir *et al.,* 2001b). The fact that the direction of Dicer processing polarity influences strand selection is not clear (Preall *et al.,* 2006; Rose *et al.,* 2006). In addition to the termini, major stability differences are observed at other positions: between positions 9 to 14 from the guide strand the internal stability is low. An increased flexibility in this region where the cleavage occurs might be important

for the cleavage itself and for removing the products upon cleavage (Khvorova *et al.*, 2003). Zamore and co-workers have identified positions within a siRNA that are most sensitive to mismatches. They have reported that there are mismatches that confer siRNAs ability to discriminate between sequences that differ by a single nt (Schwarz *et al.,* 2006). Maximal discrimination is achieved when the siRNA:target RNA pairing is disrupted by a purine:purine mismatch. Mismatches in the central and 3' regions of the guide strand provide a high degree of single nt discrimination, consistent with the idea that target cleavage requires that these regions pair with their target RNA to form an A-form RNA:RNA helix (Chiu and Rana, 2002; Haley and Zamore, 2004). Mismatches in the seed region of the guide strand (2nd to 7th nt, important for target binding) do not confer discrimination power. These data are consistent with the view that mismatches between the seed and its target compromise only RISC binding, not cleavage, and can therefore be overcome by increasing the concentration of the siRNA (Haley and Zamore, 2004). Intriguingly, a purine:purine mismatch at siRNA nt 16 (guide strand) provides always a robust discrimination, suggesting that this position may play a biochemically distinct role in directing target RNA cleavage (Schwarz *et al.,* 2006).

Systemic RNAi in *C. elegans* is efficiently inherited to F1 generation and its most potent trigger is injected or ingested dsRNA (>100 bp). RDE-4 is required for inheritance and the inherited agent seems to be siRNA (Grishok *et al.*, 2000). Systemic RNAi can affect all tissues but notably neurons are an exception (Fire *et al.*, 1998). SID-1 is a transmembrane protein that enables long dsRNA uptake in target organs (Winston *et al.*, 2002). As already mentioned, RRF-1 is required for the accumulation of secondary siRNAs in *C. elegans*. Unlike the situation in plants, where transitivity occurs normally in a bidirectional way relative to the target mRNA (Vaistij *et al.*, 2002; van Houdt *et al.*, 2003), the transitivity in *C. elegans* usually occurs upstream of the targeted sequence (Sijen *et al.*, 2001; Alder *et al.*, 2003). Primary targets may be degraded, but they remain intact for a sufficient period to allow RRF-1 activity upstream – and less efficiently downstream – of the targeting site (Pak and Fire, 2007). A recent publication reports that the transitivity in *Arabidopsis* can be initiated by a primary sRNA that acts as primer for RdRP instead of directing cleavage. In this case, transitivity proceeds from 3' to 5' and the amount of produced secondary siRNAs decreases towards the 5' end of the targeted mRNA. DCL4 and DCL2 act in this process downstream from RDR6 (Moissiard *et al.*, 2007).

The lack of RdRPs in arthropods and vertebrates have prompted the idea that these organisms lack systemic RNA silencing, but this is still a question mark, since in many cases a systemic effect has been observed in mice and also in *Drosophila* and other arthropods (Voinnet, 2005b and references therein). Interestingly, when human SID-1 ortholog was overexpressed, it facilitated the rapid cellular uptake of siRNAs (Duxbury *et al.*, 2005).

1.2.2. miRNAs

miRNAs are a large family of endogenous, small regulatory RNAs with a wide range of cellular functions such as differentiation, development (Reinhart *et al.*, 2000; Grishok *et al.*, 2001; Bernstein *et al.*, 2003; Li and Carthew, 2005), metabolic homeostasis (Poy *et al.*, 2004) and memory (Ashraf *et al.*, 2006). They were first identified in *C. elegans* as “small temporary RNAs” (Lee *et al.*, 1993). Later, three different laboratories reported at the same time the identification of a large number of sRNAs of the same kind in different animals. Thus, this new class of sRNAs was named miRNAs (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Soon after this, miRNAs were discovered also in plants (Reinhart *et al.,* 2002). In addition, miRNAs are present in the single-celled eukaryote *Chlamydomonas reinhardtii* (Molnar *et al.*, 2007) and in a number of mammalian viruses, e.g. Epstein-Barr virus (Pfeffer *et al.*, 2004), human cytomegalovirus (Dunn *et al.*, 2005) and herpes simplex virus type 1 (Cui *et al.*, 2006).

Up to now, more than 5000 miRNA loci from 58 different species have been found and over 500 000 target transcripts for all miRNAs in 24 species have been predicted. Today it is clear that many miRNAs are clade- or even organism-specific and not highly conserved as thought before. miRNAs target predominantly transcription factors and in the case of predicted human miRNAs, more than 50 % of them are localized in cancer-associated genomic regions or in fragile sites (Calin *et al.*, 2004). Computational predictions of miRNA targets suggest that up to 30 % of human protein coding genes are regulated by miRNAs (Rajewsky, 2006). miRNAs are often clustered close together. Interestingly, only 5-10 % of *Arabidopsis* miRNAs overlap annotated transcripts, while 40-70 % of vertebrate miRNAs appear to locate to introns of protein- and non-coding transcripts (Griffiths-Jones *et al.*, 2007).

The biogenesis of miRNAs is different in plants and animals. In the case of animals, miRNAs are generated in two separate steps: long primary miRNAs (pri-miRNA) are processed in the nucleus by a complex containing the RNase-III enzyme Drosha and the dsRNA-binding domain protein DGCR8 (known as “Pasha” in *Drosophila*) to ~ 60-70 nt long precursor miRNA (pre-miRNA) intermediates (Denli *et al.,* 2004; Gregory *et al.,* 2004; J. Han *et al.,* 2004). These hairpin-shaped pre-miRNAs are transported through Exportin 5 to the cytoplasm, where they are cleaved by Dicer to generate ~ 22 nt long miRNAs (Hutvagner *et al.,* 2001, Ketting *et al.,* 2001). After Dicer processing, miRNAs emerge as siRNA-duplex-like molecules, designated as miRNA/miRNA*, which are incorporated into RISC. The miRNA itself is found on the 5’ or on the 3’ arm of its pre-miRNA stem-loop. Most of the animal miRNAs are only partially complementary to their target RNAs. They are thought to repress the expression of the targets through blocking translation because of imperfect pairing with the 3’ UTR region, or to accelerate target mRNA decay by recruiting components of more general RNA turnover pathways (Hutvagner and Zamore, 2002; Valencia-Sanchez *et al.*, 2006).

It appears that siRNAs and miRNAs, both in animals and plants, may be functionally interchangeable, i.e. some siRNAs may act like miRNAs repressing translation, and some miRNAs may degrade RNA of the target the way siRNAs do (Llave *et al.,* 2002; Doench *et al.,* 2003; Zeng *et al.,* 2003). The fact that animals and plants have in general more than one Dicer and many AGO proteins is partially explained considering that different Dicers and AGOs are specialized for RNA silencing involving siRNAs or miRNAs. This has been proven for *Drosophila*, where usually Dcr1 and AGO1 take part in the miRNA pathway while Dcr2 and AGO2 are the key enzymes in the siRNA pathway (Okamura *et al.,* 2004). Recently, one *Drosophila* miRNA (miR-277), which resembles a siRNA because of its practically perfect pairing feature, has demonstrated that although it is processed by Dcr1 together with Loquacious, it is afterwards bound to Dcr2 and R2D2 in order to be loaded into AGO2. This corroborates on the one hand that the dicing and the AGO-loading are two separate steps. On the other hand, it clearly shows that the kind of duplex determines to which AGO the sRNA will be bound and also which kind of silencing mechanism will follow. The mechanisms could be cleavage of the target mRNA in the case of AGO2 or repression of target's expression in the case of AGO1, which is an enzyme with less efficient Slicer activity (Förstemann *et al.*, 2007; Tomari *et al.*, 2007). Some *Drosophila* and *C. elegans* miRNAs bypass Drosha processing when they are formed from a spliced intron that mimics the loop of a pre-miRNA, called "mirtron" (Ruby *et al.*, 2007; Okamura *et al.*, 2007).

The miRNA biogenesis differs from the one occurring in animal cells. In *Arabidopsis*, it depends specifically on DCL1, which works like animal Drosha, converting pri-miRNA to pre-miRNA; afterwards DCL1 cuts the pre-miRNA to obtain the mature miRNA (Kurihara and Watanabe, 2004). The miRNA/miRNA* duplex is methylated by HEN1 and transported to the cytoplasm with a possible intervention of HASTY. In the cytoplasm the miRNA* strand is degraded while the other strand – miRNA – is incorporated into RISC (Jones-Rhoades *et al.*, 2006). Plant miRNAs tend to pair to their mRNA targets with near-perfect complementarity and act the same way siRNAs do, namely via cleavage of the target through AGO1 (Llave *et al.,* 2002; Bartel and Bartel, 2003). Nonetheless, in the case of miR172, which has a near-perfect complementarity to its single target *APETALA2*, translational repression appears to take place (Chen, 2004). The stem-loops of plant miRNAs are much more variable in size and typically larger than the ones in animals. There is also more pairing between the miRNA sequence and the other arm of the stem-loop (Bartel, 2004). It is worth mentioning that at least in the case of miR822 and miR839, the processing of the miRNA is carried out by DCL4 instead of DCL1, and it has been hypothesized that during the course of miRNA genes' evolution, an adaptive switch from DCL4 to DCL1 processing has occurred (Rajagopalan *et al.*, 2006). miRNAs have important roles, as evidenced by the strong developmental defects caused by several miRNA overexpression or loss-of-function mutants (Bartel, 2004). It has been shown that plant miRNAs accumulate spatially and temporally in a coordinated manner, proving that they have a role in

cell-fate determination and differentiation (Valoczi *et al.*, 2006). These sRNAs usually target transcription factor gene families related to developmental patterning or cell differentiation (Rhoades *et al.*, 2002). They regulate for example the accumulation of transcription factors necessary for the leaf shape (Palatnik *et al.*, 2003), abaxial-adaxial leaf asymmetry (Kidner and Martienssen, 2004), number of petals (Baker *et al.*, 2005), etc. Moreover, regulatory elements of the plant response to auxin are controlled by miRNAs (Mallory *et al.*, 2005). Surprisingly, miR393 regulates negatively mRNA for auxin receptors and this repression of auxin signaling inhibits *Pseudomonas syringae* growth in *Arabidopsis* (Navarro *et al.*, 2006). Furthermore, abiotic stresses, like oxidative stress, have been shown to induce expression of miRNAs (Sunkar *et al.*, 2006).

1.2.3. tasiRNAs

These endogenous plant sRNAs derive from specific *TAS* loci and arise by phased, DCL processing of dsRNA formed by RDR6/SGS3 activity on RNA polymerase II non-coding transcripts (Peragine *et al.*, 2004; Vazquez *et al.*, 2004a). miRNA directed cleavage is needed for initiating tasiRNAs' biogenesis because it recruits RDR6-mediated transitivity on the primary tasiRNA (pri-tasiRNA) cleavage products, allowing dsRNA production of one of the fragments (Allen *et al.,* 2005; Yoshikawa *et al.,* 2005). The phased 21 nt long tasiRNAs negatively regulate other genes targeting mRNAs the way miRNAs do, and HEN1 also takes part in this pathway (Allen *et al.*, 2005). Specific tasiRNAs from at least four families function as do miRNAs (Chapman and Carrington, 2007 and references therein). RDR6 action is linked to SDE5 in tasiRNA biogenesis (Hernandez-Pinzon *et al.*, 2007). DCL4 – together with DRB4 – is the enzyme cutting the phased tasiRNAs (Gasciolli *et al.*, 2005; Xie *et al.*, 2005; Adenot *et al.*, 2006). It can be replaced by other DCLs, but if that is the case, the correct phasing is lost (Gasciolli *et al.*, 2005; Bouche *et al.*, 2006; Howell *et al.*, 2007). Strikingly, the DCLs cut only starting from the dsRNA end corresponding to the initial miRNA cut site (Allen *et al.*, 2005). tasiRNAs co-immunoprecipitate with AGO1, and in addition, AGO7 has been shown to be involved in tasiRNAs' production (Qi *et al.*, 2005; Adenot *et al.*, 2006). Pri-tasiRNA cleavage can take place in the nucleus, because *TAS1* and *TAS2* pri-tasiRNAs reside within introns (Brodersen and Voinnet, 2006). Thus, it is not surprising that Peragine *et al.* (2004) found that HASTY is involved in this pathway and so is SERRATE (Lobbes *et al.*, 2006; Yang *et al.*, 2006).

Recently, a case was reported were one tasiRNA directed the cleavage of another pri-tasiRNA, meaning that there was a cascade in the biogenesis of sRNAs: one miRNA induced the production of a tasiRNA and this one provoked the biogenesis of another tasiRNA (Chen *et al.*, 2007).

It is noteworthy that genes that are regulated by miRNAs, the *PPR*s mRNAs, are highly represented tasiRNA targets in both *A. thaliana* and *Populus trichocarpa*. PPRs are pentatricopeptide repeat proteins, i.e. putative RNA binding proteins with repeats of 35 amino acid motives that play a role in RNA processing,

stability or translation in chloroplasts or mitochondria (Lurin *et al.*, 2004; Howell *et al.*, 2007).

tasiRNAs have been found not only in higher plants but also in moss (Axtell *et al.*, 2006; Talmor-Neiman *et al.*, 2006).

1.2.4. nat-siRNAs

This class of siRNAs arises in plants from overlapping transcripts induced by biotic or abiotic stresses. This pathway finely tunes physiological responses to for example salt stress, in the case of the first nat-siRNA described (Borsani *et al.*, 2005). Nat-siRNAs guide the cleavage of one of the two parent transcripts to induce an adaptation to stress or a defense reaction against a pathogen (Katiyar-Agarwal *et al.*, 2006).

In the case of the salt stress, DCL2 cuts a 24 nt long primary nat-siRNA from the overlapping region in such an intriguing way that only one specific endogenous sRNA is produced. For this step RDR6, SGS3 and NRPD1a are needed but the reason is not clear. Thereafter, this 24 nt long nat-siRNA guides the cleavage of the same target transcript and secondary 21 nt long nat-siRNAs are produced by DCL1. For this second step in addition to RDR6, SGS3 and NRPD1a, also HEN1 is needed. The function of these secondary nat-siRNAs is not obvious, since only the presence of the primary nat-siRNA is enough to downregulate the target RNA and control salt tolerance (Borsani *et al.*, 2005).

The nat-siRNA induced by *Pseudomonas syringae* pathovar tomato carrying the avirulence gene *avrRpt2* is 22 nt long and only DCL1 is involved in its biogenesis. No secondary nat-siRNAs were found and as in the other case, it was derived from only one specific site of the overlapping region. Apart from RDR6, SGS3, NRPD1a and HEN1, also HYL1 is needed for its biogenesis. This nat-siRNA guides the downregulation of a negative regulator of RPS2 (R protein) resistance pathway. RPS2 recognizes the effector *avrRpt2* and after this, a series of resistance responses to limit bacterial growth are induced. Also NDR1 is required for *avrRpt2*-induced resistance and both NDR1 and RPS2 proteins are indispensable for the production of this nat-siRNA. Interestingly, mutations in other resistance signaling components, like ethylene signaling component EIN2, reduced the level of produced nat-siRNA and consequently, the repression of its target mRNA was also reduced (Katiyar-Agarwal *et al.*, 2006).

Although natural *cis*-antisense transcripts (NATs) make up to 7.4 % of annotated transcription units in the *Arabidopsis* genome, they are not a major source of sRNAs in the absence of stress (Henz *et al.*, 2007).

1.2.5. lsiRNAs

The so called “long siRNAs” were identified recently in *Arabidopsis* and are 30-40 nt long (Katiyar-Agarwal *et al.*, 2007). Five of them were found to be induced in response to bacterial infection (*P. syringae* pathovar tomato carrying

avrRpt2) and one of them was specifically expressed in cell suspension culture. These sRNAs are generated from protein-coding regions and are present in various plant organs. Some of the lsiRNAs are generated as nat-siRNAs, but are longer. One of them – AtlsiRNA-1 – was studied in more detail. Its biogenesis involves DCL1 and DCL4 (probably for secondary siRNAs production), RDR6, Pol IV, SDE3, HEN1, HASTY, HYL1 and AGO7 (AGO1 role could not be tested). AtlsiRNA-1 is derived from the overlapping region of two genes, the one in sense orientation codes for a putative leucine-rich repeat receptor-like protein kinase and the one in antisense orientation, whose 3' UTR is part of the lsiRNA, encodes a protein with a putative RAP domain (AtRAP). This AtRAP protein is negatively regulated by AtlsiRNA-1 upon bacterial infection. The hypothesis is that AtRAP keeps the resistance response "off" under normal conditions. When the bacteria infect the plant, *AtRAP* mRNA is decapped and degraded. AtlsiRNA-1 seems to induce this decapping and subsequent action of the exoribonuclease XRN4, perhaps guiding its target mRNA with the help of AGO7 to the putative plant P-bodies (Xu *et al.*, 2006; Goeres *et al.*, 2007; Katiyar-Agarwal *et al.*, 2007).

1.2.6. siRNAs related to transcriptional gene silencing

The pathways described up to now take place mainly in the cytoplasm. Interestingly, sRNAs not only guide, through their incorporation into AGOs, RNA-RNA pairing but also RNA-DNA pairing, meaning that the key players of RNA silencing (sRNAs, Dicers, AGOs and RdRPs) affect gene function also at the level of genomic DNA, i.e. they are involved in TGS (Matzke and Birchler, 2005).

The TGS mechanism includes epigenetic processes, e.g. DNA methylation and/or histone modifications. RdDM has been described thoroughly in *Arabidopsis* and histone methylation with the consequent chromatin silencing has been extensively studied in fission yeast. In plants both levels of methylation take place, whereas in fission yeast there is only histone methylation (Matzke and Birchler, 2005). It is noteworthy that in mammals and in *N. crassa*, histone methylation does not necessarily mean siRNA-mediated TGS (Chicas *et al.*, 2004; Freitag *et al.*, 2004; Murchison *et al.*, 2005; Wang F. *et al.*, 2006). Transposons, retroelements and intergenic transcripts flanked by long terminal repeats are targets of chromatin silencing (Xie *et al.*, 2004).

Many components of the TGS pathway have been genetically identified but the current knowledge is still far away from understanding the process itself. As mentioned already, in the case of *Arabidopsis*, the main effectors are: Pol IV, DCL3, AGO4 and RDR2. It has also been explained that the specific DCL and AGO can be replaced by other paralogs, but interestingly, RDR2 cannot be substituted by another RDR (Kasschau *et al.,* 2007). In plants, TGS is important for taming transposons and for the maintenance of genome integrity (Zilberman *et al.*, 2003). Silencing at endogenous repeat loci involves histone 3 methylation (at lysine 9) and RdDM that is correlated with the production of homologous 24 nt long siRNAs (Cao *et al.*, 2003; Lippman *et al.*, 2003; Xie *et al.*, 2004; Zilberman *et*

al., 2004). According to a recent genome-wide analysis of *Arabidopsis* siRNAs, the most abundant sRNAs are 24 nt long, derived from repeated sequences including transposons and retroelements (Kasschau *et al.,* 2007). In addition, also miRNAs and viral siRNAs trigger RdDM (Shiba and Takayama, 2007).

One model describes the TGS process as follows: RdDM requires *de novo* methyltransferase activity (DRM1 or DRM2). DNA methylation and histone modification (deacetylation or methylation) are usually coupled in plants but it is not known, which process precedes the other one (Brodersen and Voinnet, 2006). Endogenous repeats are normally transcribed by RNA polymerase I, II or III. But if these sites have been methylated, the transcription elongation can be disturbed in such a way that the nascent transcripts are sensed as aberrant and become templates for Pol IVa. Then, these Pol IVa transcripts move to the nucleolus where RDR2, DCL3 and AGO4 do their work, i. e. dsRNA production, dicing and loading of siRNAs into AGO4. NRPD1b joins AGO4 and all the complex moves out of the nucleolus. When NRPD1b meets its NRPD2 counterpart, Pol IVb is activated and the AGO4-siRNA-Pol IVb complex targets loci in the heterochromatic regions and *de novo* cytosine methylation takes place again (Pontes *et al.*, 2006). It is not known how siRNAs act at target loci to direct RdDM. One possibility is that the siRNA pairs directly the complementary DNA (Grewal and Moazed, 2003). However, it is also possible that the pairing is between the siRNA and a nascent transcript, as suggested in the case of the methylation of *PHABULOSA*, where the target site crossed an exon-exon junction (Bao *et al.*, 2004). In fission yeast, target transcription by RNA polymerase II is required for histone methylation and the AGO complex associates with the nascent transcripts (Schramke *et al.*, 2005). The histone methylation of the human *EF1A* promoter, which was directed by siRNAs, was also dependent on RNA polymerase II transcription (Weinberg *et al.*, 2006).

Although in plants most TGS targets are transposons, a gene can also be silenced in this way in order to regulate its function as it is the case for *FWA* that controls flowering in *Arabidopsis*. It is normally expressed in the endosperm but is silenced in vegetative tissues by methylation of tandem repeats in its promoter (Kinoshita *et al.*, 2004).

Recently, FCA and FPA, two proteins previously characterized as flowering-time regulators in *Arabidopsis*, have been shown to act together with NRPD1a and RDR2. They contain many RNA recognition motives (RRM). In *C. elegans* some of the proteins involved in TGS contain also RRMs (Robert *et al.*, 2005). Surprisingly, FCA and FPA seem to regulate mainly chromatin silencing of single and low-copy genes. However, there are also some repetitive loci whose silencing is regulated by these proteins together with the other effectors of the pathway previously described (Bäurle *et al.*, 2007).

1.3. Biological roles of RNA silencing

From the aforementioned processes it can be inferred that the biological roles of RNA silencing are multiple. It is clearly an antiviral defense mechanism in both plants and animals (Voinnet, 2005a; Wang X. H. *et al.*, 2006). In the case of plants, it is known that this defense role is not restricted to viruses but extended to protect the plant genome against invading nucleic acids such as bacterial transfer-DNA (T-DNA, Dunoyer *et al.*, 2006), transposons and transgenes (Voinnet, 2005a). The defense is not only mediated by siRNAs derived from the "invaders" themselves – viruses or transgenes – but endogenous sRNAs can also trigger the defense response. For example, in the case of mammals, miRNA-mediated antiviral defense has been reported (Lecellier *et al.*, 2005). In addition, miRNAs, nat-siRNAs and lsiRNAs have been found to regulate bacterial disease resistance in plants, mediating the repression of genes involved in negative regulation of defense pathways (Navarro *et al.*, 2006; Katiyar-Agarwal *et al.*, 2006 and 2007).

The other outstanding role of RNA silencing is the regulation of gene expression. miRNAs and in the case of plants also tasiRNAs, are mostly responsible for the correct expression of thousands of endogenous genes at the precise place and time of organism development. The epigenetic modifications mediated by sRNAs also account for gene regulation. In addition, nat-siRNAs regulate gene expression in plants, orchestrating complicated physiological responses to stress. Through RNA silencing whole sets of genes can be coordinated in their expression because one single sRNA is able to target multiple genes. Thus, RNA silencing contributes to the integration of genetic regulation (Baulcombe, 2004). Another way in which RNA silencing is involved in the integration of gene regulation is through feedback loops. I have already explained how miRNAs regulate DCL1 and AGO1. Another feedback example is the hormonal regulation (by gibberellic acid and abscisic acid) of *miR159* and its targets. The targets of this miRNA are mRNAs from hormone-dependent MYB transcription factors, important for floral development and seed germination in *Arabidopsis* (Achard *et al.*, 2004; Reyes and Chua, 2007).

1.4. RNA silencing and other defense pathways

In plants, *R*-genes confer resistance to many pathogens, including viruses. The defense pathway is initiated when the R-protein recognizes the avirulence determinant (Avr) of the pathogen. Following this detection, many important changes occur in the plant leading mostly to programmed cell death, termed as hypersensitive response (HR). Among these changes are activation of kinase cascades, generation of reactive oxygen species, production of nitric oxide and changes in ion fluxes. Cellular activities are altered to respond to the pathogen attack and several plant hormones participate in this response (salicylic acid (SA), jasmonic acid and ethylene). SA is produced during the HR and it is essential later, during the systemic acquired resistance (Soosaar *et al.*, 2005).

Many overlaps between RNA silencing and R-gene mediated response do exist. However, it is not known how deep the real croostalk between both defense pathways is. One important observation is that at least two RNA silencing suppressors are at the same time Avr factor: *Turnip crinkle virus* P38 (the coat protein) and *Tobacco mosaic virus* P126 (the replicase; Table 2). The fact that the same protein in one case induces the defense response and in the other case suppresses it is not contradictory. It could reflect an evolution of the plant genome to detect a threat to a defense pathway through another.

SA is a key player in the R-gene mediated response. However, it also participates in an independent pathway causing inhibition of long-distance viral movement (Gaffney *et al.*, 1993; Mayers *et al.*, 2005). This SA-mediated resistance and RNA silencing seem to cooperate in the battle against viruses. At least three RNA silencing suppressors have been shown to interfere with SA-mediated resistance: 2b of CMV (Ji and Ding, 2001), P1/HcPro of *Tobacco etch virus* (TEV; Alamillo *et al.,* 2006) and P6 of CaMV (Love *et al.*, 2007). Interestingly, P6 suppressor also inhibits ethylene signaling (Geri *et al.*, 2004). Alamillo *et al.* (2006) demonstrated that SA-mediated resistance cooperates with RNA silencing in the case of plum pox potyvirus. The authors propose that the SA-mediated response prevents cell-to-cell movement of the virus from the site of inoculation, while RNA silencing – enhanced through SA-mediated defense – prevents unloading of the virus from the vasculature into mesophyll cells.

It is noteworthy that several RdRPs (RDR1, *Nt*RdRP1, *Nb*RdRP1m) are inducible by both SA and certain viruses (Xie *et al.*, 2001; Yu *et al.*, 2003; Yang *et al.*, 2004; Alamillo *et al.*, 2006).

In the case of mammalian viruses, the viral dsRNA triggers a number of immune responses: Toll-like receptor-mediated innate immunity, RIG-I and MDA-5 type I interferon induction, PKR pathway, 2'-5'-oligoadenylate (2-5A) system, etc. (reviewed in Sen and Peters, 2007).

One of the very first dsRNA-dependent interferon-induced anti-viral pathways discovered was the mammalian 2-5A system (Brown *et al.*, 1976; Kerr and Brown, 1978). The complete system is characteristic only of higher vertebrates. Some of its components have also been found in invertebrates, but not outside the animal kingdom (Wiens *et al.*, 1999). Summing up, the 2-5A system works as follows. Upon induction by interferons, the oligoadenylate synthetase (OAS) binds dsRNA. This binding changes the conformation of the enzyme activating it. Thus, the synthetase converts ATP into oligomers of adenosine, linked by phophodiester bonds in the unusual conformation of 2' to 5'. The 2'-5' oligoadenylates bind then to the endoribonuclease RNase L and a dimerization of the enzyme follows with its subsequent activation. Activated RNase L cleaves mRNAs (e.g. viral RNA) and also ribosomal RNAs (28S; Sen and Peters, 2007).

A tiny relationship between RNA silencing and the 2-5A system is that RDE-3, a protein needed for the RNA silencing of endogenous sRNAs in *C. elegans* (Lee *et al.*, 2006) shares conserved domains with OASs (Chun-Chieh *et al.*, 2005).

Interestingly, the least characterized component of the pathway, the protein initially named RNase L inhibitor (RLI; Bisbal *et al.*, 1995), is encoded by genomes of all eukaryotes as well as archaea (Kerr, 2004). Human RLI was cloned more than 10 years ago as a protein, which inhibits the 2-5A pathway by blocking the activation of RNase L (Bisbal *et al.*, 1995). Apart from being involved in the 2-5A system, RLI's essential functions include in some organisms its role in ribosome biogenesis and in translation initiation (Dong *et al.*, 2004; Kispal *et al.*, 2005; Yarunin *et al.*, 2005). RLI is essential for the normal development of several organisms which do not code RNase L, like *C. elegans* and *Saccharomyces cerevisiae* (Winzeler *et al.*, 1999; Gonczy *et al.*, 2000; Estevez *et al.*, 2004). Braz *et al.* (2004) proposed that RLI plant ortholog could be involved in RNA silencing.

The innante immune responses induced by viral dsRNA are modulated by host proteins, such as PACT and Tar-binding protein (TRBP) that activate and inhibit the immune response, respectively (Gupta *et al.*, 2003; Garcia-Sastre and Biron, 2006). Importantly, PACT and TRBP are needed for RNA silencing in mammals and both are required for human miRNA biogenesis and activity (Lee *et al.*, 2006; Tolia and Joshua-Tor, 2007).

A further junction point between RNA silencing and mammalian innate immunity is that interferon antagonist proteins were identified as suppressors of RNA silencing. These were vaccinia virus E3L, human influenza virus (A, B and C) NS1 and La Crosse virus NSs proteins (Li *et al.*, 2004; Soldan *et al.*, 2005). Notably, in the case of E3L and NS1, their dsRNA-binding domains are required for both RNA silencing suppression and to inhibit innate antiviral immunity (Li *et al.*, 2004).

While RNA silencing plays a major role in protecting plants and insects against viruses, DNA viruses infecting mammals appear to have evolved ways of using this pathway to their own advantage. Thus, viral miRNAs modulate both viral and host genes (Dölken *et al.*, 2007). Simian virus 40 (SV40) encoded miRNAs represent the best link between an RNA silencing pathway and the innate immune response. These SV40-encoded miRNAs mediate slicing of the perfectly complementary SV40 early transcripts. This decreases viral T antigen expression and so, the susceptibility to cytotoxic T cells is attenuated and the virus yield is maintained (Sullivan *et al.*, 2005). Through viral miRNAs, the viruses are also able to regulate their latent and lytic stages, as has been observed in the case of mouse cytomegalovirus (Dölken *et al.*, 2007) and herpes simplex virus-1 (Gupta *et al.*, 2006).

Nevertheless, the host also makes use of the miRNA-pathway to defend itself. Therefore, cellular miRNAs have been shown to have an antiviral role (Lecellier *et al.*, 2005). In the case of the primate foamy retrovirus (PFV-1) and vesicular stomatitis virus, the miRNA target is the virus itself (Lecellier *et al.*, 2005; Otsuka *et al.*, 2007). However, when the animal is infected with HIV, the miRNA target is a host factor, critical for the viral gene expression (Triboulet *et al.*, 2007).

Finally, the human adenoviral non-coding RNA VA1, known as a PKR-antagonist, inhibits miRNA processing and nuclear export (Lu and Cullen, 2005).

1.5. Viral suppressors of RNA silencing

RNA silencing prevents virus accumulation in plants, animals and fungi. Accordingly, viruses – as obligate parasites – have evolved various strategies to counteract this defense mechanism. The counterdefense involves suppressor proteins of RNA silencing which are encoded by the genomes of both RNA and DNA viruses.

In the case of plants, more than 35 different suppressors have been identified from all plant virus types. Moreover, every virus that has been closely examined to date encodes a suppressor (Table 2; Li and Ding, 2006; Ding and Voinnet, 2007). Many of these were identified first as pathogenicity determinants (Brigneti *et al*., 1998; Voinnet *et al*, 1999) or as viral cell-to-cell or long distance movement proteins (Voinnet *et al*., 1999; Roth *et al*., 2004). Viral symptoms involving developmental defects are probably due to silencing suppressors interfering with endogenous sRNA pathways (Baulcombe, 2004). Closely related viruses may show different silencing suppression activity in different hosts (Voinnet *et al*., 1999; Voinnet, 2001). This indicates specific interactions of the suppressor molecules with their targets, and strong selective co-evolution between virus and host (Lehto and Siddiqui, 2005).

Many viral proteins suppress RNA silencing by capturing siRNAs or longer dsRNA molecules (Merai *et al.*, 2005; Lakatos *et al.*, 2006). Although the strategy is the same, the way the different suppressors bind the dsRNA molecules is different, due in part to the diverse proteins' structures (Ding and Voinnet, 2007). For example, P19 of *Cymbidium ringspot virus* binds 21 nt long siRNA duplexes forming a head-to-tail homodimer (Vargason *et al*., 2003; Ye *et al*., 2003), whereas P21 of *Beet yellows virus* forms an octameric ring that binds with equal affinity short, long, ssRNA or dsRNA (Ye and Patel, 2005). It should be noted that there is discrepancy in the octamer of P1 needed or not for the suppressor activity (Lakatos *et al.*, 2006). Other suppressors like P0 of *Beet western yellows virus* (BWYV) and 2b of CMV target directly AGO1 (Zhang *et al*., 2006; Baumberger *et al*., 2007). Some viruses encode more than one suppressor, suggesting that these proteins have different counteracting strategies or that each suppressor is needed in a precise moment or space. This is the case for *Citrus tristeza virus* and for *African cassava mosaic virus* (ACMV; Lu *et al.*, 2004; Vanitharani *et al.*, 2004). Several viral suppressors that target primary siRNAs also interfere with transitivity, with the production of secondary siRNAs (Moissiard *et al.*, 2007). This could mean that the suppression of silencing movement takes place ahead of the infection (Ding and Voinnet, 2007).

It is noteworthy that also many animal viruses code suppressors of RNA silencing (Li and Ding, 2006) and lately, viral suppressors have been reported also for fungal viruses (Segers *et al.*, 2006; Hammond *et al.*, 2008).

Table 2. Examples of viral suppressors in plants (adapted from Li and Ding, 2006)

Virus genus	Virus name	Suppressor	Implicated motif
Positive-strand RNA viruses			
Aureusvirus	*Pothos latent virus*	P14	dsRNA binding
Benyvirus	*Beet necrotic yellow vein virus*	P31	
Carmovirus	*Turnip crinkle virus*	P38	
Closterovirus	*Beet yellows virus* *Citrus tristeza virus* *Grapevine leafroll-associated virus-2* *Beet yellow stunt virus*	P21 P20 P23 CP P24 P22	dsRNA binding
Crinivirus	*Sweet potato chlorotic stunt virus*	P22 RNase3	 RNaseIII
Comovirus	*Cowpea mosaic virus*	small CP	
Cucumovirus	*Cucumber mosaic virus* *Tomato aspermy virus*	2b 2b	dsRNA binding
Furovirus	*Soil-borne wheat mosaic virus*	19K	Cysteine-rich
Hordeivirus	*Barley stripe mosaic virus*	γb	Cysteine-rich
Pecluvirus	*Peanut clump virus*	P15	Cysteine-rich
Polerovirus	*Beet western yellows virus*	P0	
Potexvirus	*Potato virus X*	P25	
Potyvirus	*Tobacco etch virus* *Potato virus Y* *Turnip mosaic virus*	P1/HcPro HcPro P1/HcPro	
Sobemovirus	*Rice yellow mottle virus*	P1	
Tobamovirus	*Tobacco mosaic virus* *Tomato mosaic virus*	P126 P130	
Tobravirus	*Tobacco rattle virus*	16K	Cysteine-rich
Tombusvirus	*Tomato bushy stunt virus* *Cymbidium ringspot virus*	P19 P19	dsRNA binding
Tymovirus	*Turnip yellow mosaic virus*	P69	
Vitivirus	*Grapevine virus A*	P10	
Negative-strand RNA viruses			
Tenuivirus	*Rice hoja blanca virus*	NS3	
Tospovirus	*Tomato spotted wilt virus*	NSs	
Double-stranded RNA viruses			
Phytoreovirus	*Rice dwarf virus*	Pns10	
DNA viruses			
Begomovirus	*Tomato leaf curl virus* *African cassava mosaic virus* (KE)/(CM) *Mungbean yellow mosaic virus*	C2 AC2/AC4 AC2	DNA binding, NLS DNA binding,NLS/ miRNA binding DNA binding, NLS
Caulimovirus	*Cauliflower mosaic virus*	P6	

NLS: Nuclear localization signal; CP: coat protein.

1.5.1. P1 of sobemoviruses

Sobemoviruses encode a small P1 protein from the 5'-terminal ORF1. The ORF1 nucleotide sequences as well as the P1 primary sequences of the different members of the genus *Sobemovirus* are not similar (Tamm and Truve, 2000b).

P1 of *Rice yellow mottle virus* (RYMV) is an important pathogenicity determinant in rice (Bonneau *et al.,* 1998) and therefore it was a good candidate for RNA silencing suppressor. This protein is indispensable for the systemic spread of the virus and dispensable for its replication (Bonneau *et al.,* 1998). Voinnet *et al.* (1999) reported the suppressor effect of RYMV P1 in *N. benthamiana*, a non-host species. In that work, they inserted P1 into a *Potato virus X* (PVX) vector and infected GFP-silenced plants. The green fluorescence visualized after the infection proved that P1 interferes with the maintenance or amplification of RNA silencing. After that, the same protein was tested also in *N. benthamiana* but this time plants were transgenic for GFP and P1 was agroinfiltrated (Hamilton *et al.*, 2002; Himber *et al.*, 2003). Those studies concluded that P1 prevented the systemic spread of the silencing signal but not its short-range movement at the edge of the infiltrated patches.

1.5.2. P0 of poleroviruses

Poleroviruses, from the family *Luteoviridae*, are restricted to the phloem in their hosts (Mayo and Ziegler-Graff, 1996). The 5'-terminal ORF codes P0, a protein which is poorly expressed, in part as a consequence of the suboptimal translation initiation context of the protein start codon (Pfeffer *et al.*, 2002). It has been demonstrated that this low expression of P0 is clearly selected, as mutations to turn the context more favorable are lost rapidly (Pfeffer *et al.*, 2002).

P0 of BWYV, *Cucurbit aphid-borne yellows virus* (CABYV) and *Potato leafroll virus* (PLRV) were identified as RNA silencing suppressors in a transient assay agroinfiltrating GFP-transgenic *N. benthamiana*. PLRV was less efficient than the other two poleroviruses' P0 suppressors at the local level in this assay, although PLRV infects naturally *N. benthamiana*, whereas CABYV does not. The sequence identities among the P0s are low (Pfeffer *et al.*, 2002).

BWYV and CABYV infect *Arabidopsis*. In a two-hybrid screening of an *Arabidopsis* cDNA library, the P0 of these viruses were found to interact by means of an F-box-like motif with orthologs of S-phase kinase-related protein 1 (SKP1; Pazhouhandeh *et al.*, 2006). SKP1 is a core subunit of the multicomponent SCF E3 ubiquitin ligase, which can direct ubiquitination of target proteins to be degraded by the 26S proteasome (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). The identity between poleroviral P0s is low. Nevertheless, the F-box-like motif consisting of only five amino acid residues was conserved among all poleroviruses and turned to be essential for the suppressor activity of P0. These results suggested a model in which P0 was acting as an F-box protein that targets an essential

component of the RNA silencing machinery to degradation in the proteasome (Pazhouhandeh *et al.*, 2006).

A good candidate for being the ubiquitinated protein was AGO1. Two recent reports describe the interaction of P0 with AGO1 and its subsequent destabilization or degradation. However, contrary to what was expected, the destabilization of AGO1 does not depend on the proteasome (Baumberger *et al.*, 2007). Not only AGO1 but also all other tested AGO paralogs were destabilized by P0. The destabilization depends on the F-box-like motif and on the other hand on the PAZ and N-terminal domains of AGOs (Baumberger *et al.*, 2007). It is noteworthy, that a physical interaction between P0 and AGO1 has been proven *in vivo*. The interaction takes place in the nucleus, where SCF ubiquitination primarily occurs (Bortolamiol *et al.*, 2007).

P0 does not affect the accumulation of primary siRNAs, as it acts downstream of DCLs, but it considerably reduces the accumulation of secondary siRNAs, in accordance to the need of AGO for amplification (Baumberger *et al.*, 2007; Bortolamiol *et al.*, 2007). The mobile signal of silencing is not blocked by P0, indicating that AGOs are not required for this signal (Baumberger *et al.*, 2007).

Transgenic *Arabidopsis* expressing P0 of BWYV exhibited developmental aberrations and consistently, several miRNA levels were found to be enhanced (Bortolamiol *et al.*, 2007).

1.5.3. 2b of cucumoviruses

CMV-encoded 2b protein was among the first RNA silencing suppressors identified (Brigneti *et al*., 1998). 2b is a pathogenicity determinant, i.e. a factor dispensable for virus replication within single cells but needed for its accumulation in systemic tissues. As a logical consequence, this suppressor has been reported to interfere with the long-distance movement of the silencing signal (Guo and Ding, 2002). Interestingly, it was demonstrated that the nuclear localization of 2b is strictly required for its suppressor activity (Lucy *et al.*, 2000).

More recently, the molecular way of suppressing RNA silencing was investigated. Zhang *et al.* (2006) showed that 2b directly interacts with AGO1 *in vitro* and *in vivo*, blocking the cleavage of the target mRNA. The interaction takes place on one surface of the PAZ-domain and part of the PIWI-domain of AGO1. The surface of the PAZ-domain where the interaction occurs corresponds to the place where AGO1 harbors the small RNA and its target mRNA-binding groove. Intriguingly, the 2b-AGO1 interaction takes place in the nucleus, as in the case of P0-AGO1.

CMV has a wide range of strains with clear differences between mild and severe ones. 2b from the mild Q strain has little or no effect on miRNA functions (Chapman *et al*., 2004), while 2b from the severe strain FNY inhibits miRNA pathways altering the accumulation of different miRNAs (Zhang *et al*., 2006). When plants were transformed with 2b from FNY strain, approximately 80 % of the transgenics displayed developmental abnormalities, whereas from the plants

transformed with 2b from the mild Q strain this percentage was very low (0.6 %). Curiously, although the mRNA levels of 2b in both kinds of transgenics were more or less the same, the 2b Q protein could barely be detected in very few plants. Transgenic *Arabidopsis* expressing 2b from FNY phenocopied *ago1 Arabidopsis* mutants and were also similar to transgenic plants expressing P21, P19 or P1/HcPro (Chapman *et al.*, 2004, Dunoyer *et al.*, 2004; Zhang *et al.*, 2006).

Another group also transformed *Arabidopsis* with 2b of a mild strain (LS) and with 2b of FNY. Only 2b FNY transgenics exhibited strong symptom-like phenotypes and interfered with miRNA pathways. However, both 2b proteins suppressed siRNA-mediated RNA silencing with the same efficiency (Lewsey *et al.*, 2007).

A recent study compared an attenuated CMV strain (CM95) versus a spontaneous mutant of it that induces severe symptoms. The severe mutant was a stronger suppressor of RNA silencing. Although the difference was only in one amino acid, the mutant could strongly bind siRNAs, whereas the CM95 could barely do it (Goto *et al.*, 2007).

An elegant study analyzed CMV infection in *Arabidopsis dcl* and *rdr* mutants. In addition, the analysis was done with a CMV lacking almost the complete 2b, which is able to infect *Arabidopsis* locally. CMV infection produces 21, 22 and 24 nt long siRNAs. This study concludes that 2b suppression depended on inhibition of the accumulation of all three classes of siRNAs. The silencing suppressor activity of 2b was needed to establish the infection but it was dispensable in eliciting symptoms (Diaz-Pendon *et al.*, 2007). Notably, 2b can block SA-mediated virus resistance (Ji and Ding, 2001). Using the mutants just described, it was shown that the production of CMV siRNAs without 2b suppression depends on RDR1, inducible by SA (Diaz-Pendon *et al.*, 2007).

1.5.4. P19 of tombusviruses

P19 is a protein of approximately 19 kDa needed for virus movement. It is a determinant of pathogenicity (Scholthof *et al.*, 1995 a & b). This protein is highly conserved among tombusviruses (Scholthof, 2006). It is dispensable for infecting *N. clevelandii*, however it is indispensable for the infection of pepper and spinach (Dalmay *et al.*, 1993; Scholthof *et al.*, 1995b).

Already in 1999, its function as suppressor was detected (Voinnet *et al.*, 1999) and three years later, its precise molecular role was reported. Namely, P19 specifically binds siRNA duplexes having 2 nt 3’ overhangs (Silhavy *et al.*, 2002). The crystallization of P19 homodimer in complex with 21 nt siRNAs revealed a perfect sequestering of the siRNAs of that size and therefore a perfect adaptation of the virus to its host RNA silencing machinery (Vargason *et al*., 2003). Thus, it was the first suppressor whose molecular way of blocking RNA silencing was elucidated.

P19 has the intrinsic capacitiy of inhibiting the step between DCL and RISC. RISC cannot be activated because the siRNA has been captured. Consistently with

this, P19 is able to bind any kind of 21 nt long siRNA duplex in a wide range of organisms: non-host plants, insect and human cells (Dunoyer *et al.*, 2004; Li *et al.*, 2004; Lecellier *et al.*, 2005). This is possible because P19 contacts the sugar-phosphate backbone of the siRNA and is therefore unaffected by its sequence (Vargason *et al.*, 2003; Ye *et al.*, 2003). Recently, it was reported that the binding of P19 from *Carnation Italian ringspot virus* (CIRV) to the siRNA duplex is sensitive to pH and salt concentration, the optimal pH range being between 6.2 and 7.6 (Koukiekolo *et al.*, 2007).

This protein captures not only siRNA duplexes, but also miRNA/miRNA* duplexes, methylated or not. Thus, it is not surprising that transgenic *Arabidopsis* expressing *Tomato bushy stunt virus* (TBSV) P19 exhibits malformations in leaves and flowers (Dunoyer *et al.*, 2004; Yu *et al.*, 2006). The transgenic expression of P19 inhibits the methylation of miRNAs (Yu *et al.*, 2006).

The way P19 efficiently suppresses silencing has the disadvantage of being a stoichiometric 1:1 reaction. However, if P19 is re-used after the sequestration step, then the efficiency may be even higher. Some host proteins have been found to interact with P19 and could help in the recycling of the suppressor (Scholthof, 2006). These host proteins are from the ALY/REF group (Park *et al.*, 2004; Uhrig *et al.*, 2004). ALY/REF proteins serve as transcriptional co-factors and are involved in subsequent RNA transport. The interaction of some ALY with P19 relocate these proteins from the nucleus to the cytoplasm (Uhrig *et al.*, 2004). More recently, it has been shown that other ALY proteins translocate P19 from the cytoplasm to the nucleus. Interestingly, this translocation inhibits the suppressor activity of P19. In the same study it was found that the C-terminal part of the RNA recognition motif of ALY is responsible for the interaction with P19 (Canto *et al.*, 2006). In this sense, the interaction seems to be more a strategy from the host to inhibit the viral suppression. The situation is also complex as different ALY proteins have diverse expression patterns in *Arabidopsis*. Actually, the biological role of these interactions with different ALY proteins is not clear (Canto *et al.*, 2006).

Calabrese and Sharp (2006) have reported that P19 of CIRV binds rRNA in mouse embryonic stem cells. Further, they show that P19 is able to bind only dsRNAs, where the ds fragment is 19 nt long. This means that the bound RNA molecule itself can be longer than a sRNA. Also in the 21 nt siRNA duplex the dsRNA region is 19 nt long.

The tombusviral suppressor is an attractive biotechnology tool because it is able to increase the expression of a foreign protein 50 times if it is coexpressed transiently (Voinnet *et al.*, 2003).

1.5.5. HcPro of potyviruses

Potyviruses encode the helper component-proteinase (HcPro), a multifunctional protein required for maintenance of genome replication, long-distance movement, polyprotein processing and aphid transmission (Klein *et al.*, 1994; Cronin *et al.*,

1995; Kasschau *et al*., 1997; Llave *et al*., 2000). In addition, it was the first suppressor of RNA silencing identified (Anandalakshmi *et al.,* 1998).

This protein has three domains and for its suppression activity two of them are required: the central domain, important for maintenance of genome amplification, and the C-terminal domain, necessary mainly for proteolityc activity (Varrelmann *et al.*, 2007). The central region contains several motifs that are highly conserved in all potyviruses, including the FRNK box. In the case of *Zucchini yellow mosaic virus* this motif is associated to symptom severity (Gal-On and Raccah, 2000). Recently, the N-terminus of HcPro was shown to interact *in vivo* with three subunits of the *Arabidopsis* 20S proteasome (Jin *et al.*, 2007). The inhibition of the 20S proteasome endonuclease activity *in vitro* by HcPro had been shown previously (Ballut *et al.*, 2005).

In plants where dsRNAs are directly produced from an inverted repeat or from a viral amplicon, HcPro leads to a significant accumulation of the longer species of siRNAs (24 nt). This result was explained saying that HcPro, as a cytoplasmic protein, does not interfere in the nuclear pathway connected to DCL3/RDR2/AGO4, where the 24 nt long siRNAs are produced. At the same time, HcPro-mediated suppression of transgene silencing does not eliminate the transgene DNA methylation or the systemic signaling, processes probably dependent on 24 nt siRNAs (Mallory *et al.*, 2001, 2002, 2003, Dunoyer *et al.*, 2004; Li and Ding, 2006). In addition, HcPro reduced the accumulation of the 21 nt long siRNAs (Mallory *et al.*, 2002; Dunoyer *et al.*, 2004) and HcPro expression caused a more pronounced inhibition of the 3' end methylation in the case of this class of siRNAs than in the case of the 24 nt long siRNAs. This means that HcPro destabilizes the shorter siRNAs with preference (Ebhardt *et al.*, 2005). More recently, the binding of HcPro to siRNA duplexes and miRNA/miRNA* was reported and the above mentioned observations are in accordance to it. Namely, HcPro binds 21 nt long siRNA duplexes containing 3' overhangs (2 nt) with higher affinity than to 24 nt siRNA duplexes or to duplexes lacking overhangs (Lakatos *et al.*, 2006). A mutational analysis of the FRNK box of HcPro has just demostrated that this motif is required for sRNA binding and therefore for the suppressor activity of HcPro of some potyviruses (Shiboleth *et al.*, 2007).

The effective sequestration of the siRNA duplexes required the presence of non-identified cellular factor(s). This seems to be the main difference between the suppressor strategy of P19 and the one of HcPro (Lakatos *et al.*, 2006). The association of HcPro to a plant protein, identified as an endogenous suppressor of RNA silencing, was reported several years ago (Anandalakshmi *et al.*, 2000).

In addition, HcPro expressed transgenically inhibits the methylation of miRNAs (Yu *et al.*, 2006).

1.5.6. P25 of potexviruses

P25 is one of the three triple gene block proteins of PVX required for its cell-to-cell movement (Beck *et al*., 1991; Angell *et al*., 1996). It is also an RNA helicase (Kalinina *et al*., 2002) and modifies plasmodesmata (Angell *et al*., 1996).

As RNA silencing suppressor, it prevents the systemic spread of the silencing signal (Voinnet *et al*., 2000; Hamilton *et al*., 2002). Interestingly, P25 silencing suppression is required for short-distance movement of the virus. It seems that when P25 is present, the silencing signal is not produced or it is not able to target the viral RNA and therefore the virus is able to spread (Bayne *et al*., 2005).

1.5.7. AC2 of geminiviruses

The AC2 protein encoded by begamoviruses, which are DNA viruses, functions as a transactivator of viral transcription and is therefore sometimes also abbreviated as TrAP and in addition as C2, or AL2. This protein possesses three domains typical of transcription activators: a bipartite nuclear localization signal (NLS), a non-classical Zn finger and an acidic activator domain (Hartitz *et al*., 1999; Trinks *et al*., 2005). It has been shown that AC2 activates the viral late promoters (Sunter and Bisaro, 2003; Shivaprasad *et al.*, 2005; Trinks *et al.*, 2005) and acts as a pathogenicity determinant (Hong *et al.*, 1996, 1997). In the case of ACMV (Kenyan strain), *Mungbean yellow mosaic virus*, *Tomato golden mosaic virus* (TGMV) and *East African cassava mosaic Cameroon virus*, AC2 has been shown to be a suppressor of RNA silencing (Voinnet *et al.*, 1999; Vanitharani *et al.*, 2004; Trinks *et al.*, 2005; Wang *et al.*, 2005). Is is noteworthy that the three domains of AC2 are required for both promoter activation and silencing suppression. These two functions cannot be separated, which suggests that the suppressor activity is causally coupled to the transcription factor activity. AC2 is a nuclear protein and interestingly, its way of suppressing RNA silencing is through modification of the host transcriptome. Silencing suppression might become activated by AC2, when AC2 represses genes that are positively involved in the silencing process (Trinks *et al*., 2005) or if it activates negative regulators of RNA silencing, i.e. other suppressors. It has been reported that AC2 induces a common set of approximately 30 host mRNAs and among these is the *WERNER EXONUCLEASE-LIKE 1* (*WEL1*) transcript. WEL1 is a putative endogenous suppressor of RNA silencing (Trinks *et al*., 2005).

The only reported exception, where one of the three AC2 domains is not needed for RNA silencing suppression, is the activator domain of TGMV. Interestingly, in this case, the suppression capacity of AC2 depends on inactivation of adenosine kinase (ADK) by a direct protein-protein interaction (Wang *et al.*, 2005). It has been reported that if ADK activity is drastically reduced, then plants exhibit reduced methylation (Moffatt *et al.*, 2002). Therefore AC2 could be interfering with RdDM.

1.6. Endogenous suppressors of RNA silencing

RNA silencing pathways are multiple not only in plants but also in other organisms. The different pathways, as explained in the case of plants, are interconnected and share common factors. Therefore, it is logical to infer an endogenous regulation that keeps different pathways under control. Positive and negative regulators coded by the organism itself should coordinate this regulation. Indeed, some endogenous suppressors have been already found in different organisms and for sure much more will be identified in the future.

The first endogenous suppressor of RNA silencing was identified in tobacco and was called "regulator of gene silencing-calmodulin-like protein" (rgs-CaM). Rgs-CaM interacts with the potyviral suppressor HcPro and is believed to function through a calcium-dependent pathway (Anandalakshmi *et al*., 2000). Also two other endogenous suppressors of plants – still putative – are dependent on a viral suppressor. These proteins were identified in *Arabidopsis* and are the Werner exonuclease-like 1 (WEL-1) and the cold- and abscisic acid-inducible protein KIN1 (Trinks *et al*., 2005). In this case, it is the geminiviral protein AC2, a transcriptional-activator protein, which seems to activate WEL-1 and KIN1 (Trinks *et al*., 2005). All these three proteins blast nicely with proteins in different plants. Some fungi and animals share certain level of identity with tobacco rgs-CaM and *A. thaliana* WEL-1.

The *Arabidopsis* cytoplasmic exoribonuclease XRN4, mentioned before in the lsiRNA pathway, was firstly identified as an endogenous suppressor. XRN4 antagonized RNA silencing induced by a sense-transgene and it was proposed that this suppressor degrades templates for RDR6 (Gazzani *et al.,* 2004). Interestingly, target transcripts of known miRNAs accumulated to higher levels in *xrn4* T-DNA insertion mutants, suggesting that these targets are also degraded by XRN4 (Souret *et al.*, 2004). This all means that XRN4 acts as suppressor in the case of the miRNA pathway and in the sense-transgene induced RNA silencing pathway. It is possible that it also inhibits other silencing pathways dependent on RDR6. However, in the case of lsiRNA it seems to be a needed component for the proper activity of that pathway, which also depends on RDR6 but at the same time on PolIV. The common and different components of each pathway may explain the positive or negative action of the same protein and the coordination between the pathways.

In addition, "enhanced silencing phenotype" (ESP) proteins were identified as endogenous suppressors. The authors propose that these ESP proteins, which are involved in RNA processing and 3' end formation, prevent transgene and endogenous mRNA from entering RNA silencing pathways, if they have aberrant 3' termini. In other words, they don't let RDRPs convert aberrant mRNAs into dsRNA (Herr *et al.*, 2006).

Finally, three new endogenous suppressors have been identified in *Arabidopsis*. Two are nuclear exonucleases (XRN2 and XRN3), which together degrade miRNA/miRNA* duplexes before they enter RISC. They also degrade transgene-

or virus-derived aberrant mRNAs that would otherwise be RDR6 templates. The third identified suppressor is FIERY1, a nucleotidase/phophatase that regulates XRNs levels (Gy *et al.*, 2007).

In *C. elegans*, two proteins were reported as endogenous suppressors of RNA silencing: RRF-3 and ERI-1. It was thought that the RdRP RRF-3 suppressed silencing by blocking the generation of secondary siRNAs dependent on EGO-1 and RRF-1, other RdRPs. Thus, it was understood that RRF-3 prevented amplification of the silencing signal (Simmer *et al*., 2002). Nowadays, after discovering that *C. elegans* has different RNA silencing pathways (Duchaine *et al.*, 2006) and after understanding how tightly connected they are (Lee *et al.*, 2006), it is clear that RRF-3 suppresses the RNA silencing pathways related to exogenous siRNAs. RRF-3 itself is the RdRP needed for one endogenous RNA silencing pathway and it competes with other exogenous RNAi pathways for common components (Lee *et al.*, 2006).

ERI-1 (named after "enhanced RNAi") is a highly conserved protein among vertebrates, including humans, and it is also present in fission yeast. ERI-1 of *C. elegans* and its human ortholog partially degrade siRNAs with 2 nt 3' overhangs *in vitro*. Therefore, it was thought that this was the way ERI-1 suppressed RNA silencing (Kennedy *et al*., 2004). However, after a large proteomic analysis carried out by C. Mello's laboratory, it has been shown that ERI-1 forms a complex with RRF-3 and DCR-1. Apparently, ERI-1 facilitates the production of dsRNA substrates for DCR-1 in an endogenous RNA silencing pathway, making use of its nucleic acid binding domain (Duchaine *et al.*, 2006). Thus, in principle, it can be considered a suppressor of exogenous RNA silencing pathways, the same way RRF-3 is.

HeIF is a putative RNA helicase from *Dictyostelium*. Its gene shares homology with the "dicer-related helicase" (drh-1) gene from *C. elegans* and with helicases domains of Dicers or Dicer-like proteins in *C. elegans* and *Arabidopsis*. The protein is located in nuclear foci and suppresses RNA silencing when it is induced by hairpin constructs. However, when *Dictyostelium* was transformed with constructs carrying antisense sequences of some endogenes, no effect was detected in *heIF* knock-outs. These mutants exhibited abnormal development in later stages. It is not known how this suppressor interferes with RNA silencing pathways (Popova *et al.*, 2006).

2. AIM OF THE STUDY

The aim of this study was to gain knowledge about RNA silencing in plants through the identification of new suppressors and the analysis of the effects of transgenically expressed suppressors on plant phenotype and viral infection.

For this purpose the following tasks were carried out:

1. Identification of the RNA silencing suppressor of *Cocksfoot mottle virus* (CfMV), a virus which has been studied for more than ten years in our laboratory.
2. Analysis and comparison of different viral suppressors expressed from transgenes in *N. benthamiana* and *N. tabacum*.
3. Determination of a role for the RLI protein, present in different kingdoms, possibly related to RNA silencing.

3. MATERIALS AND METHODS

The methods used are described in detail in the articles presented in this thesis (especially in publications I and III) and mentioned here as follows:

3.1. Plant material and growing conditions

A. thaliana wt and transgenic lines were grown at 22 °C in a plant room under a 16 h photoperiod (publication I). *N. benthamiana* as well as *N. tabacum* wt and transgenic lines were grown in a plant room at 24 °C or in a greenhouse at 25 °C under a 16 h photoperiod (publications I, II and III).

3.2. Standard cloning

AtRLI2 cDNA, binary construct pC35S-RLI as well as pC-RLI2prom, and the expression vector pGEX6P-2 (Amersham) containing the coding sequence of *AtRLI2* were obtained as described in publication I. Cloning of CfMV ORF1 into pET 43.1a(+) vector (Novagen) to express NUS-P1 and cloning of NUS-ORF1 as well as ORF1 alone into pBIN61 binary vector is explained in publication II. All obtained clones were sequenced for validation.

3.3. Production of transgenic plants

The different *N. benthamiana* and *N. tabacum* transgenic lines analyzed in the publication III were obtained via transformation of leaf discs with *Agrobacterium tumefaciens* carrying the sequences of interest, according to Smith *et al.* (1994). The transformants were selected on Murashige-Skoog medium containing kanamycin and propagated to R2 generation. This work was carried out by S. A. Siddiqui from the University of Turku.

A. thaliana was transformed via floral dipping with *A. tumefaciens* harboring pC-RLI2prom or pCAMBIA 1301 and T3 (i.e. R3) hygromycin-resistant transformants were analyzed for GUS expression (publication I).

3.4. Expression and purification of recombinant proteins

GST-tagged AtRLI2, GST-tagged human RNase L, His-tagged NUS-P1 (of CfMV) and His-tagged *Influenza A virus* NS1 proteins were expressed in *Escherichia coli* and purified as described in publications I and II.

3.5. Agroinfiltration and GFP imaging

Fresh over-night cultures of *A. tumefaciens* harboring different binary constructs were infiltrated into *N. benthamiana* and *N. tabacum* leaves as

previously described (Hamilton *et al.*, 2002). The culture's final density was always 1.0 at OD_{600} (publications I, II and III). Plants expressing GFP were photographed under UV-light with Olympus CAMEDIA digital camera at different dpi and the pictures were processed using Adobe Photoshop 6.0 (publications I and II). In publication III the digital camera was Canon EOS 20D SLR and the analysis of the fluorescence is detailed there.

3.6. RNA isolation and Northern blot analysis

RNA was extracted from *N. benthamiana* and *N. tabacum* agrobacteria-infiltrated leaf patches at different dpi as described by Szittya *et al.* (2002; publications I and II). The isolation from *A. thaliana* total RNA followed the protocol from Logemann *et al.* (1987; publication I). Total RNA extraction from *N. benthamiana* and *N. tabacum* was done according to Sijen *et al.* (1996; publication III).

Northern blot from infiltrated leaf patches of *N. benthamiana* and *N. tabacum* is described in publication I. Detection of GFP mRNA and siRNA was done with a ^{32}P-labeled probe in publication I and II, but in the case of publication II, GFP mRNA was detected with a DIG-labeled probe.

The RNA from *A. thaliana*, *N. benthamiana* and *N. tabacum* organs was separated electrophoretically and blotted as described by Sambrook and Russell (2001; publications I and III). For *AtRLI2* detection a ^{32}P-labeled probe was used (publication I), while in the case of the transgenes' detection a DIG-labeled probe was used (publication III).

3.7. PCR and RT-PCR

DNA fragments from the different transgenic *N. benthamiana* and *N. tabacum* plants were PCR-amplified to verify the presence of the transgenes (publication III). RNA extracted from *Arabidopsis* was used for RT-PCR to amplify *AtRLI2* 3' UTR (publication I).

3.8. RNase protection assay

Total RNA extracted from different *Arabidopsis* organs was analyzed through RPA to quantify the expression of *AtRLI2* (publication I).

3.9. siRNA binding assay

GST-RLI and GST-RNase L (publication I) as well as NUS-P1 (publication II) purified proteins were tested for binding to 21 nt long siRNA duplexes as described by Bucher *et al.* (2004). Crude extract prepared from *N. benthamiana* leaves agro-infiltrated with CfMV P1 was analyzed for binding to 21 and 26 nt long siRNA duplexes and to 49 nt dsRNA according to Merai *et al.* (2005; publication II).

3.10. Microscopy

Thin sections of leaves from HcPro and AC2 transgenic *N. benthamiana* and *N. tabacum* plants were cut with a Reichert ultramicrotome and examined with a Reichert Zetopan microscope (publication III). This work was carried out by our colleagues of the University of Turku.

4. RESULTS AND DISCUSSION

4.1. P1 of *Cocksfoot mottle virus* suppresses RNA silencing [1]

It is believed that almost all plant viruses encode one or more suppressors of RNA silencing in order to counteract the defense pathway of the host.

CfMV and RYMV belong to the genus *Sobemovirus* and infect only monocots. As RYMV P1 was one of the first viral suppressors discovered (Voinnet *et al.*, 1999) and because CfMV is the most studied virus in our laboratory, we wanted to test if P1 of CfMV is also a suppressor of RNA silencing.

The sobemovirus genome consists of polycistronic positive-strand ssRNA (Tamm and Truve, 2000b). ORF1 of CfMV encodes P1 that is required for systemic infection but is dispensable for replication (Meier *et al.*, 2006) as is also the case for RYMV P1 and *Southern cowpea mosaic virus* P1 (Bonneau *et al.*, 1998; Sivakumaran *et al.*, 1998). In addition, P1 of RYMV is reported to be a pathogenicity determinant (Bonneau *et al.*, 1998). The molecular masses of different P1s range between 11.7 and 24.3 kDa and the similarity between the P1s' amino acid sequences is low, making this region the most variable one in the genome of sobemoviruses (Ngon A Yassi *et al.*, 1994; Mäkinen *et al.*, 1995; Othman and Hull, 1995).

The 5'-terminal half of the genomes of sobemoviruses and of poleroviruses are similar in their organization (Hull and Fargette, 2005). The 5'-terminal ORF of poleroviruses encodes P0, which like P1 of sobemoviruses, is required for virus accumulation (Sadowy *et al.*, 2001; Meier *et al.*, 2006). P0 and P1 are the most divergent proteins among poleroviruses and sobemoviruses, respectively, and have no homology with other known proteins (Mayo and Miller, 1999; Tamm and Truve, 2000b). Another common feature of P0 and P1 is their poor translation initiation codon context (Pfeffer *et al.*, 2002; Dwyer *et al.*, 2003). Finally, the P0 proteins encoded by BWYV, PLRV and CABYV have been shown to be suppressors of RNA silencing (Pfeffer *et al.*, 2002).

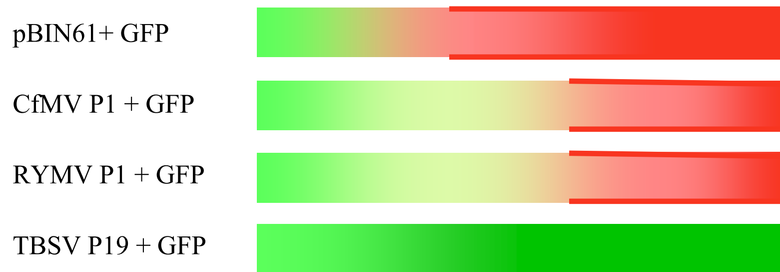
Since RYMV P1 acts as a silencing suppressor in *N. benthamiana*, a non-host species (Voinnet *et al.*, 1999), we investigated the suppressor activity of CfMV P1 in *N. benthamiana* (wt and GFP-transgenic line) and in *N. tabacum* (wt) using the agrobacterium-mediated transient assay (Hamilton *et al.*, 2002). We co-infiltrated the leaves with *A. tumefaciens* carrying the *GFP* in combination with the *A. tumefaciens* containing *CfMV P1* or *RYMV P1*. As negative control the co-infiltration was done with the *A. tumefaciens* carrying the empty binary vector pBIN61 and as positive control the one containing the gene from the strong

[1] During the preparation of this manuscript (publication II) the longest siRNA class was considered as 25 nt long. However, now it is known that the actual size is 24 nt, due to the detailed analysis of DCL3 product (Xie *et al.*, 2004). Therefore, throughout this section the mentioned length is 24 nt.

suppressor P19 of TBSV. All genes were under the control of the strong constitutive promoter 35S.

The differences in the GFP fluorescence visualized in the infiltrated patches at different time points showed a clear suppressor effect of CfMV P1, RYMV P1 and TBSV P19 at the local level. CfMV P1 effect was similar to the one of RYMV P1, whose suppression in the same system has been partially described (Himber *et al.*, 2003). As expected, the area infiltrated with TBSV P19 was intensely green after 11 dpi, due to its strong suppressor effect (Voinnet *et al.*, 2003). However, the suppressor activity could not be followed in *N. tabacum* infiltrated with P19, because the inoculated tissue started dying at 3 dpi, due to the local necrotic lesions that this protein elicits in this plant (Scholthof *et al.*, 1995). In addition, in the case of infiltrated GFP-transgenic *N. benthamiana*, we observed the appearance of a red ring at the border of the patch in the plants infiltrated with the empty vector already at 5 dpi, whereas the same ring appeared later (7 dpi) in the case of CfMV P1 and RYMV P1 (Fig.4; publication II, Fig.1A a-k). The red ring is a hallmark for the cell-to-cell short distance movement of the silencing signal. This process is initiated from the infiltrated cells located exactly at the border of the patch and spreads 10-15 cells in the absence of amplification (Himber *et al.*, 2003).

***N. benthamiana* (16c)**



***N. benthamiana* (wt)**

***N. tabacum* (wt)**

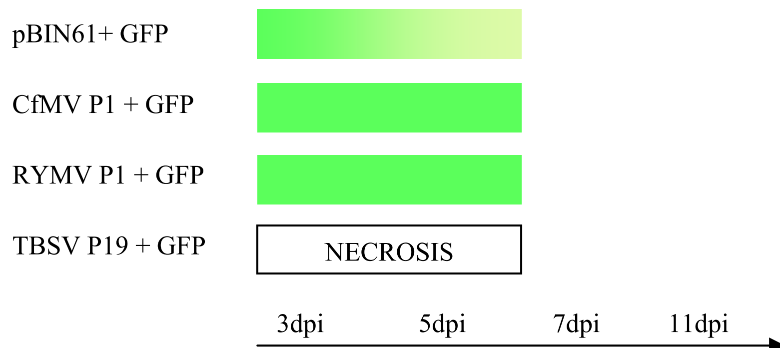


Fig. 4. Changes of GFP fluorescence in leaves agroinfiltrated with different RNA silencing suppressors
Red: autofluorescence of chlorophyll under UV-light (GFP-silenced tissue). Green: GFP fluorescence under UV-light (GFP expression). Red border: red ring at the border of the infiltrated patch. Colors turning lighter: decrease in fluorescence. Color turning darker: increase in fluorescence. 16c: GFP-transgenic line; wt: wild-type; dpi: days post-infiltration.

To confirm that the observed results were due to RNA silencing suppression, we determined the levels of GFP siRNAs in the infiltrated patches of *N. benthamiana* GFP-transgenic plants. At 5 dpi, both classes of GFP siRNAs were abundant in the pBIN61 control patch, whereas in the presence of any suppressor the siRNA levels were reduced. With P19 no siRNA was detected, as expected (Hamilton *et al.*, 2002), with RYMV P1 only the shortest class of siRNAs were above the detection limit, as previously described (Hamilton *et al.*, 2002), and with CfMV P1 both siRNAs classes were reduced (publication II, Fig. 1B i). At 7 dpi, the levels of siRNAs detected in the case of CfMV P1 and RYMV P1, were higher than before. This time, the patch infiltrated with RYMV P1 showed clearly both classes of siRNAs (publication II, Fig. 1B ii). This correlates with the observed change in fluorescence of infiltrated patches from 5 to 7 dpi (Fig. 4). Himber *et al.* (2003) reported in the same system a lack of the longest siRNAs even at 7 dpi, although the infiltrated patch appeared – as our infiltrated area – red. A plausible explanation for these diverse results could be slight differences in the growing conditions of the plants. Also Northern blot analysis of the GFP mRNA from the infiltrated patches at 5 dpi confirmed the observed results, for both *N. benthamiana* GFP-transgenics and *N. tabacum* wt plants (publication II, Fig. 1C).

In conclusion, CfMV P1 suppressed local RNA silencing similarly to RYMV P1, with an effect that persisted for a short period of time in two non-host plants. Cell-to-cell movement of the silencing signal was delayed by both suppressors because the red ring was absent at 5 dpi and because the amount of the 21 nt siRNAs, which are a component of the cell-to-cell silencing signal (Dunoyer *et al.*, 2005), was reduced. A noteworthy difference between sobemoviral suppressors was that only in the case of RYMV P1, the longest siRNAs could not be easily detected when the suppression was strongest (5 dpi).

When the tested suppressors were infiltrated without the RNA silencing inducer (GFP) into a *N. benthamiana* transgenic line where GFP-silencing was already active, both sobemoviral suppressors reversed the silencing. However, this interference with the maintenance of the silencing was evident only in 42 % of the plants, while in the case of P19 all plants show this reversion (publication II, Fig. 1A l-o).

RNA silencing suppression was also followed at the systemic level in the GFP-transgenic *N. benthamiana* and the results are shown in Fig. 5 (and in publication II, Fig. 2). P1 of RYMV is clearly stronger than P1 of CfMV at the systemic level. This may indicate that phloem-dependent movement of the silencing signal, which is sometimes related to 24 nt siRNAs (Hamilton *et al.*, 2002), is more efficiently blocked by RYMV P1 than by CfMV P1, resulting in stronger interference with systemic silencing. The stronger effect of RYMV P1 partially correlates with the observed specific reduction of 24 nt siRNAs, especially at 5 dpi.

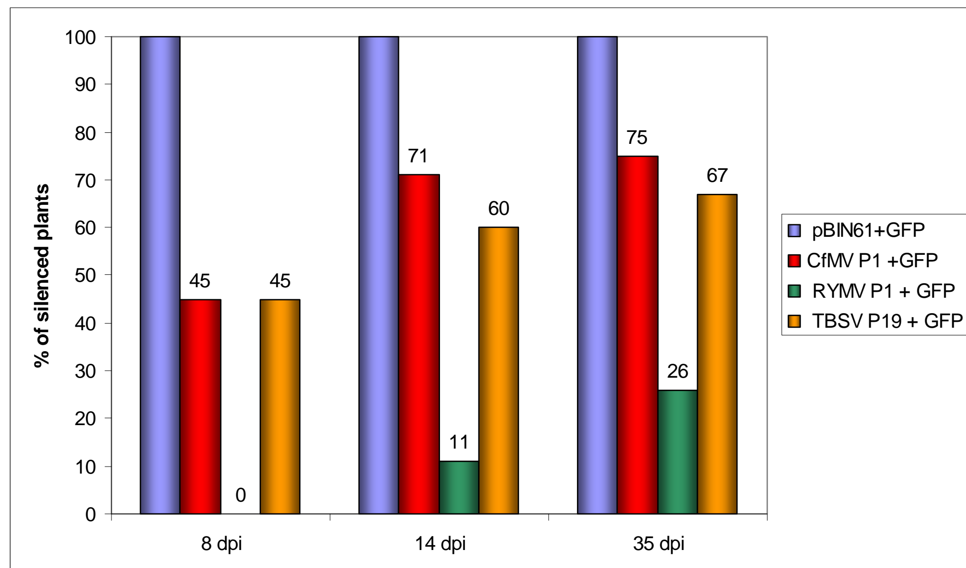


Fig. 5. Systemic silencing in *N. benthamiana* GFP-transgenic plants
Plants were considered systemically silenced when any kind of red tissue appeared outside the infiltrated area. The percentage was calculated from four independent experiments, including each time 6 or 7 plants per suppressor or control.

As it is known that P1 of CfMV binds ssRNA in a sequence-independent manner (Tamm and Truve, 2000a) and because its action as a suppressor was observed early in silencing, we tested the capacity of this protein to bind siRNAs and a 49 nt long dsRNA. For this purpose we performed gel shift assay with crude extracts from leaves infiltrated with CfMV P1 and also with this protein purified after its expression in *E. coli*. The assays were carried out as described in Merai *et al.* (2005) and Bucher *et al.* (2004), respectively, using the needed controls. Although the positive controls clearly shifted the bands showing binding to the 21 and 26 nt siRNAs (publication II, Fig. 3) and to the 49 nt dsRNA (data not shown), P1 of CfMV did not bind any of those RNA molecules.

Our future aim concerning this suppressor is to follow the silencing dynamics in a host plant and to find host factors interacting with it.

4.2. Effects caused by different viral RNA silencing suppressors in *N. benthamiana* and *N. tabacum*

The comparative study of RNA silencing suppressors helps to gain knowledge about the silencing process itself. The finding of two different classes of siRNAs (Hamilton *et al.*, 2002) and the understanding of a relay-amplification process in the movement of the silencing signal (Himber *et al.*, 2003) are examples of how research based on silencing suppressors has contributed to the understanding of RNA silencing.

One way of comparing silencing suppressors is through their transgenic expression. Such comparative analyses have previously been published for *Arabidopsis* (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004). Our aim was to compare seven different suppressors, belonging to six different viral genera, in two *Nicotiana* species. The genes of RNA silencing suppressors transformed to *N. benthamiana* and *N. tabacum* were the following: *P1* of RYMV, *P1* of CfMV, *P19* of TBSV, *P25* of PVX, *HcPro* of *Potato virus Y* (PVY, strain N), *2b* of CMV (strain Kin) and *AC2* of ACMV. All genes were inserted into pBIN61 binary vector. As a control, empty pBIN61 was also transformed into both *Nicotiana* species.

For each transgene, two independent homozygote R2 lines were selected, based on their 100 % germination rate on kanamycin-containing medium. The presence of the transgene in all lines was confirmed by PCR (publication III, Fig. 1B). The mRNA of the transgene was detected by Northern blot for many lines, whereas for other lines the mRNA remained below detection level (Tables 3 and 4; publication III, Fig. 1A). Interestingly, some lines where the mRNA could not be detected displayed clear variations in the phenotype of the plants, suggesting that the transgenes were indeed expressed (Tables 3 and 4).

Simplifying the results obtained, it is possible to distinguish between two groups: suppressors affecting the normal plant phenotype (HcPro, AC2, P19 and RYMV P1) versus suppressors not having significant effects on the phenotype (CfMV P1, P25 and 2b). The latter group includes suppressors counteracting systemic silencing and/or local silencing but only weakly (2b is from the mild strain Kin) therefore this result is not surprising.

Phenotypic variation compared to the control plants (wt/non-transformed and transformed with pBIN61) was especially evident in leaves and flowers. This could mean that the RNA silencing suppressors were interfering with the miRNA pathways responsible for the correct development of these organs. In addition to malformation in leaves and flowers, flowering was frequently delayed. miRNA levels play a crucial role in flowering time and in floral patterning. For example, miR172 is known to repress *APETALA2*. Through this negative regulation, miR172 defines the expression domain for *APETALA3* and *PISTILLATA* (Aukerman and Sakai, 2003; Chen, 2004; Zhao *et al.*, 2007). In fact miR172 is a good candidate for further analysis, at least in *N. benthamiana* transgenics, as it is known to be conserved and because it has been studied by others in this same *Nicotiana* species.

It is noteworthy that the flower malformation reported by Mlotshwa *et al.* (2006) for an *APETALA2* transgenic *N. benthamiana* phenocopies the flowers from HcPro *N. benthamiana* line 1 (publication III, Fig. 2D), suggesting that the miR172 level in that HcPro line may be reduced.

Another recurrent phenotypic variation was the stunting exhibited by several transgenic lines. This kind of plant growth alteration has also been linked to deregulated miRNA levels. For example, miR159 predominantly regulates transcription factor MYB33 and MYB65 genes. *Arabidopsis* T-DNA insertion mutants of miR159 displayed a stunted growth (Allen *et al.*, 2007).

Previous works with transgenic *N. benthamiana* reported no phenotypic alterations for plants transformed with HcPro from PVA (Savenkov and Valkonen, 2002) or PVY-N (Mlotshwa *et al.*, 2002), respectively. Differences between these and the above mentioned results could be due to different transgene mRNA or/and protein levels. We can also not rule out that the site of the transgene insertion plays a role. These reasons may also explain the marked differences between the homozygote lines 1 and 2 for some of the transgenes, including for example RYMV P1 in *N. benthamiana* or P19 in *N. tabacum*.

Another important observation is the fact that AC2 and HcPro, two suppressors known to function in different ways counteracting RNA silencing, cause macroscopically very similar malformations in the leaves of *N. tabacum* (Table 4). However, when observed at the cellular level, the alterations were not the same. Moreover, these two suppressors affected the leaf structures in an opposite way, with hyperplasia in the case of HcPro and hypoplasia caused by AC2 (publication III, Fig. 4). These effects were the same in both *Nicotiana* species, although the malformation in *N. benthamiana* looked macroscopically different (Table 3). Having the same malformations provoked by the same suppressor in two different species may reflect the conservation of miRNAs and their targets.

Compared to *N. tabacum*, the phenotype of *N. benthamiana* is much more affected by RNA silencing suppressors expressed transgenically (Tables 3 and 4). This *Nicotiana* species is also more sensitive to viral infections than *N. tabacum*. This suggests that the RNA silencing machinery of *N. benthamiana* is more vulnerable and more easily counteracted by viral suppressors. Thus, although closely related, these species have an important difference that directly or indirectly affects their defense capacity.

Having described the influence of the transgenically expressed suppressors on plant growth and development, we further wanted to analyze the effect on the RNA silencing as a defense mechanism. For this we challenged the transgenic lines with crTMV, but comparing only one R2 line for each suppressor. For each transgene the line chosen for the inoculation was the one displaying the most pronounced phenotypic variation, or at least where the transgene mRNA levels were higher. Table 1 and Fig. 3 from publication III describe and show these lines in detail.

For the crTMV inoculation we used a virus where the coat protein was replaced by GFP (Marillonnet *et al.*, 2004). This virus was able to infect and spread slowly in *N. benthamiana* but not in *N. tabacum* (data not shown). We agroinfiltrated the

leaves of the transgenic *N.benthamiana* and analyzed the changes in the fluorescence of GFP as well as the spread of the fluorescent area or appearance of new fluorescent spots far from the infiltrated area. The GFP brightness reflected the accumulation of the virus and the increase of the fluorescent area indicated the spreading of the virus (publication III, Fig. 5). AC2 clearly enhanced both the accumulation and spread of crTMV while 2b and also both of the P1s enhanced only the movement of the virus. P19 favoured the accumulation of crTMV and repeatedly caused spread of the infection into new foci. Surprisingly, HcPro and P25 both reduced the spread of the virus, with P25 doing so more strongly. These results seem to be in accordance with previous results of Pruss *et al.* (2004), showing that expression of the HcPro of TEV in transgenic tobacco plants enhances their resistance against TMV and against *Tomato black ring virus*.

The fact that almost all suppressors enhanced the accumulation and/or spread of crTMV reflects the synergistic effect of both viral suppressors (crTMV suppressor is p122; Csorba *et al.*, 2007) due to the well counteracted RNA silencing defense pathway.

For the future it would be needed to analyze how different miRNA levels are affected by the suppressors expressed in these transgenic plants.

Table 3. Transgene mRNA detection and variation in the phenotype of *N. benthamiana* R2 transgenic lines compared to wt

lines	mRNA	leaf	flower	growth
wt	---------	normal	normal	normal
pBIN61	---------	normal	normal	normal
HcPro **1**	+	curling, hairy, elongated vein pattern, no petioles	short petals and stamens, protruding pistils, no petioles, sterile	creeping stems, stunting
HcPro 2	++	curling	small, reduced seed set	stunting
AC2 1	+++	blistering	normal	normal
AC2 **2**	bdl	occasionally cup-shaped, blistering	reduced seed set	stunting
P19 1	bdl	blistering	occasionally bending stalks, reduced seed set	normal
P19 **2**	bdl	blistering, mildly serrated, hairy	occasionally bending stalks, reduced seed set	normal
RP1 **1**	bdl	occasionally cup-shaped	malformed petals, bending stalks, few flowers, sterile	stunting, reduced germination
RP1 2	bdl	curling	normal, reduced seed set	normal
CP1 1	+	normal	normal	normal
CP1 **2**	++	normal	occasionally bending stalks	normal
P25 1	bdl	curling	normal	normal
P25 **2**	++++	normal	smaller, not fully opened, reduced seed set	early senescence, stunting
2b **1**	++++	normal	normal	normal
2b 2	++	normal	normal	normal

bdl: below detection level; relative amounts of mRNA detected are symbolized by number of "+"; RP1: RYMV P1; CP1: CfMV P1; numbers in bold represent the line described in publication III, Table 1.

Table 4. Transgene mRNA detection and variation in the phenotype of *N. tabacum* R2 transgenic lines compared to wt

lines	**mRNA**	**leaf**	**flower**	**growth**
wt	---------	normal	normal	normal
pBIN61	---------	normal	normal	normal
HcPro **1**	++	thick, hairy, dark green, blistering	reduced seed set late flowering	stunting, short internodes
HcPro 2	+	thick, hairy, dark green, blistering	reduced seed set late flowering	stunting, short internodes
AC2 **1**	+	thick, hairy, blistering, occasionally cup-shaped	moderate malformation, stamens/sepals transformed into petals, less stamens, reduced seed set, late flowering	stunting, short internodes
AC2 2	+	normal	severely malformed, all stamens transformed into petals, reduced seed set	normal
P19 **1**	bdl	normal	occasionally severely malformed, late flowering	normal
P19 2	++	normal	normal	normal
RP1 **1**	++	normal	late flowering	normal
RP1 2	++	normal	normal	normal
CP1 1	bdl	normal	normal	normal
CP1 **2**	bdl	normal	reduced seed set	normal
P25 1	bdl	normal	normal	normal
P25 **2**	bdl	normal	normal	normal
2b **1**	+++	normal	late flowering	normal
2b 2	+++++	normal	normal	normal

bdl: below detection level; relative amounts of mRNA detected are symbolized by number of "+";RP1: RYMV P1; CP1: CfMV P1; numbers in bold represent the line described in publication III, Table 2.

4.3. AtRLI2 is an endogenous suppressor of RNA silencing

RNA silencing, as already explained, is also regulated by endogenous suppressors. This is one of the less studied fields of RNA silencing and therefore testing the role of RLI from *Arabidopsis* as an endogenous suppressor was attractive.

As 2-5A pathway has been an object of investigation in our department for a long time, it was tempting to find out if AtRLI is implicated in RNA silencing. AtRLI was a good candidate for being an endogenous suppressor because Braz *et al.* (2004) found that its expression in silenced plants was slightly increased. In addition, it has been reported that the overexpression of human RLI leads to an increased susceptibility to some viruses (Martinand *et al.*, 1998 and 1999).

Human RLI was cloned 13 years ago as a protein which inhibits the interferon-induced 2-5A antiviral pathway by blocking the activation of RNase L, the enzyme responsible for the degradation of RNA (Bisbal *et al.*, 1995). Surprisingly, although RNase L is only present in mammals, birds and reptiles (Player and Torrence, 1998), RLI is present in all eukaryotes and even in archaea (Kerr, 2004). Several functions of RLI not related to the 2-5A pathway have been described. Essential functions include its role in ribosome biogenesis and in translation initiation as well as in translation termination (Dong *et al.*, 2004; Yarunin *et al.*, 2005; Kispal *et al.*, 2005; Le Roy *et al.*, 2005; Chen *et al.*, 2006; Andersen and Leevers, 2007). Moreover, the knock-outs of RLI in yeast, *C. elegans* and *Trypanosoma brucei* are lethal (Winzeler *et al.*, 1999; Gonczy *et al.*, 2000; Estevez *et al.*, 2004).

The highly conserved RLI protein contains at its N-terminus a cystein rich motif binding two iron-sulfur clusters (FeS) followed by two important nucleotide-binding domains (NBDs; Barthelme *et al.*, 2007; Karcher *et al.*, 2007). The NBDs form a heterodimer with an interface where the ATPase active site is located. ATP binding or ADP dissociation could induce conformational changes of the NBDs and consequently of RLI (Karcher *et al.*, 2005).

According to Braz *et al.* (2004) *A. thaliana* has three RLI paralogous sequences: *AtRLI1* (At3g13640), *AtRLI2* (At4g19210) and *AtRLI3* (At4g30300), the last one being a truncated protein. We do not think that *AtRLI3* can be considered a functional paralog, since it consists only of an ATP-binding domain, does not contain any introns and according to The Arabidopsis Information Resource (TAIR, www.arabidopsis.org) database there are no ESTs corresponding to this sequence. *AtRLI2* displays the most consistent phylogenetic position and an overall higher expression level (Braz *et al.*, 2004), that is why we centered our analysis on this gene (Fig. 6).

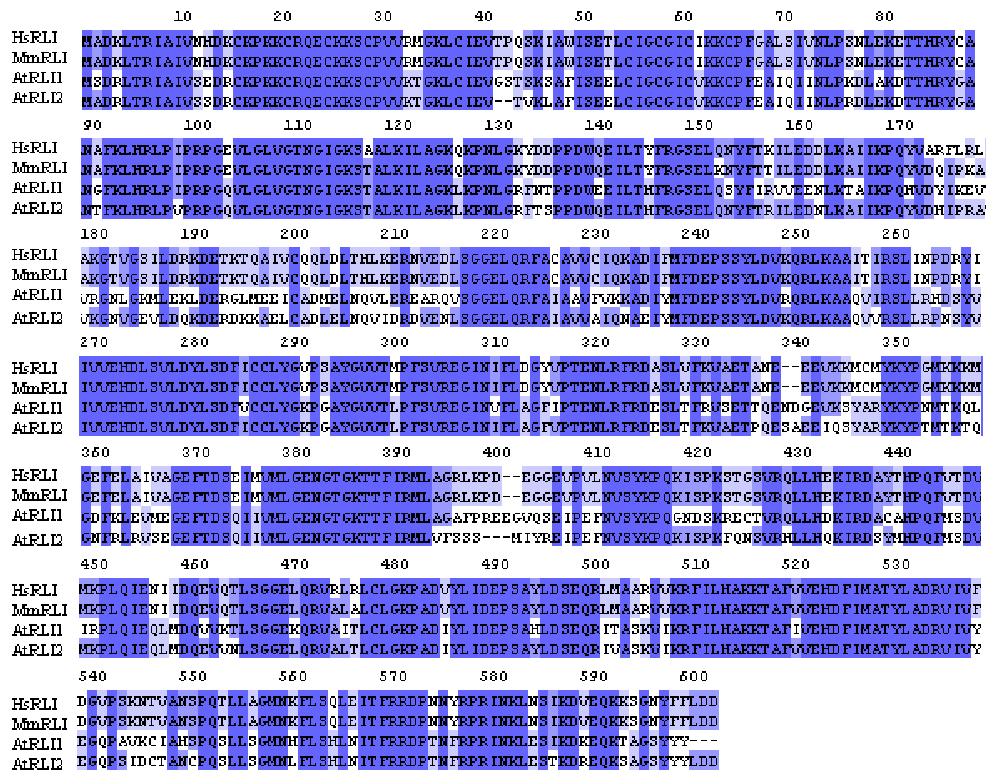


Fig. 6. Multiple ClustalW sequence alignment of RLI (courtesy of L. Nigul)
Hs: *Homo sapiens*, Mm: *Mus musculus*, At: *A. thaliana*, dark blue: identical amino acid residues, light blue: similar (conserved substitutions) amino acid residues.

First of all, the cDNA of *AtRLI2* was obtained, the predicted gene structure was confirmed and the level of expression in different plant organs determined. We showed that an intron is present at the 3' UTR, a seldom characteristic of eukaryotic genes (Deutsch and Long, 1999). In addition, we determined that *AtRLI2* is ubiquitously expressed and although Braz *et al.* (2004) obtained generally the same result, our analysis showed an increased expression in flowers and siliques but not in leaves.

To further analyze AtRLI2 expression related to RNA silencing, we carried out RPA of RNA isolated from the rosette leaves of a flowering (3-weeks-old) wt *A. thaliana* and of the GFP-silenced *A. thaliana* line GxA. We could not detect any significant difference at the *AtRLI2* expression level between both lines. Braz *et al.* (2004) had reported a slight increase of the expression in different silenced lines, all of them containing an inverted repeat as a transgene. The diverse results could be explained taking into consideration the existing differences between the silencing produced by a hairpin versus the one induced by a virus (Dunoyer *et al.*, 2007). The GxA line contains a PVX:GFP-amplicon transgene (Dalmay *et al.*,

2000) and therefore the GFP silencing in this line could be compared to a virus-induced silencing.

Thereafter we tested the putative role of AtRLI2 as RNA silencing suppressor in *N. benthamiana*, co-infiltrating AtRLI2 with GFP into *N. benthamiana* GFP-transgenic line. The result was that AtRLI2 suppressed the local as well as the systemic silencing, to a certain extent. The most striking outcome was the almost complete disappearance of the GFP siRNAs. We also observe the presence of the red ring bordering the infiltrated patch. All this implies that – at the conditions of the experiment – GFP silencing was affected at the amplification step and the cell-to-cell movement of the silencing signal was not compromised.

In order to know if the GFP siRNAs were drastically reduced because of a sequestering action of AtRLI2, we challenged the capacity of this suppressor to bind synthetic siRNA duplexes *in vitro* and the result was negative. The fact that AtRLI2 is not able to bind siRNAs in such a gel shift assay does not rule out the possibility that it can do it *in vivo*. We can also not exclude a possible binding to dsRNA or even ss siRNA. RLI is known as a protein which suffers conformational changes easily. Besides this, RLI is known to interact with ribosomal subunits and to affect translation initiation and termination in different eukaryotes, meaning that a putative association of RLI as part of a ribonucleoprotein complex is feasible. The conditions for the specific binding of RLI to siRNAs *in vivo* might be impossible to reproduce in an *in vitro* assay. This is because different molecules could be necessary to trigger a conformational change that induces the direct or indirect interaction with RNAs, exposing – in one or another way – the needed binding surfaces. Another possible limitation of the gel shift assay is the use of synthetic siRNAs that only mimic the real siRNA duplex but for example are not methylated at the 3' end. With the obtained results we do not know if AtRLI2 is able to bind any DCL, AGO or RDR.

It is noteworthy that in the case of an endogenous suppressor, a clear temporal and spatial regulation of its expression is needed and even more so if the suppressor is a multifunctional protein, as is the case for RLI. RLI is a very flexible protein, e.g. the complexes it forms with ribosomal subunits are weak, sensitive to salt concentration (Kispal *et al.*, 2005; Yarunin *et al.*, 2005). Thus, it is possible that although this protein is ubiquitously expressed, it is active as a suppressor only in the needed time and place, when the surroundings enable the corresponding conformational status for the suppressor activity or when it is recruited to some specific subcellular compartment. All this is overlooked in the agroinflitration assay, where RLI is transiently overexpressed.

RLI is a protein taking part in at least two defense pathways that have as trigger dsRNA, namely the 2-5A and the RNA silencing pathways. This is not surprising as it is known that different defense pathways are related. At the same time, it is of outstanding interest to continue the investigation on this protein in order to understand how the relationship between those pathways is actually established and how and when is one or another pathway switched on or off. Moreover, as RLI is a protein with an ancient origin, its investigation could give clues about the evolution

of different defense pathways. The better we understand the defense pathways, the better we can exploit them, especially with a therapeutic aim. In this sense, it is extremely important to test if human RLI is also a suppressor of RNA silencing. We are moving forward in order to clarify this.

CONCLUSIONS

1. P1 of CfMV is a suppressor of RNA silencing in *N. benthamiana* and in *N. tabacum*, two non-host plants. It interferes with the initiation and amplification of RNA silencing, affecting it at both the local and the systemic levels. Comparing this suppressor with the previously discovered sobemoviral suppressor RYMV P1, the effect of the CfMV suppressor on systemic RNA silencing is weaker.

2. The transgenic expression of the suppressors HcPro of PVY, AC2 of ACMV, P19 of TBSV and P1 of RYMV, induced malformations in leaves and flowers as well as frequent variation in plant growth in both *N. benthamiana* and *N. tabacum*. On the contrary, P25 of PVX, P1of CfMV and 2b of CMV (Kin strain) expression exhibited minimal phenotypic variation in both *Nicotiana* species.

3. HcPro expressed transgenically leads to hyperplasia in the leaf tissues of *N. benthamiana* and *N. tabacum*, whereas AC2 expression gives rise to hypoplasia in the leaves of the same plant species.

4. The transgenic expression of AC2, P19, 2b, CfMV P1 and RYMV P1 in *N. benthamiana*, enhanced the accumulation and/or spread of crTMV, whereas HcPro and P25 reduced the spread of the virus.

5. AtRLI2 is an endogenous suppressor of RNA silencing. It affects sense-transgene silencing at the local and systemic levels when expressed transiently in *N. benthamiana*. In this system, RLI reduces drastically the amount of siRNAs and at the same time it diminishes the extension of systemic silencing.

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PUBLICATION I

AtRLI2 is an endogenous suppressor of RNA silencing

Sarmiento, C., Nigul, L., Kazantseva, J., Buschmann, M. and Truve, E.

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PUBLICATION II

Cockcfoot mottle virus* P1 suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum

Sarmiento, C., Gomez, E., Meier, M., Kavanagh, T. A. and Truve, E.

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PUBLICATION III

Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic Nicotiana benthamiana and N. tabacum

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ABSTRACT

Suppressors of RNA silencing in plants

RNA silencing collectively refers to the suppression of gene expression through sequence-specific interactions that are mediated by RNA. This mechanism is involved in gene regulation, maintenance of genome stability and antiviral response in both animals and plants.

Viruses and their hosts have co-evolved and this is reflected by the diverse range of viral proteins coded to counteract the RNA silencing mechanism. These proteins are known as viral suppressors of RNA silencing. During the last ten years, many different viral suppressors have been described, especially for plant viruses. The study of these proteins has provided important knowledge about the RNA silencing mechanism itself. Therefore, the research on viral suppressors is not only meant to develop tools for controlling the viral infections but it is also a suitable way to gain an understanding about a mechanism which can be exploited as a powerful biotechnology instrument with countless promising applications.

The fact that RNA silencing is involved in many different processes implies that it is finely tuned to act when, where and how it is needed. As a consequence, there are also negative regulators coded by the host itself, known as endogenous suppressors of RNA silencing. Up to now, few such suppressors have been described in both plants and animals.

This thesis deals with both kinds of RNA silencing suppressors in plants: viral and endogenous ones. The suppressor of cocksfoot mottle sobemovirus (CfMV), a virus infecting only monocots, was identified. It is P1, a non-conserved protein of the *Sobemovirus* genus. This protein partially blocks RNA silencing in the non-host species *Nicotiana benthamiana* and *Nicotiana tabacum*. P1 of rice yellow sobemovirus (RYMV) was already known as a suppressor and when compared to cocksfoot mottle sobemoviral P1, it was clearly stronger at the systemic level.

Further, this thesis presents a comparative study of seven viral suppressors expressed transgenically in both *N. benthamiana* and *N. tabacum*: *P1* of RYMV, *P1* of CfMV, *P19* of *Tomato bushy stunt virus* (TBSV), *P25* of *Potato virus X* (PVX), *HcPro* of *Potato virus Y* (PVY, strain N), *2b* of *Cucumber mosaic virus* (CMV, strain Kin) and *AC2* of *African cassava mosaic virus* (ACMV). The suppressors inducing malformations in leaves and flowers as well as variation in the plant growth were HcPro, AC2, P19 and RYMV P1. Compared to *N. tabacum*, the phenotype of *N. benthamiana* is much more affected by RNA silencing suppressors. This variation in the phenotype is most probably due to interference with the microRNA pathways caused by the suppressors. The transgenic plants were also challenged with a tobamovirus (crTMV) to analyze how the antiviral mechanism was counteracted. Almost all suppressors enhanced the accumulation and/or spread of crTMV. The exceptions were HcPro and P25.

Finally, a new endogenous suppressor was identified and described in *Arabidopsis*. It is AtRLI2, a homolog of RNase L inhibitor (RLI), a protein which inhibits the interferon-induced 2-5A antiviral pathway in mammals. RLI is highly conserved and present in all eukaryotes and even in archae although RNase L is

only present in mammals, birds and reptiles. As RNA silencing is present in all eukaryotes, the role of RLI as endogenous suppressor could be its universal function.

KOKKUVÕTE

RNA vaigistamise supressorid taimedes

Termin RNA vaigistamine tähistab RNA-vahendatud geeniekspressiooni allasurumist läbi järjestusspetsiifiliste interaktsioonide. See mehhanism osaleb nii loomades kui ka taimedes geeniregulatsioonis, genoomi stabiilsuse säilitamises ning antiviraalsetes reaktsioonides.

Viirused ja nende peremehed evolutsioneeruvad koos ning seda kajastavad ka väga erisugused viraalsed valgud, mis kõik funktsioneerivad kui erineval viisil RNA vaigistamist takistavad molekulid. Neid valke tuntakse RNA vaigistamise supressoritena. Viimase kümne aasta jooksul on kirjeldatud palju erinevaid viraalseid supressoreid, seda eriti taimeviirustest. Nende valkude uurimine on andnud olulist informatsiooni RNA vaigistamise mehhanismi enda kohta. Seetõttu ei saa tööd nende valkudega võtta üksnes kui abinõud uute strateegiate rakendamiseks viirusinfektsioonide kontrollimisel, vaid kui paljulubavat biotehnoloogilist tööriista väga erinevate atraktiivsete rakenduste elluviimiseks.

Tõsiasi, et RNA vaigistamine on osa nii paljudest erinevatest bioloogilistest protsessidest, viitab sellele, et vajalikud on mehhanismid, mis täpselt määratlevad, millal, kus ja kuidas vaigistamine töötab. Seetõttu kodeerivad rakud ka endogeensete supressoritena tuntud vaigistamise negatiivseid regulaatoreid. Seniajani on kirjeldatud üksikuid selliseid valke nii taimedest kui ka loomadest.

Käesolev dissertatsioon tegeleb nii viraalsete kui ka endogeensete RNA vaigistamise supressoritega taimedes. Esmalt identifitseeriti supressor keraheina laiguviirusest (*Cocksfoot mottle virus*, CfMV) – üksnes teatud kõrrelisi nakatavast viirusest. Tegemist oli valguga P1, mis on äärmiselt erinev kõikidel perekonna *Sobemovirus* liikidel. See valk blokeerib osaliselt RNA vaigistamist tubakas ning *Nicotiana benthamiana*'s – kahes taimeliigis, mis pole selle viiruse jaoks peremeesteks. Riisi kollalaiksuse viiruse (*Rice yellow mottle virus*, RYMV) P1 valku tunti juba varem kui supressorit. Võrreldes süsteemsel tasemel CfMV P1-ga, on selle valgu näol tegemist oluliselt tugevama vaigistamise supressoriga.

Edasi kirjeldab käesolev dissertatsioon võrdlevat analüüsi, mille käigus ekspresseeriti seitset erinevat viraalset RNA vaigistamise supressorit transgeensetes tubaka ning *N. benthamiana* taimedes. Nendeks supressoriteks olid RYMV P1, CfMV P1, tomati põõsasja kääbusviiruse (*Tomato bushy stunt virus,* TBSV) P19, kartuliviiruse X (*Potato virus X,* PVX) P25, kartuliviiruse Y (*Potato virus Y,* PVY, tüvi N) HcPro, kurgi mosaiigiviiruse (*Cucumber mosaic virus,* CMV, tüvi Kin) 2b ning aafrika manioki mosaiigiviiruse (*African cassava mosaic virus,* ACMV) AC2. HcPro, AC2, P19 ning RYMV P19 põhjustasid taimede lehtede ja õite arenguhäireid. Sealjuures olid *N. benthamiana*'s avalduvad tunnused märksa tugevamad. Selliseid muudatusi põhjustasid ilmselt supressorite avaldatud mõjud erinevatele mikroRNA-de biokeemilistele radadele. Transgeenseid taimi testiti ka nende vastuvõtlikkuse osas tobamoviiruste (crTMV) infektsioonile. Enamus supressoreid soodustas crTMV paljunemist ja/või levimist. Erandina ei teinud seda HcPro ning P25.

Töö viimases osas kirjeldame me uudse endogeense supressori, AtRLI2, identifitseerimist ning iseloomustamist müürloogast. Tegemist on RNaas L-i inhibiitori (RLI) homoloogiga. Imetajates inhibeerib RLI interferoon-indutseeritavat 2-5A rada. RLI on kõrgelt konserveerunud valk, mis esineb peaaegu kõikides eukarüootides ning isegi arhedes. Samas on RNaas L ise teadaolevalt olemas üksnes imetajate, lindude ning roomajate rakkudes. Arvestades seda, et ka RNA vaigistamine toimib peaaegu kõikides eukarüootides, võiks RLI kui endogeense supressori kirjeldamise kaudu olla paika pandud ka selle valgu universaalne roll eluslooduses.

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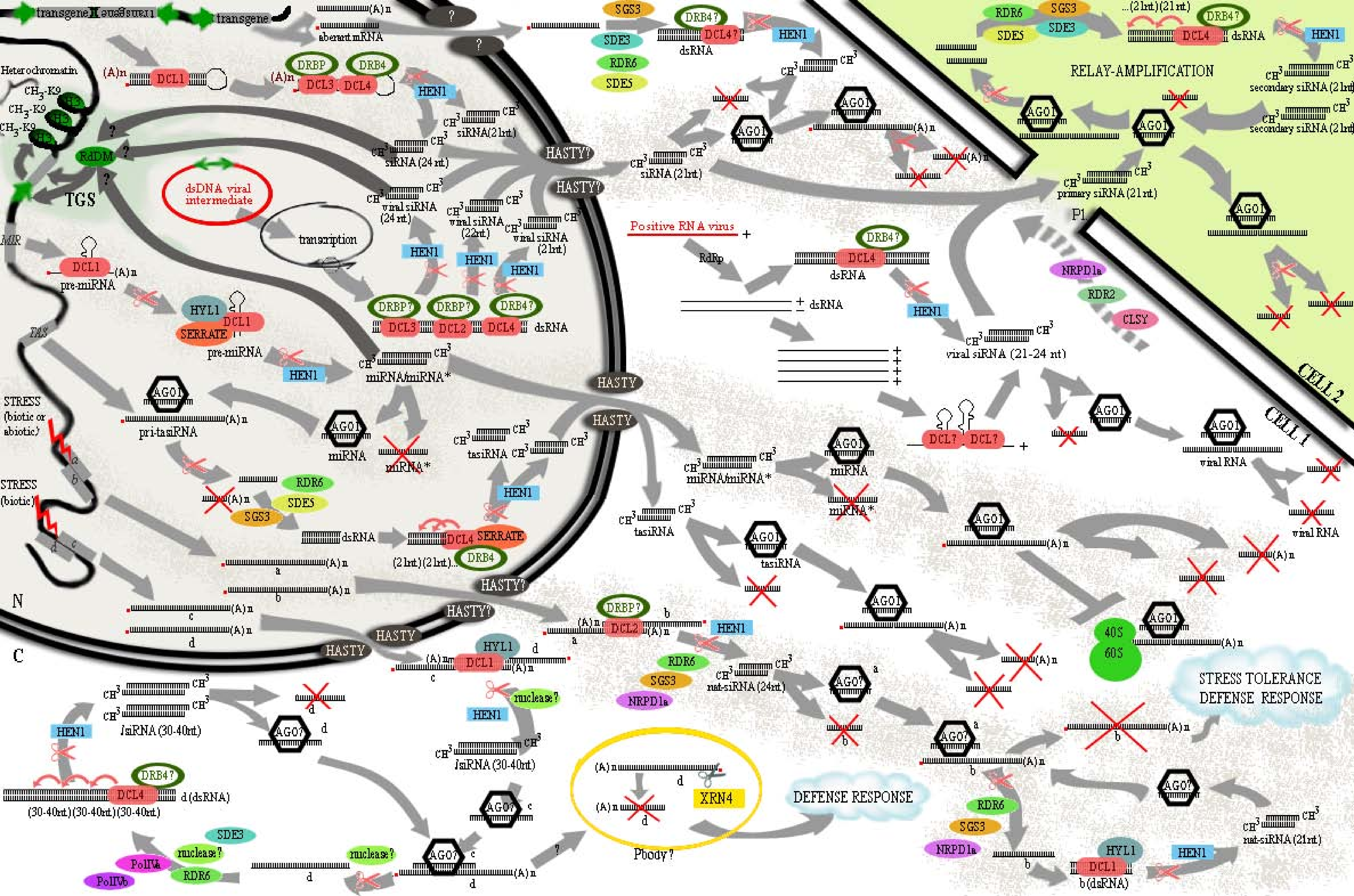

transgene
aberrant mRNA
Heterochromatin
DCL1
DRBP
DRB4
DCL3
DCL4
HEN1
siRNA (21nt)
siRNA (24nt)
RdDM
TGS
dsDNA viral intermediate
transcription
viral siRNA (24nt)
viral siRNA (22nt)
viral siRNA (21nt)
HASTY?
MIR
pre-miRNA
HYL1
SERRATE
TAS
miRNA/miRNA*
AGO1
pri-tasiRNA
miRNA
miRNA*
STRESS (biotic or abiotic)
STRESS (biotic)
RDR6
SDE5
SGS3
dsRNA
tasiRNA
HASTY
N
C
SDE3
DRB4?
dsRNA
RELAY-AMPLIFICATION
secondary siRNA (21nt)
primary siRNA (21nt)
P1
Positive RNA virus
RdRp
NRPD1a
RDR2
CLSY
viral siRNA (21-24 nt)
DCL?
viral RNA
CELL 1
CELL 2
DRBP?
DCL2
nat-siRNA (24nt)
AGO?
40S
60S
STRESS TOLERANCE DEFENSE RESPONSE
lsiRNA (30-40nt)
nuclease?
XRN4
Pbody?
DEFENSE RESPONSE
d (dsRNA)
PolIVa
PolIVb
nat-siRNA (21nt)
b (dsRNA)

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AtRLI2 is an endogenous suppressor of RNA silencing

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Key words: agroinfiltration, *Arabidopsis thaliana*, expression analysis, RNA interference, RNase L inhibitor, siRNA

Abstract

RNA silencing is a mechanism involved in gene regulation during development and anti-viral defense in plants and animals. Although many viral suppressors of this mechanism have been described up to now, this is not the case for endogenous suppressors. We have identified a novel endogenous suppressor in plants: RNase L inhibitor (RLI) of *Arabidopsis thaliana*. RLI is a very conserved protein among eukaryotes and archaea. It was first known as component of the interferon-induced mammalian 2′–5′ oligoadenylate (2–5A) anti-viral pathway. This protein is in several organisms responsible for essential functions, which are not related to the 2–5A pathway, like ribosome biogenesis and translation initiation. *Arabidopsis* has two RLI paralogs. We have described in detail the expression pattern of one of these paralogs *(AtRLI2)*, which is ubiquitously expressed in all plant organs during different developmental stages. Infiltrating *Nicotiana benthamiana* green fluorescent protein (GFP)-transgenic line with *Agrobacterium* strains harboring *GFP* and *AtRLI2*, we proved that AtRLI2 suppresses silencing at the local and at the systemic level, reducing drastically the amount of GFP small interfering RNAs.

Abbreviations: 2–5A, 2′–5′ oligoadenylate; Ago, Argonaute protein; *AtRLI2*, *Arabidopsis thaliana* gene At4g19210; dpi, days post-infiltration; dsRNA, double-stranded RNA; GFP, green fluorescent protein; nt, nucleotide; RISC, RNA-induced silencing complex; RdRP, RNA-dependent RNA polymerase; RLI, RNase L inhibitor; RNAi, RNA interference; RPA, RNase protection assay; siRNA, small interfering RNA; UTR, untranslated region

Introduction

RNA silencing or RNA interference (RNAi) fulfils diverse biological roles, including – at least in animals and plants – defense against viruses (Voinnet, 2005). It is therefore not surprising that viruses encode proteins to suppress various stages of RNA silencing. Although RNA silencing also bears a clear role in development (Bartel, 2004), very little is known about endogenous RNA silencing suppressors.

Double-stranded RNA (dsRNA)-mediated anti-viral defense mechanisms were characterized a long time ago, particularly in animals. One of the very first dsRNA-dependent anti-viral pathways discovered was the mammalian 2′–5′ oligoadenylate (2–5A) system (Brown *et al.*, 1976; Kerr and Brown, 1978). The complete system is characteristic only of higher vertebrates. Some of its components have also been found in invertebrates, but not outside the animal kingdom (Wiens *et al.*, 1999). Interestingly, the least characterized component of

the pathway, the protein initially named RNase L inhibitor (RLI) (Bisbal *et al*., 1995), is encoded by genomes of all eukaryotes as well as archaea (Kerr, 2004). Its common function in all these different organisms is unknown, but it cannot be related to RNase L which is present only in mammals, birds and reptiles (Player and Torrence, 1998). A recent report proposed that RLI plant ortholog could be involved in RNA silencing (Braz *et al*., 2004).

In the genome of higher plants, like in rice and *Arabidopsis thaliana*, the RLI gene has been duplicated (Braz *et al*., 2004; Kerr, 2004). The analysis of EST databases indicates that two *Arabidopsis* sequences, highly similar to RLI, are functional genes. One is located on chromosome 3 (At3g13640, *AtRLI1*) and the other on chromosome 4 (At4g19210, *AtRLI2*). *AtRLI2* expression is easily detectable in all organs during different developmental stages whereas *AtRLI1* expression is not (Braz *et al*., 2004).

In this study we report a detailed expression pattern of *AtRLI2*. In addition, exploiting the agroinfiltration assay and small interfering RNA (siRNA) detection, we prove that in our system AtRLI2 acts as an endogenous suppressor of RNA silencing.

Materials and methods

Plant material

Wild-type *A. thaliana* ecotype Columbia and *A. thaliana* green fluorescent protein (GFP)-silenced line GxA (kindly provided by D. Baulcombe, The Sainsbury Laboratory, UK) were grown in a plant room at 22 °C under a 16-h photoperiod. Wild-type *Nicotiana benthamiana* and *N. benthamiana* GFP transgenic line 16c (kindly provided by D. Baulcombe) were grown in a plant room at 24 °C under a 16-h photoperiod.

AtRLI2 *cDNA*

cDNA of *AtRLI2* was obtained from the Arabidopsis Biological Resource Center as EST clone 232A23T7 (GenBank Accession No. N65784). The clone was cut with restriction enzyme *Sal*I. The resulting three fragments were subcloned into pUC57 plasmid and sequenced.

RNA isolation, Northern analysis and RT-PCR

Total *Arabidopsis* RNA from 0.2 g of different organs was isolated as described by Logemann *et al*. (1987).

Ten micrograms of total RNA were separated electrophoretically through agarose gel containing formaldehyde following the capillary transfer of RNAs onto Hybond-N filter (Sambrook and Russell, 2001). ^{32}P-labeled cDNA probes corresponding to the 1200 nucleotide (nt) long central part of *AtRLI2* were used for the subsequent hybridization. Radioactive signals were detected with Molecular Imager GS-525 (BioRad).

Primers 3′UTR5′ (5′-cggggtaccgacaactaccaagag-3′) and 3′UTR3′ (5′-cggggtaccgtaaaatccaaagtagta-3′) were used for RT-PCR. Two out of four separate RNA isolations were treated with DNase I for 30 min. 1 μl out of 20 μl of the total RNA preparation in water was taken for the first strand cDNA synthesis. The reaction was carried out at 42 °C for 50 min in the presence of primer 3′UTR3′. PCR reaction was carried out in 30 cycles in the presence of both 3′UTR primers at 56 °C (annealing temperature). Control reactions without reverse transcriptase were carried out with RNAs not treated with DNase I.

RNase protection assay

The procedure was carried out using the Ambion RPA III kit according to the manufacturer's recommendations with the following modifications: only RNase T1 was used and the hybridization was carried out at 45 °C. ^{32}P-labeled *AtRLI2* riboprobe was synthesized by *in vitro* transcription of a linearized plasmid containing the sequence specific to the 300 nt region of *AtRLI2* 3' untranslated region (UTR). Ten micrograms of the total RNA from various plant organs were hybridized using *AtRLI2* riboprobe. The radioactive signal was detected with Molecular Imager GS-525.

Construction of binary vectors and plant transformation

AtRLI2 cDNA was cut out from the original clone 232A23T7 with *Eco*105I and *Kpn*I. 35S promoter of *Cauliflower mosaic virus* (CaMV) was cut out from pANU5 (Mäkinen *et al*., 1995) with *Hin*dIII and *Kpn*I. pCAMBIA1301 was linearized with

*Hin*dIII and *Ecl*136II. Consequent triple ligation resulted with the binary construct pC35S-RLI, where *AtRLI2* cDNA was under the control of 35S promoter.

Approximately 1 kb region upstream from the start of *AtRLI2* 5′UTR, presumably containing the promoter region of *AtRLI2*, was PCR amplified from *Arabidopsis* total DNA with primers RLI2prom5′ (5′-ctcggtaccggtatggccatgcccc-3′) and RLI2prom3′ (5′-cggggtacctaaggaaccagcggag-3′). The resulting fragment was ligated into the pTZ57R/T vector (InsT/Aclone PCR Product Cloning Kit; MBI Fermentas) yielding plasmid pTZ-RLI-P. cDNA of the reporter gene *GUS* with *nos*-terminator was cut out from pANU5 with *Ecl*136II and *Hin*dIII and ligated to *Sma*I and *Hin*dIII sites of pTZ-RLI-P giving the construct pRLI2prom1. In order to obtain pRLI2prom, pRLI2prom1 was cut with *Pst*I and ligated into empty vector pTZ57R/T, previously linearized with *Pst*I. This same fragment from pRLI2prom was also cloned into pCAMBIA1300 *Pst*I site to obtain pC-RLI2prom.

pCAMBIA1301 as a positive control, pC35S-RLI and pC-RLI2prom were transformed into *Agrobacterium tumefaciens* strain C58C1 by electroporation and selected in Luria–Bertani medium containing kanamycin at 50 μg/ml, tetracycline at 5 μg/ml and rifampicin at 100 μg/ml.

Arabidopsis thaliana plants were transformed with pC-RLI2prom and pCAMBIA1301 as described by Clough and Bent (1998). Putative hygromycin-resistant transformants were selected. T3 generation plants were verified to be *uidA* (β-glucuronidase)-transgenics carrying out the histochemical GUS assay as described by Nigul *et al.* (2004).

Agroinfiltration assay

Equal volumes of *A. tumefaciens* culture containing p35S-GFP (kindly provided by D. Baulcombe) and *A. tumefaciens* culture harboring pC35S-RLI were mixed before infiltration. As controls, *A. tumefaciens* carrying the empty pCAMBIA1301 sequence or pBIN61-P19 (provided by D. Baulcombe) were also co-infiltrated with *A. tumefaciens* containing p35S-GFP. Agroinfiltrations with only one *A. tumefaciens* culture (harboring pC35S-RLI or pBIN61-P19) were carried out in parallel. The final density for each *A. tumefaciens* culture was always 1.0 at OD_{600}. These cultures were prepared for infiltration and then also infiltrated to *N. benthamiana* line 16c as previously described (Hamilton *et al.*, 2002). Infiltrated plants were kept in a plant room at 24 °C under a 16-h photoperiod. GFP fluorescence was monitored visually using a hand-held 100 W, long-wave UV lamp (Black-Ray B-100AP, Ultraviolet Products). Plants were photographed with Olympus CAMEDIA digital camera and the pictures were thereafter processed using Adobe Photoshop 6.0.

RNA isolation and Northern analysis from infiltrated tissue

Total RNA was extracted from the agroinfiltrated patches as described previously (Szittya *et al.*, 2002). RNA gel blot analysis of 21- to 26-nt RNAs was performed according to Szittya *et al.* (2002), with the following modifications: RNA electroblotting onto Hybond-N membranes was done in 0.5 Tris–borate–EDTA buffer for 1 h at 500 mA and RNA was fixed by UV-crosslinking. Northern analysis of higher molecular weight RNAs was carried out the same way, but the electrophoresis was run longer and the membrane was washed with greater stringency. ^{32}P-labeled *in vitro* transcript corresponding to the anti-sense strand of GFP was used as probe. Radioactive signals were detected with Molecular Imager GS-525.

Expression and purification of recombinant AtRLI2

The coding sequence of *AtRLI2* was PCR-amplified from clone 232A23T7 using primers RLIexpr5′ (5′-ctcggtaccgcagatcgattgacacg-3′) and RLIexpr3′ (5′-ccggggtaccctaatcatccaagtagtag-3′) and cloned into pTZ57R/T vector. The coding sequence was then cut out with *Sma*I and *Xho*I and cloned into the same sites of pGEX6P-2 (Amersham). Transformed *E. coli* strain BL21(DE3)pLysS cells were grown from overnight cultures in 2xYT medium in the presence of ampicillin (100 μg/ml) at 30 °C to the optical density of $A_{600} = 0.6$ and induced by adding 0.2 mM IPTG, followed by incubation for 3 h. The cells were harvested by centrifugation and GST-fusion protein was purified as described by Dong and Silverman (1997) with the following modifications. PBSc buffer containing 10 mM NaH_2PO_4, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM ATP, 10% glycerol, 1 mM PMSF

(pH 7.4) was used for resuspending the cells. Suspended cells were disrupted with a French press. After sedimenting cell debris and other insoluble material, GST-tagged AtRLI2 was purified from supernatant by Sepharose 4B affinity chromatography (Amersham) according to the manufacturer's instructions. Human RNase L and influenza A virus NS1 proteins were purified from recombinant *E. coli* strains DH5α and XL1-Blue, respectively (kindly provided by R. Silverman and M. Prins) according to Dong and Silverman (1997) and Bucher *et al.* (2004).

siRNA binding assay

5×10^{-3} pmol of ^{32}P-labeled synthetic luciferase GL3 double-stranded small interfering RNA (siRNA) (Qiagen) was mixed with 0.5 pmol of protein (GST-RNaseL, GST-RLI or His-NS1) in RNA-binding solution (Wang *et al.*, 1999). GST and BSA were also mixed with siRNA as controls. Incubation, native gel electrophoresis and detection were done as described by Bucher *et al.* (2004).

Results

AtRLI2 *is expressed in all organs throughout* Arabidopsis thaliana's *lifecycle*

The sequencing of 232A23T7 subcloned fragments revealed the full-length cDNA of *AtRLI2*. The resulting sequence confirmed that the predicted gene structure available at The Arabidopsis Information Resource (TAIR) database is correct, including the predicted intron in the 3′UTR of *AtRLI2* gene. We were able to prove the splicing of this intron in rosette leaves (Figure 1) as well as in all other plant organs (data not shown).

Northern analysis showed that *AtRLI2* is expressed in leaves, stems, roots, flowers and siliques (Figure 2).

To quantify the expression of *AtRLI2*, we used RNase protection assay (RPA). RPA showed that the mRNA is present in all *Arabidopsis* organs at different developmental stages. Increased expression of *AtRLI2* was observed at the flowering stage (3-week-old plants) in different shoot organs, especially in flowers and siliques (Figure 3).

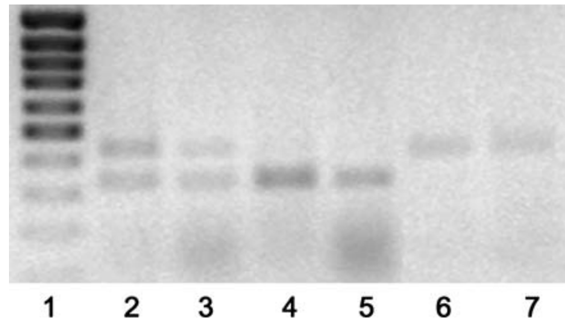

Figure 1. RT-PCR of wild-type *A. thaliana* rosette leaves. 1, DNA size-marker (GeneRuler™ 100 bp DNA Ladder, Fermentas); 2 and 3, two different RNA isolations, not treated with DNase I; 4 and 5, two different RNA isolations, DNase I-treated; 6 and 7, same RNA isolations as on lines 2 and 3, without reverse transcriptase.

Additional evidence for *AtRLI2* being an ubiquitously expressed gene, comes from the analysis of transgenic plants, where *GUS* reporter gene was under the control of *AtRLI2* promoter region. Before transforming *Arabidopsis*, we checked our construct pRLI2prom by bombarding it in parallel with the control plasmid pANU5 into *N. benthamiana* young leaves. Histochemical GUS assay of bombarded leaves showed that the cloned promoter region of *AtRLI2* is capable of directing the expression of the reporter gene with comparable efficiency to that of 35S promoter (data not shown). After subsequent selections for hygromycin resistance, we analyzed 10 T3 plants from 2 independent transgenic lines. In all plants analyzed, GUS expression was easily detectable in all organs of two-week-old plants. The intensity of GUS staining was comparable to that of GUS expression driven by CaMV 35S promoter in pCAMBIA1301 (Figure 4).

AtRLI2 suppresses RNA silencing in plants

To elucidate if AtRLI2 plays a role in RNA silencing as it has been recently proposed (Braz

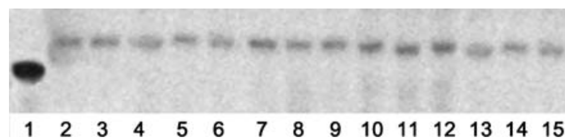

Figure 2. Northern analysis of *AtRLI2*. 1, *AtRLI2* partial cDNA (1200 nt); 2 and 3, leaves; 4–6, roots; 7–9, flowers; 10–12, siliques; 13–15, stems.

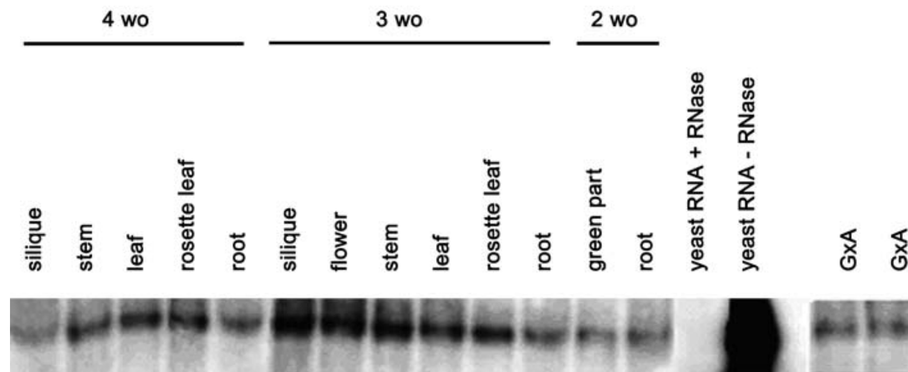

Figure 3. RPA of different *A. thaliana* organs from different developmental stages and of *A. thaliana* GFP-silenced line GxA. wo, week-old.

et al., 2004), we applied the broadly used *Agrobacterium*-mediated transient expression system (Voinnet *et al.*, 2000; Johansen and Carrington, 2001). A week after the infiltration of *N. benthamiana* 16c line with *Agrobacterium* strain carrying the *GFP* silencing initiator reporter gene in combination with one expressing the *AtRLI2* gene product, the infiltrated patch remained green as the non-infiltrated leaf tissue itself. Only a minimal red zone, indicating silencing, was observed at the edge of the patch. This patch was clearly different from the red one, where *Agrobacterium* harboring the *GFP* gene together with one containing the empty binary vector were infiltrated. It also differed from the strong green fluorescence emitted by the patch co-infiltrated with *GFP* and *Tomato bushy stunt virus P19*, gene coding a strong plant viral silencing suppressor (Hamilton *et al.*, 2002; Figure 5A and B).

Ten days post-infiltration (10 dpi), when systemic RNA silencing had been established and red veins could be detected under UV light in the newly emerged leaves of plants infiltrated with *GFP* and the empty binary vector (control plants), some plants infiltrated with *GFP* and *AtRLI2* also showed red veins, while the ones infiltrated with *GFP* and *P19* did not (Figure 5C). In contrast, 14 dpi, taking into consideration any kind of red tissue appearing in a place different from the infiltrated area, systemic silencing could be detected in 73% of the control plants, 63% of the plants infiltrated with *GFP* and *P19*, and 65% of the ones infiltrated with *GFP* and *AtRLI2*. Thirty-five days post-infiltration, the percentages raised to 100%, 86% and 83%,

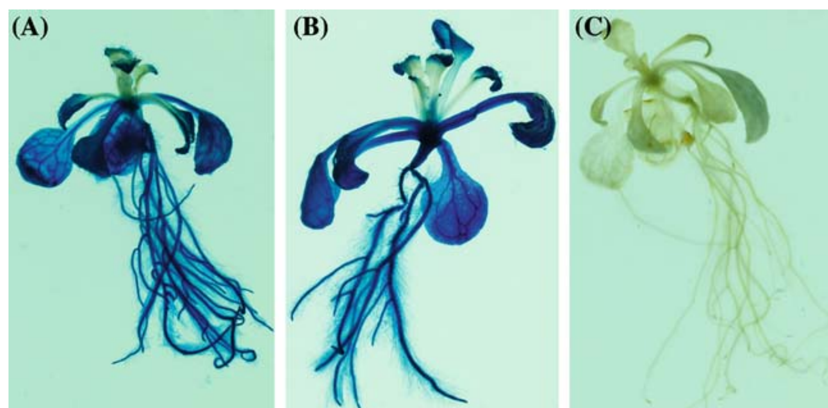

Figure 4. Histochemical GUS assay of two-week-old *A. thaliana*. (A) Transgenic plant (T3) carrying the *GUS* reporter gene under the control of *AtRLI2* promoter region. (B) pCAMBIA1301 transgenic plant (T3) carrying the *GUS* reporter gene under the control of CaMV 35S promoter. (C) Wild-type *A. thaliana*.

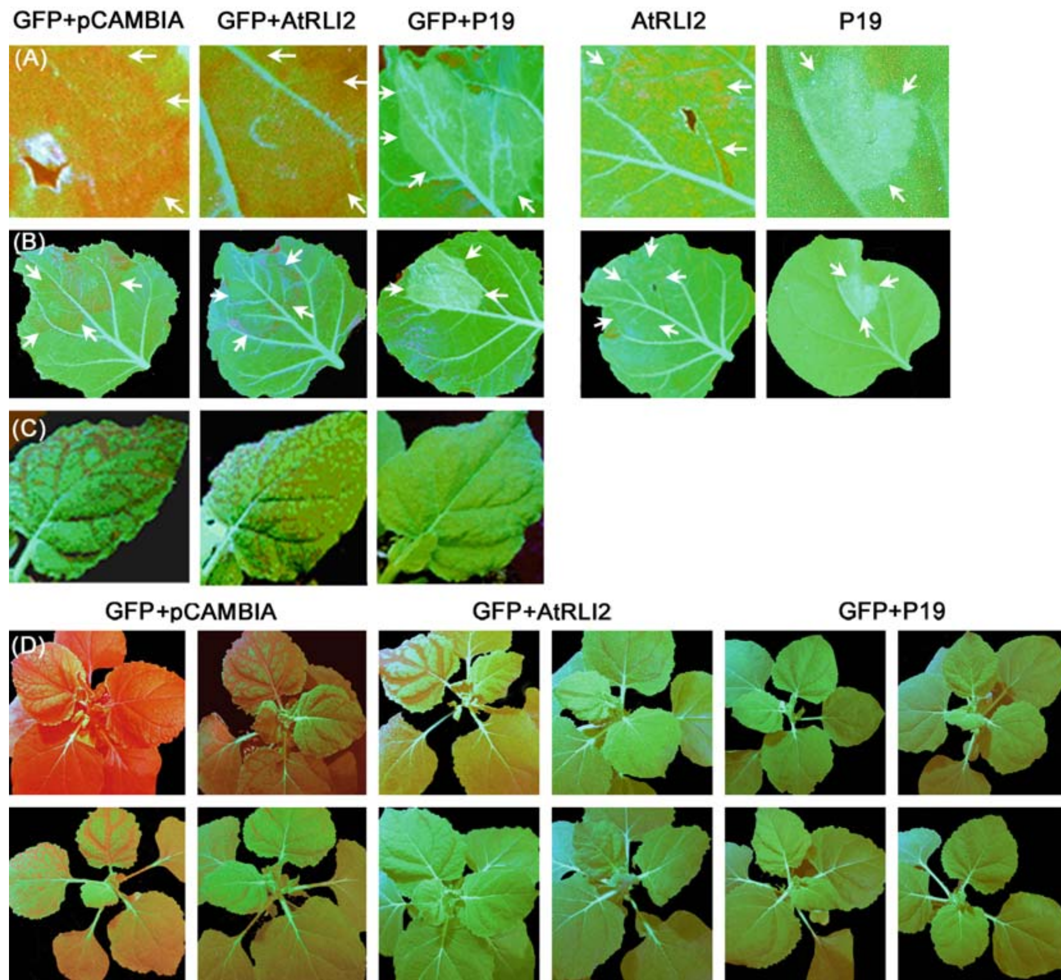

Figure 5. GFP-silencing in *N. benthamiana* 16c line. Plants were infiltrated with *A. tumefaciens* harboring the sequences shown on the upper part of the panels. Local silencing (A and B). Systemic silencing (C and D). (A) Close-ups of infiltrated patches 7 dpi. (B) Infiltrated leaves 7 dpi. (C) Leaves showing systemic silencing 10 dpi. (D) Plants showing systemic silencing 14 dpi. A representative sample of 4 plants taken from 3 independent experiments with 7–8 plants each. All photographs were taken under UV light. Arrows (in A and B) indicate the boundary of the infiltrated patches.

respectively. All these results are the outcome of three independent experiments with 7–8 plants each. There was also a clear difference when we compared the amount of silenced (red) tissue, which appeared in the different cases: among the control plants we could find some of them almost completely red, while the plants infiltrated with *GFP* and *P19* showed in general very few red veins. The plants infiltrated with *GFP* and *AtRLI2* had more red than the latter ones but clearly less than the control plants (Figure 5D).

To further analyze the suppressor effect of AtRLI2, we infiltrated the same GFP-transgenic *N. benthamiana* line with the well-known *P19* suppressor or with *AtRLI2*, now in absence of the RNA silencing initiator. In this case, only P19 boosted GFP expression (Figure 5A and B).

AtRLI2 strongly reduces siRNAs accumulation

Total RNA was extracted from the infiltrated patches and Northern blot analysis was carried out to detect *GFP* mRNA and *GFP* siRNA levels. The presence of AtRLI2 drastically reduced the accumulation of *GFP*-specific siRNAs in three independent experiments, indicating that this protein suppresses the RNA silencing pathway at an important point. A week after infiltration (7 dpi),

only faint remainders of approximately 21- and 25-nt siRNA bands could be detected in patches co-infiltrated with *GFP* and *AtRLI2*, while in patches infiltrated with *GFP* and the empty vector, both siRNA bands were clearly present. In the case of the patch co-infiltrated with *GFP* and *P19*, no siRNAs could be found. The co-infiltration of *GFP* with *AtRLI2* raised the levels of *GFP* mRNA above the levels of the *GFP* mRNA detected in the silenced control plants (infiltrated with *GFP* and the empty vector) but remained lower than the levels detected in a non-infiltrated GFP-transgenic *N. benthamiana*. The co-infiltration of *GFP* with *P19* boosted drastically the accumulation of *GFP* mRNA (Figure 6). These differences in the levels of *GFP* mRNA were confirmed by semi-quantitative RT-PCR (data not shown).

AtRLI2 does not bind siRNAs

Since AtRLI2 reduced drastically the levels of siRNAs, hinting a feasible direct interaction of AtRLI2 protein with siRNAs, we decided to check if AtRLI2 binds siRNAs *in vitro*. As a positive binding control we used influenza A virus NS1 protein, reported recently to be a silencing suppressor which binds siRNAs (Bucher *et al.*, 2004). AtRLI2 was expressed in *E. coli* as GST-AtRLI2 and therefore we also included GST protein in the binding assay as a control. BSA was taken as the negative binding control. Human RNase L, also expressed as a GST-tagged fusion, was included in the assay because it is the key enzyme in the 2–5A pathway in mammals and it has been reported to interact directly with RLI (Bisbal *et al.*, 1995). A sample containing only labeled siRNA was also added to have a reference of free siRNAs on the gel.

The different proteins were incubated together with synthetic radiolabeled siRNAs in a ratio of 100:1 and afterwards, a native gel electrophoresis was run and exposed for the detection of radioactive signal. Only one band was shifted to the top of the gel, indicating that NS1 bound siRNAs, whereas AtRLI2, as well as the other proteins tested, did not (Figure 7).

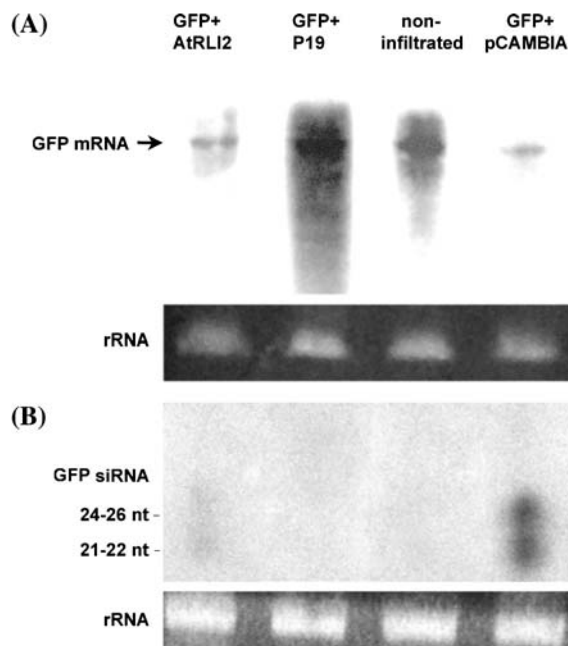

Figure 6. Northern analysis of the RNA isolated from the *N. benthamiana* 16c infiltrated patches (7 dpi). GFP mRNA detection (A). GFP siRNA detection (B). GFP mRNA and GFP siRNA were detected using a ^{32}P-labeled GFP-anti-sense riboprobe. Ethidium bromide staining of rRNA as a loading control for each Northern analysis (A and B).

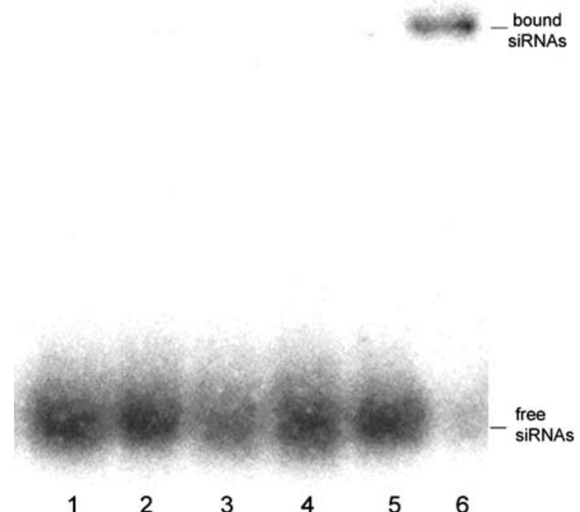

Figure 7. siRNA-binding assay. Radiolabeled synthetic siRNAs (5×10^{-3} pmol) incubated with the following purified proteins (0.5 pmol) and visualized by phosphoimaging after native gel electrophoresis: 1, no protein was added for the incubation; 2, human RNase L; 3, AtRLI2; 4, GST; 5, BSA; 6, influenza virus A NS1.

AtRLI2 *expression in a transgene-silenced* A. thaliana *line*

In order to compare the expression level of *AtRLI2* in a transgene-silenced *A. thaliana* line with the expression in wild-type plants, we carried out RPA of the RNA isolated from the rosette leaves of a flowering (3-week-old) *A. thaliana* wild-type and of the GFP-silenced *A. thaliana* line GxA, where the silencing is due to a cross between a GFP-transgenic line and a PVX:GFP-amplicon transgenic line (Dalmay *et al*., 2000). We could not detect any significant difference at the *AtRLI2* expression level between both lines in two independent experiments (Figure 3).

Discussion

In previous years, our understanding of RNA silencing in plants and animals has narrowly linked its anti-viral/defensive role with its role in endogenous gene regulation (Voinnet, 2005). It is therefore logical to assume, that this multi-branched mechanism has needed a fine tuning involving positive and negative regulators. Many positive regulators (components of the RNA silencing machinery) have been characterized to some extent up to now. The first suppressor of RNA silencing – negative regulator – was described already in 1998 (Anandalakshmi *et al*., 1998; Kasschau and Carrington, 1998). Today, the list of viral suppressors includes more than 20 proteins (Voinnet, 2005). However, when we try to find out how many endogenous suppressors have been described, it is surprising that there are only three: rgs-CaM (regulator of gene silencing-calmodulin-like protein) in tobacco (Anandalakshmi *et al*., 2000), RRF-3 (Simmer *et al*., 2002) and ERI-1 (Kennedy *et al*., 2004) both in *Caenorhabditis elegans*. Recently, two good candidates for host suppressors were identified in *A. thaliana*: Werner exonuclease-like 1 (WEL-1) and the cold- and abscisic acid-inducible protein KIN1 (Trinks *et al*., 2005).

Rgs-CaM interacts with the potyviral suppressor HC-Pro and is believed to function through a calcium-dependent pathway (Anandalakshmi *et al*., 2000). Also the two other endogenous suppressors of plants – still putative – are dependent on a viral suppressor. In this case, it is the geminiviral protein AC2, a transcriptional-activator protein, which seems to activate WEL-1 and KIN1 (Trinks *et al*., 2005). All these three proteins blast nicely with proteins in different plants. Some fungi and animals share certain level of identity with tobacco rgs-CaM and *A. thaliana* WEL-1.

RRF-3 of *C. elegans* is a RNA-dependent RNA polymerase (RdRP), which suppresses silencing most likely by blocking the generation of secondary siRNAs dependent on EGO-1 and RRF-1, other RdRPs. Thus, it prevents amplification of the silencing signal (Simmer *et al*., 2002). Few eukaryotic but no human proteins share certain level of identity with this suppressor. ERI-1 (named after "enhanced RNAi") is a highly conserved protein among vertebrates, including humans, and it is also present in fission yeast. ERI-1 of *C. elegans* and its human ortholog partially degrade siRNAs with 2-nt 3′ overhangs *in vitro* (Kennedy *et al*., 2004).

Recently, a report by Braz *et al*. (2004) suggested a role for AtRLI2 in RNA silencing. Human RLI was cloned 10 years ago as a protein, which inhibits the interferon-induced 2–5A antiviral pathway by blocking the activation of RNase L, the enzyme responsible for the degradation of RNA (Bisbal *et al*., 1995). Several functions of RLI not related to this pathway have been described in the last years and RLI orthologs – but not RNase L orthologs – were found in different organisms.

RLI is essential for the normal development of several organisms which do not code RNase L: the knock-out of *RLI* gene in *Saccharomyces cerevisiae* (Winzeler *et al*., 1999), *C. elegans* (Gonczy *et al*., 2000) and *Trypanosoma brucei* (Estevez *et al*., 2004) is lethal. Petersen *et al*. (2004) partially silenced the *RLI* gene in *N. tabacum* and *N. benthamiana* by transforming the plants with a construct harboring a fragment of the *N. tabacum* ortholog in an inverted-repeated orientation. They obtained only one *N. tabacum* transformant showing silencing of *RLI*, which looked stunted, having distorted leaves with white spots. RLI may influence development indirectly through interactions with other proteins, playing a chaperone role, as it has been described for human RLI in the case of immature HIV-1 capsids' assembly (Zimmerman *et al*., 2002). Other RLI's essential functions include in some organisms its role in ribosome biogenesis and in translation initiation (Dong *et al*., 2004; Kispal *et al*., 2005; Yarunin *et al*., 2005).

As several RLI essential functions have been reported up to now, it was our interest to find out if the proposed role of this protein in RNA silencing (Braz *et al.*, 2004) was due to a suppressor action. Previous studies analyzed if viral infection was affected by a change in the expression level of RLI. Indeed, in the case of EMCV (Martinand *et al.*, 1998) and HIV (Martinand *et al.*, 1999) an overexpression of RLI lead to an increased susceptibility to these viruses. Thus, at least for HIV and EMCV, the reported data could reflect the role of RLI as suppressor of RNA silencing.

Although there are still many questions related to the conservation of the RNA silencing mechanism along evolution, some components of this pathway have been found already in archaea (Parker *et al.*, 2004; Song *et al.*, 2004; Ma *et al.*, 2005) as for example Ago2, the ribonuclease known as Slicer (Liu *et al.*, 2004) and siRNA (Parker *et al.*, 2005). RLI, which at least in plants acts as silencing suppressor, is present in all eukaryotes and archaea (Kerr, 2004).

According to Braz *et al.* (2004) *A. thaliana* has three RLI paralogous sequences: *AtRLI1* (At3g13640), *AtRLI2* (At4g19210) and *AtRLI3* (At4g30300), the last one being a truncated protein. We do not think that *AtRLI3* can be considered a functional paralog, since it consists only of an ATP-binding domain, does not contain any introns and according to TAIR database there are no ESTs corresponding to this sequence. *AtRLI2* displays the most consistent phylogenetic position and an overall higher expression level (Braz *et al.*, 2004), that is why we centered our analysis on this gene. Our first interesting result was to prove the presence of the 3'UTR intron of *AtRLI2* in *Arabidopsis*, because only 2% of eukaryotic genes contain introns in UTRs (Deutsch and Long, 1999). We detected the expression of *AtRLI2* in all different organs (Figures 2 and 3), and this result was confirmed by the *AtRLI2* promoter transgenic plants (Figure 4). Also Braz *et al.* (2004) reported an ubiquitously expression of this gene, but our RPA analysis showed increased expression at the flowering stage, especially in flowers and siliques, while the mentioned authors estimated by real-time PCR a higher expression in leaves (they do not report the developmental stage of the plant). RLI is also a constitutively expressed gene in rice (Du *et al.*, 2003) and in humans (Aubry *et al.*, 1996).

RLI seems to be an endogenous suppressor expressed in all kind of tissues during different developmental stages. This means, that probably its function as suppressor is not continuous, because if so, it would harm the organism itself. We propose that it functions transiently as suppressor. This is in agreement with the fact that RLI interaction with other proteins or ribosomal subunits has been described as weak or transient (Martinand *et al.*, 1998; Zimmerman *et al.*, 2002; Karcher *et al.*, 2005; Yarunin *et al.*, 2005) and with RLI conformational changes, which enable its mechanochemical role in RNA/protein complex formations (Karcher *et al.*, 2005).

The reason why Braz *et al.* (2004) suggest a role of RLI in RNA silencing is their observation of an increased level of RLI expression in *A. thaliana* silenced with hairpin constructs for two endogenes (*chs* and *ein*2) and one heterologous gene (*nptII*). We tested if also in GFP-silenced *A. thaliana* GxA line this was the case. Our RPA data did not show any difference in expression compared to wild type *A. thaliana* (Figure 3). One explanation for these different results could be, that RLI suppression takes place at a specific level of determinated Dicer(s)-Ago protein(s) combination. The GFP-silenced *A. thaliana* GxA line contains one PVX:GFP-amplicon transgene, which could generate through silencing not perfectly matched siRNA-duplexes. On the other hand, a hairpin construct generates only perfectly matched siRNA-duplexes. This difference in the produced miRNAs/siRNAs during silencing is related to different Dicers or Argonaute (Ago) proteins (Voinnet, 2005).

Our results show that at the local level, as well as at the systemic level, AtRLI2 suppresses silencing in GFP-transgenic *N. benthamiana* agroinfiltrated with 35S-GFP and 35S-AtRLI2 (Figure 5). The suppression was also molecularly evident: a clear decrease in GFP siRNA levels and a moderate increase in GFP mRNA levels could be detected (Figure 6). Comparing AtRLI2 suppression to the one due to P19, this last one was stronger. It is known that P19 sequesters siRNA-duplexes (Vargason *et al.*, 2003), and is therefore a potent suppressor. AtRLI2 is not blocking the silencing at this crucial step: AtRLI2 did not bind

siRNAs (Figure 7). In addition, P19 was able to revert the silencing established in the same GFP-transgenic *N. benthamiana* line, while AtRLI2 was not (Figure 5). This all could mean that AtRLI2 is inhibiting to some extent the amplification of the silencing signal, as silencing in the infiltrated patch (except short-range movement of silencing, evident in the red ring surrounding the patch) as well as systemic silencing, are dependent on amplification (Himber *et al.*, 2003).

It is worth mentioning, that our results show that AtRLI2, an *A. thaliana* protein, is able to act as suppressor in *N. benthamiana*, which suggests that it may also work in other dicots. RLI is up to now the most conserved endogenous suppressor described, with an ancient origin. It remains an important task to find out if RLI also has an evident suppressor effect in mammalian cells.

Acknowledgments

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Short communication

Cocksfoot mottle virus P1 suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum*

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Abstract

The *Sobemovirus* genome consists of positive sense, single-stranded polycistronic RNA. The 5′-terminal ORF, encoding the protein P1, is its most variable region. Sobemoviral P1 has been described as dispensable for replication but indispensable for systemic infection. The P1 of *Rice yellow mottle virus*-Nigerian isolate (RYMV-N) is the only RNA silencing suppressor reported for sobemoviruses until now. Using an agrobacterium-mediated transient assay, we demonstrate here that P1 of *Cocksfoot mottle virus*-Norwegian isolate (CfMV-NO) suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum*, two non-host plants. CfMV-NO P1 was able to suppress the initiation and maintenance of silencing. The suppression of systemic silencing was weaker with CfMV-NO P1 than in the case of RYMV-N P1. In the case of suppression at the local level, the reduction in the amount of 25-nucleotide small interfering RNAs (siRNAs) was less pronounced for CfMV-NO P1 than it was when RYMV-N P1 was used. At the same time, we show that CfMV-NO P1 did not bind siRNAs.
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Keywords: *Cocksfoot mottle virus*; *Sobemovirus*; P1; siRNA; RNA silencing; Suppressor

Plants have developed a number of defense mechanisms against pathogens. One of these is RNA silencing, a sequence-specific RNA degradation process conserved among eukaryotes (Voinnet, 2005). RNA silencing is induced by dsRNA, which is processed to 21–26-nt small interfering RNAs (siRNA) (Hamilton and Baulcombe, 1999) that mediate degradation of identical RNA molecules (Hammond et al., 2000). In plants, RNA silencing is initially activated at the single-cell level and a mobile silencing signal is generated. This signal moves systemically through plasmodesmata and also through the phloem reaching distant organs (systemic silencing; Palauqui et al., 1997).

The majority of plant viruses have genomes of (+)-ssRNA (Hull, 2002) and are thought to trigger RNA silencing by dsRNA replicative intermediates and by highly structured ds regions in genomic RNA (Molnar et al., 2005). However, viruses have evolved suppressor proteins to counteract RNA silencing (Voinnet, 2005). The identification of viral suppressors and the elucidation of their mode of action are important for understanding RNA silencing mechanisms.

The *Sobemovirus* genome consists of polycistronic (+)-ssRNA (Tamm and Truve, 2000b). ORF1 of *Cocksfoot mottle virus* (CfMV), which infects only monocots, encodes a protein (P1) of 12 kDa that is required for systemic infection but is dispensable for replication (Meier et al., 2006) as is also the case for *Rice yellow mottle virus* (RYMV) P1 and *Southern cowpea mosaic virus* P1 (Bonneau et al., 1998; Sivakumaran et al., 1998). Moreover, P1 of RYMV is reported to be a pathogenicity determinant (Bonneau et al., 1998) and in the case of the Nigerian isolate (RYMV-N) it has been also described as an RNA silencing suppressor (Voinnet et al., 1999). Surprisingly, however, there is no similarity between the amino acid sequences of sobemoviral P1s (Ngon A Yassi et al., 1994; Mäkinen et al., 1995; Othman and Hull, 1995). Since RYMV-N P1 acts as a silencing suppressor in *Nicotiana benthamiana*, a non-host species (Voinnet et al., 1999), we investigated the suppressor activity of P1 of CfMV-Norwegian isolate (CfMV-NO; Mäkinen et al., 1995) in *N. benthamiana* using the agrobacterium-mediated transient assay (Hamilton et al., 2002).

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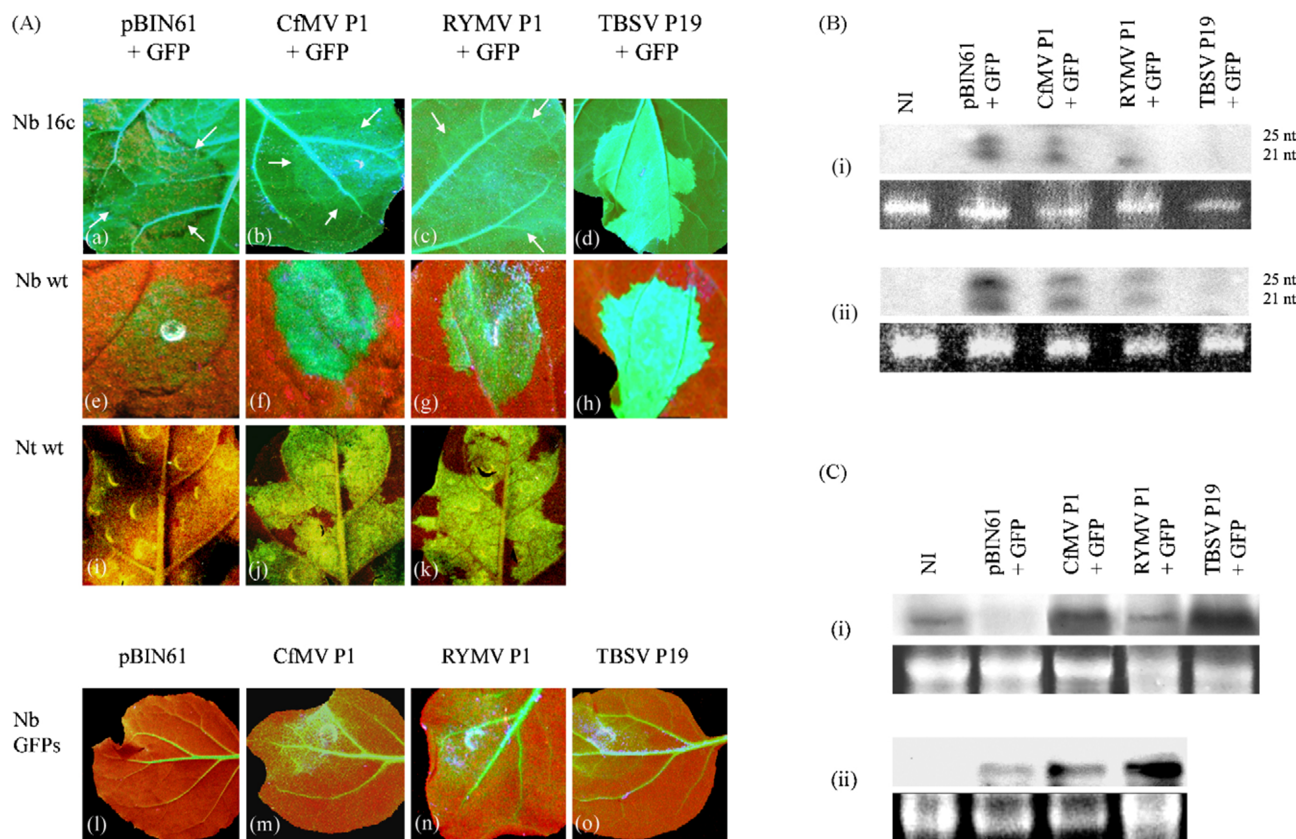

Fig. 1. Effect of CfMV-NO P1 on local RNA silencing in *N. benthamiana* and *N. tabacum*. (A) Infiltrated patches (5 dpi). *Agrobacterium* transformed with constructs indicated on the top were infiltrated into: *N. benthamiana* 16c (Nb 16c, a–d), wild-type *N. benthamiana* (Nb wt, e–h), wild-type *N. tabacum* (Nt wt, i–k), GFP-silenced *N. benthamiana* (Nb GFPs, l–o). Infiltration was carried out as previously described (Hamilton et al., 2002). Plants were photographed with Olympus CAMEDIA digital camera and pictures were processed using Adobe Photoshop 6.0. (B and C) Northern-blot analysis showing GFP siRNAs (B (i) at 5 dpi, B (ii) at 7 dpi) and GFP mRNA (C) extracted from *N. benthamiana* 16c (for B and C (i)) or *N. tabacum* (for C (ii)) patches infiltrated with the indicated strains. siRNA detection with ^{32}P-labeled in vitro transcript corresponding to the anti-sense strand of GFP was carried out as described (Sarmiento et al., 2006). DIG-labeled GFP-specific PCR fragment was used for mRNA detection at 5 dpi as described (Bucher et al., 2004). Ethidium bromide-stained rRNA is shown as loading control. NI: non-infiltrated leaf. Arrows indicate edges of infiltrated patches.

CfMV-NO ORF1 was amplified with primers 5′-CCTAGATCTAGCTTAGATGTGCGAACCTCC-3′ and 5′-GAGCTGCAGAACAACCCATTCTTGGTCACCCT-3′ and inserted into pTZ57R/T (Fermentas) to generate pTZ:CfMVORF1. CfMV-NO ORF1 (nt 62–441) was excised with *Xba*I and *Bam*HI from pTZ:CfMVORF1 and cloned into pBIN61 between the 35S promoter and Nos terminator to give pBIN61-P1. 35S-C_P1 refers to *Agrobacterium tumefaciens* (C58C1) containing pBIN61-P1.

To test if CfMV-NO P1 acts as a suppressor of RNA silencing, we first infiltrated leaves of GFP-transgenic *N. benthamiana* line 16c (Ruiz et al., 1998) with both 35S-C_P1 and 35S-GFP (*A. tumefaciens* carrying the GFP gene). In parallel, we co-infiltrated the 16c line with 35S-GFP together with pBIN61 (*A. tumefaciens* containing the empty pBIN61) or 35S-P19 (*A. tumefaciens* containing the strong suppressor P19 of TBSV). *A. tumefaciens* carrying P1 of RYMV-N (called 35S-R_P1) was also co-infiltrated with 35S-GFP to compare the effect of both sobemoviral proteins. At 5 days post-infiltration (dpi), GFP silencing has just been established and could be visualized by the appearance of a red ring at the border of the patch infiltrated with 35S-GFP plus pBIN61 and the weak red fluorescence inside this patch (Himber et al., 2003; Fig. 1A a). In contrast, the patches infiltrated with 35S-GFP and 35S-C_P1 or 35S-R_P1 still showed GFP expression (green fluorescence) at 5 dpi (Fig. 1A (b and c)). The patch infiltrated with 35S-GFP and 35S-P19 emitted at this time a very strong green fluorescence (Fig. 1A (d)). At 7 dpi, the patch infiltrated with 35S-GFP and 35S-C_P1, as well as the one co-infiltrated with 35S-R_P1, turned red (also a red ring appeared at the border of the patch) and by 11 dpi it was as red as the patch infiltrated with 35S-GFP and pBIN61 (data not shown). As expected, the area infiltrated with 35S-GFP and 35S-P19 remained intensely green after 11 dpi, due to the strong suppressor effect of P19 (Voinnet et al., 2003). The difference in the fluorescence of the patches indicates that CfMV-NO P1 suppressed GFP silencing in *N. benthamiana* 16c. At the local level, C_P1 was similar to R_P1, whose suppression in the same system has been partially described (Himber et al., 2003). To confirm these results we determined the levels of GFP siRNAs in the infiltrated patches. At 5 dpi, 21 and 25-nt GFP siRNAs were abundant in the pBIN61 control patch, whereas in the presence of any suppressor the levels changed: with P19 no siRNA was detected, as expected (Hamilton et al., 2002), with R_P1 only the shortest class of siRNAs were above the detection limit,

as previously described (Hamilton et al., 2002), and with C_P1 both siRNAs classes were reduced (Fig. 1B (i)). At 7 dpi, the levels of siRNAs detected in the case of C_P1 and R_P1, were higher than before. This time, the patch infiltrated with R_P1 showed clearly both classes of siRNAs (Fig. 1B (ii)). This correlates with the change in fluorescence of the infiltrated patches from 5 to 7 dpi (described above). Himber et al. (2003) reported in the same system a lack of the 25-nt siRNAs even at 7 dpi, although the infiltrated patch appeared – as our infiltrated area – red. A plausible explanation for these diverse results could be slight differences in the growing conditions of the plants. Also Northern-blot analysis of the infiltrated patches at 5 dpi confirmed the observed results: in the completely GFP-silenced tissue (infiltrated with 35S-GFP and pBIN61) no GFP mRNA could be detected, while in the case of tissues infiltrated with 35S-C_P1 or 35S-R_P1, the levels of GFP mRNA resembled the level of GFP mRNA in a non-infiltrated leaf. The infiltration with 35S-P19 increased the levels of GFP mRNA (Fig. 1C (i)).

In conclusion, CfMV-NO P1 suppressed local RNA silencing similarly to RYMV-N P1: clearly less than TBSV P19 and with an effect that appeared early in silencing and persisted for a short period of time. Moreover, cell-to-cell movement of the silencing signal was delayed by both suppressors. This was visualized by the absence of the red ring at 5 dpi (Fig. 1A (b–c)) and by the reduced amount of 21-nt siRNAs (Fig. 1B (i)), a component of the cell-to-cell silencing signal (Dunoyer et al., 2005). However, a noteworthy difference between sobemoviral suppressors was that only in the case of RYMV P1, the 25-nt siRNAs could not be easily detected when the suppression was strongest (5 dpi).

We further analyzed CfMV-NO P1 suppression in wild-type *N. benthamiana*. After 5 days, the patch infiltrated with 35S-GFP and pBIN61 showed low expression of GFP (weak green fluorescence, Fig. 1A (e)). This indicates that after reaching the peak of GFP expression – at about 2 or 3 dpi (Voinnet and Baulcombe, 1997) – RNA silencing was locally activated and the green fluorescence started to decline. However, if a suppressor was co-infiltrated, then GFP fluorescence was maintained (for C_P1 and R_P1, Fig. 1A (f and g)) or even elevated (for P19, Fig. 1A (h)). Thus, CfMV-NO P1 acts also as a suppressor in wild-type *N. benthamiana*. Suppression was also investigated in wild-type *Nicotiana tabacum* plants. In the case of infiltration with 35S-GFP and 35S-P19, the inoculated tissue started dying at 3 dpi, due to the local necrotic lesions that TBSV P19 elicits in *N. tabacum* (Scholthof et al., 1995). At 5 dpi, the GFP fluorescence observed in the patches infiltrated with C_P1, R_P1 or pBIN61 was comparable to that observed in *N. benthamiana* and in agreement with the levels of GFP mRNA detected by Northern-blot analysis: the green fluorescence was increased by the suppressors C_P1 and R_P1 (Fig. 1A (i–k)), as was the GFP mRNA level (Fig. 1C (ii)). Thus, both sobemoviral suppressors challenge the RNA silencing machinery in two non-host plants.

We further investigated whether CfMV-NO P1 was able to reverse already established RNA silencing and if this interference with the maintenance of silencing was similar to the other suppressors. For this, we infiltrated in parallel a GFP-silenced *N. benthamiana* line (Bucher et al., 2003) with pBIN61 (control), 35S-C_P1, 35S-R_P1 and 35S-P19. All three suppressors reversed the established GFP-silencing at 5 dpi: under UV-light, the infiltrated patches turned slightly green, contrasting with the red autofluorescence of chlorophyll (Fig. 1A (l–o)). The difference between the suppressors was that the reversion caused by P19 occurred in 100% of the cases, while C_P1 and R_P1 reversion occurred only in 42% of the cases (5 plants out of 12).

RNA silencing suppression also occurred at the systemic level: six or seven *N. benthamiana* 16c plants were co-infiltrated in parallel with 35S-GFP plus pBIN61 as control, or with 35S-GFP plus one of the three tested suppressors. This assay was performed four times and plants were monitored under UV-light. At 8 dpi, all the plants infiltrated with pBIN61 showed systemic silencing (Fig. 2a), while only 45% of the plants infiltrated with 35S-C_P1 (Fig. 2b: suppressed plant) or with 35S-P19 (Fig. 2d: suppressed plant) were systemically silenced. None of the plants infiltrated with 35S-R_P1 were by this time silenced (Fig. 2c). At 14 dpi, the percentages of suppressed plants decreased to 29% in the case of C_P1, 40% for P19 and 89% for R_P1. At 35 dpi, these percentages reached 25, 33 and 74%, respectively (Fig. 2f–h show suppressed plants). Thus, comparing both

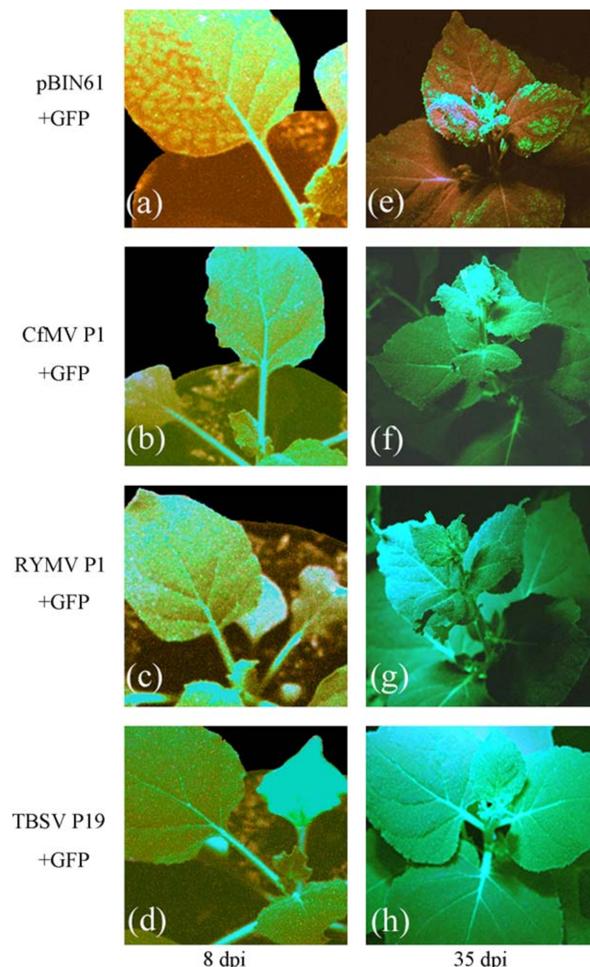

Fig. 2. Effect of CfMV-NO P1 on systemic RNA silencing. *N. benthamiana* 16c plants were co-infiltrated with the indicated *Agrobacterium* strains. GFP fluorescence was monitored for 35 dpi with a hand-held long-wavelength UV lamp. Percentages of suppressed plants were: at 8 dpi (a) 0%, (b) 55%, (c) 100% and (d) 55%; at 35 dpi (e) 0%, (f) 25%, (g) 74% and (h) 33%.

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sobemovirus suppressors it is clear that RYMV-N P1 is stronger than CfMV-NO P1 at the systemic level. This may indicate that phloem-dependent movement of the silencing signal, which is sometimes related to 25-nt siRNAs (Hamilton et al., 2002), is more efficiently blocked by RYMV-N P1 than by CfMV-NO P1, resulting in stronger interference with systemic silencing. The stronger effect of R_P1 partially correlates with the observed specific reduction of 25-nt siRNAs, especially at 5 dpi.

As it is known that P1 of CfMV-NO binds ssRNA in a sequence-independent manner (Tamm and Truve, 2000a) and because its action as a suppressor was observed early in silencing, we tested the capacity of C_P1 to bind siRNAs. For this purpose, gel-shift experiments were performed with C_P1 protein expressed in *N. benthamiana* agro-infiltrated leaves as described previously (Merai et al., 2005). Crude extracts were prepared from leaves infiltrated with 35S-C_P1 as well as with 35S-GFP and 35S-P14 (*A. tumefaciens* harboring the sequence of *Pothos latent virus* P14; Merai et al., 2005). However, we were not able to detect ds siRNA binding in the P1 extract, nor in the GFP extract, while P14 extract clearly shifted 21- and 26-nt siRNAs duplexes (Fig. 3A). The same gel-shift assay was performed with 49-nt dsRNA and the results were similar (data not shown).

In addition, we decided to express C_P1 in *Escherichia coli*, where we could quantify the protein and determine if the absence of binding was due to inherent characteristics of C_P1 or to an insufficient amount of it. C_P1 was PCR amplified with primers 5′-CGGGATCCATGTGCGAACCTCCC-3′ (carrying a *Bam*HI site) and 5′-ACGCGTCGACTACTCTGTCCTGCC-3′ (containing a *Sal*I site) corresponding to the first and last 15 nt of C_P1, respectively. The PCR product was cloned into pET 43.1a (+) vector (Novagen) and expressed in *E. coli* as a NUS-P1 fusion protein, in order to recover P1 in the soluble protein fraction. To be sure that this fusion was not affecting the suppressor activity of CfMV P1, we cloned NUS-P1 into a binary vector and the transformed *A. tumefaciens* was infiltrated to *N. benthamiana* 16c plants together with 35S-GFP. The suppression of silencing in the case of co-infiltration with 35S-GFP and *A. tumefaciens* carrying NUS-P1 was as strong as when 35S-GFP was infiltrated together with 35S-C_P1 (data not shown).

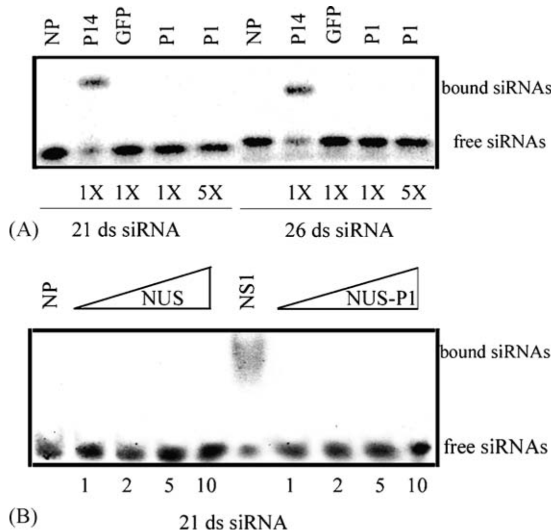

Fig. 3. CfMV-NO P1 does not bind siRNAs. (A) Shift assay with extracts prepared at 3 dpi from *N. benthamiana* leaves infiltrated with indicated *Agrobacterium* strains. Labeled duplexes of 21-nt or 26-nt siRNAs with 2-nt 3′overhangs were used. X: relative amount of total protein. (B) Labeled duplex of 21-nt siRNA (2 pM) incubated with 1, 2, 5 or 10 μg of NUS or NUS-P1 expressed in *E. coli*. NS1 as positive control (1 μg). NP: no protein was added for the reaction.

NUS-P1 expressed in *E. coli* was purified with His-tag cartridges (Novagen) and tested for siRNA-binding (21-nt long with 2-nt 3′ overhangs) using purified NUS as a negative control and purified *Influenza A virus* NS1 as a positive control following the protocol of Bucher et al. (2004). NUS-P1 did not, however, bind siRNAs even when added to the reaction in a molar excess of more than 10-fold (Fig. 3B). Thus, we conclude that CfMV-NO P1 does not bind siRNAs or 49-nt dsRNAs.

In summary, we show that CfMV-NO P1 is able to suppress the initiation and maintenance of RNA silencing, with an effect at both the local and the systemic level. We also show that the previously reported sobemoviral suppressor, RYMV-N P1 (Himber et al., 2003; Voinnet et al., 1999), interferes with silencing initiation and maintenance. The difference between the suppression properties of both sobemoviral proteins seems to be clear at the systemic level.

The 5′-terminal half of the genomes of sobemoviruses and of poleroviruses are similar in their organization (Hull and Fargette, 2005). The 5′-terminal ORF of poleroviruses encodes P0, which like P1 of sobemoviruses, is required for virus accumulation (Sadowy et al., 2001; Meier et al., 2006). P0 and P1 are the most divergent proteins among poleroviruses and sobemoviruses, respectively, and have no homology with known proteins (Mayo and Miller, 1999; Tamm and Truve, 2000b). Another common feature of P0 and P1 is their poor translation initiation codon context (Pfeffer et al., 2002; Dwyer et al., 2003). And finally, the P0 proteins encoded by *Beet western yellows virus*, *Potato leafroll virus* and *Cucurbit aphid-borne yellows virus* have been shown to be suppressors of RNA silencing (Pfeffer et al., 2002).

It is remarkable that ORFs which do not share any amino acid sequence similarity but have the same position in their respective viral genomes have a common function: they encode silencing suppressors. This is not only the case for sobemovirus P1 and polerovirus P0, but also for tombusvirus P19 and aureusvirus P14 (Merai et al., 2005) as well as for tospoviruses NS_S and tenuiviruses NS3 (Bucher et al., 2003).

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Phenotypes and Functional Effects Caused by Various Viral RNA Silencing Suppressors in Transgenic *Nicotiana benthamiana* and *N. tabacum*

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RNA silencing suppressor genes derived from six virus genera were transformed into *Nicotiana benthamiana* and *N. tabacum* plants. These suppressors were *P1* of *Rice yellow mottle virus* (RYMV), *P1* of *Cocksfoot mottle virus*, *P19* of *Tomato bushy stunt virus*, *P25* of *Potato virus X*, *HcPro* of *Potato virus Y* (strain N), *2b* of *Cucumber mosaic virus* (strain Kin), and *AC2* of *African cassava mosaic virus* (ACMV). HcPro caused the most severe phenotypes in both *Nicotiana* spp. AC2 also produced severe effects in *N. tabacum* but a much milder phenotype in *N. benthamiana*, although both HcPro and AC2 affected the leaf tissues of the two *Nicotiana* spp. in similar ways, causing hyperplasia and hypoplasia, respectively. P1-RYMV caused high lethality in the *N. benthamiana* plants but only mild effects in the *N. tabacum* plants. Phenotypic alterations produced by the other transgenes were minor in both species. Interestingly, the suppressors had very different effects on crucifer-infecting *Tobamovirus* (crTMV) infections. AC2 enhanced both spread and brightness of the crTMV-green fluorescent protein (GFP) lesions, whereas 2b and both P1 suppressors enhanced spread but not brightness of these lesions. P19 promoted spread of the infection into new foci within the infiltrated leaf, whereas HcPro and P25 suppressed the spread of crTMV-GFP lesions.

Additional keywords: leaf and flower malformation, transgenic tobacco plants, viral suppressors

RNA silencing is a versatile and complex gene regulation and defense mechanism occurring in a broad range of eukaryotic organisms. It is activated in cells by double-stranded (ds)RNAs, followed by cleavage of the inducing RNAs into short (21- to 24-nucleotide) fragments. These, in turn, mediate multiple different regulatory and defense functions in the cells (Brodersen and Voinnet 2006). In post-transcriptional gene silencing (PTGS), they target the RNA-induced silencing complex (RISC) to degrade homologous RNA transcripts or to arrest their translation. Many transcription factors mediating the differentiation of multicellular organisms are regulated via these pathways, and severe developmental disturbances are associated with malfunctions of the silencing pathways (Bartel 2004; Deleris et al. 2006). Small RNAs function also as epigenetic agents targeting de novo cytosine or histone methylation to their homologous DNA sequences to induce transcriptional gene silencing (TGS) and chromatin silencing (Chan et al. 2004, 2006; Xie et al. 2004). Both PTGS and TGS processes mediate effective defense mechanisms against invading genetic elements, such as viruses, transposons, and transgenes (Buchon and Vaury 2006). In plants, the small RNAs are involved in both cell-to-cell and long-distance movement of the silencing signal together with other proteins from the diverse RNA silencing pathways, such as DCL4 and CLASSY 1 (Dunoyer et al. 2005; Smith et al. 2007).

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Virus-encoded silencing suppressors.

RNA silencing pathways function as inducible defense reactions against viral pathogens and produce a sequence specific, single-strand (ss)RNA-specific ribonuclease (Omarov et al. 2007). To counteract this defense mechanism, many plant viruses encode for specific silencing suppressors which allow the viruses to proliferate in their specific hosts. The importance of these suppressors is reflected by the fact that many of them previously have been identified as pathogenicity factors or as viral cell-to-cell or long-distance movement proteins, essential for infectivity in indicated hosts (Voinnet 2005; Xie and Guo 2006). Silencing suppressor proteins encoded by unrelated RNA and DNA viruses bear no similarity to each other in either coding sequence or protein structure, suggesting separate origins and variable functional mechanisms for each suppressor type. In recent years, the interactions of different silencing suppressors with the RNA silencing pathways have been studied intensively. In transgenic *Arabidopsis*, severe developmental disturbances are induced by many suppressors (e.g., P1/HcPro of *Turnip mosaic virus* [TuMV, *Potyvirus*], P19 of *Tomato bushy stunt virus* [TBSV, *Tombusvirus*], P15 of *Peanut clump virus* [PCV, *Pecluvirus*), and P21 of *Beet yellows virus* [BYV, *Closterovirus*]), and the malformed phenotypes appear related to the inhibition of the miRNA-mediated cleavage of their target mRNAs (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003). On the other hand, P25 cell-to-cell movement protein of *Potato virus X* (PVX, *Potexvirus*), P38 coat protein of *Turnip crinkle virus* (TCV, *Carmovirus*), and P50 movement protein (MP) of the *Apple chlorotic leaf spot virus (*ACLSV, *Trichovirus*) cause no phenotypic changes in the plant, and primarily prevent the short- or long-distance spread of the silencing signal (Bayne et al. 2005; Deleris et al. 2006; Dunoyer et al. 2004; Yaegashi et al. 2007). The phenotype caused by the 2b protein of *Cucumber mosaic virus* (CMV, *Cucumovirus*) varies from none to severe depending on the viral strain from which the transgene was isolated, and is related to binding of

this protein to Argonaute 1 (Lewsey et al. 2007; Zhang et al. 2006). It has been suggested that the silencing suppressor activities of many viral proteins are related to their 21-nucleotide short-interfering (si)RNA-binding activity, while the suppressors of other viruses, even closely related, do not show such binding activity (Merai et al. 2005, 2006). However, thus far, direct relation between siRNA-binding and silencing suppression activity has been shown in vivo only for P19 of different tombusviruses, P21 of BYV, and HcPro of *Tobacco etch virus* (TEV) (Lakatos et al. 2006; Silhavy et al. 2002).

As the silencing suppressors compromise the silencing-mediated host defense, they also may enhance other viral infections, in a way similar to the synergistic effects of double viral infections (Pruss et al. 1997, 2004; Vanitharani et al. 2004). Surprisingly, in transgenic tobacco plants, the HcPro of TEV also enhanced the *N*-gene-mediated resistance against *Tobacco mosaic virus* (TMV) and induced a strong resistance against *Tomato black ring virus* (TBRV, *Nepovirus*) (Pruss et al. 2004). The mechanisms of these induced resistance reactions are not yet well understood.

Most studies concerning the interactions of plants with viral silencing suppressors have been focused on the suppressor effects on the processing of selected miRNA target genes in transgenic *Arabidopsis* plants. Thus, many features of the suppressor functions remain unknown; for instance, what is the degree of host specificity of different silencing suppressors, do they cause similar physiological or phenotypic effects in different hosts, or do they exert similar or different effects upon heterologous viral pathogens. To elucidate such questions, we have produced transgenic *Nicotiana benthamiana* and *N. tabacum* plants which express well-characterized silencing suppressors derived from six different virus genera. The observed features indicate that the two tobacco species react differently to the expression of these silencing suppressors. In addition, the suppressors have different effects on the replication and spread of a crucifer-infecting *Tobamovirus* (crTMV) amplicon expressing the green fluorescent protein (GFP) marker gene.

RESULTS

N. benthamiana and *N. tabacum* were transformed with silencing suppressor genes derived from six different viral genera (*P1* of *Rice yellow mottle virus* [RYMV] and *Cocksfoot mottle virus* [CfMV], *P19* of TBSV, *P25* of PVX, *HcPro* of *Potato virus Y* [PVY], *2b* of CMV, and *AC2* of *African cassava mosaic virus* [ACMV]). To avoid any possible tissue culture effects and to have stable lines, two or three independent homozygote R2 lines for each of the transgene were selected based on their 100% germination on kanamycin (Km)-containing medium. These were used for the observation of plant phenotypes and for analyzing the plant responses to the crTMV-GFP infections. Transgene mRNA expression (Fig. 1A) and variable altered phenotypes were detected in these R2 plants for most of the selected *N. benthamiana* lines. The transgene mRNA remained below detection level in both lines carrying the *P1*-RYMV and *P19* transgenes as well as in one of the lines harboring the *P25* and *AC2* transgenes. However, a clearly altered phenotype also was observed in one of the lines expressing each of the *P1*-RYMV and *P19* genes, and also in the mRNA-negative *AC2* transgenic line, suggesting that these lines were expressing the transgene. For each transgene, the line or lines with the most pronounced phenotype also showed the strongest effect on the spread and accumulation of the crTMV-GFP, as described below, and the transgene-positive status of the selected lines was confirmed by polymerase chain reaction (PCR) (Fig. 1B).

In transgenic *N. tabacum* lines, transgene mRNA was detected in all selected homozygote lines except in one of the lines containing the *P19* transgene, and in either of the two lines carrying the *P25* and *P1*-CfMV transgenes (Fig. 1A). Thus, at least one transgene-expressing *N. tabacum* line was

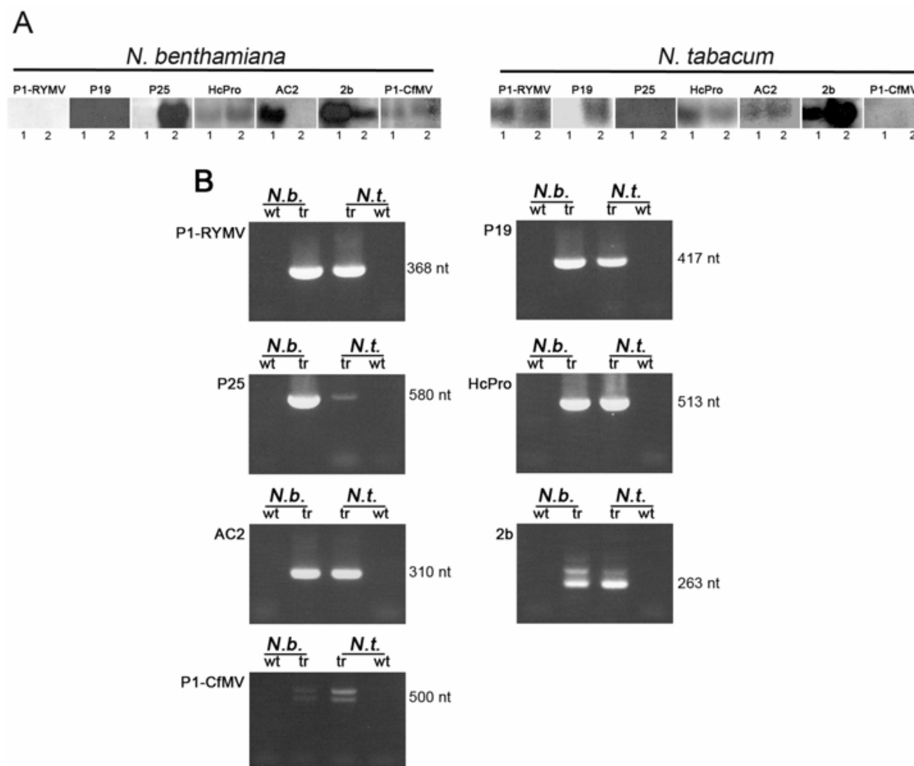

Fig. 1. A, Northern blot detection of different transgene mRNAs in *Nicotiana benthamiana* (N.b.) and *N. tabacum* (N.t.) and **B,** polymerase chain reaction-mediated detection of the transgene DNA in the selected lines of the transgenic plants. RYMV = *Rice yellow mottle virus* and CfMV = *Cocksfoot mottle virus*.

obtained for each of the transgenes except for the *P1*-CfMV and *P25*; however, these still were maintained for the analysis due to their effects in *N. benthamiana*. One of the *P25*-harboring *N. tabacum* lines showed reduction of the accumulation of PVX RNA in PVX-inoculated plants (data not shown), suggesting that the *P25* sequence was targeted by RNA silencing in this line. The presence of the transgene in all *N. tabacum* lines was confirmed by PCR (Fig. 1B).

Phenotypes of transgenic plants.

The phenotypes observed in the transgenic plants expressing the different silencing suppressor genes varied between the transgenes and also between the two tobacco species (most-pronounced phenotypes are summarized in Tables 1 and 2). The most striking phenotypes were caused by *HcPro* in both *Nicotiana* spp. In *N. benthamiana,* phenotypes caused by this transgene were similar in both selected lines, although more pronounced in one of the lines. In this line, the stems were strongly bending and twisting, causing a creeping growth habit. Leaves were strongly rolled and hairy, with elongated vein organization. Flowers had very short petals and stamens and protruding pistils, and were sterile. In addition, leaves and flowers grew without petioles (Fig. 2A through E; Table 1). Creeping stems and low seed production also were observed in most of the *HcPro* expression lines in the R0 and R1 generations (data not shown). Both HcPro transgenic *N. tabacum* lines also had a strong phenotype with distinctly thick, dark, and hairy leaves with moderate blistering. Stems were very hairy with short, thick internodes, and the plants were severely stunted (Fig. 3A). Flowering in these plants was strongly delayed; however, the flowers were fertile (Table 2).

One of the *AC2* transgenic lines in *N. tabacum* exhibited a phenotype very similar to the *HcPro* lines (Fig 3B) except that, occasionally, the leaves were rolled into cup-shaped forms (Fig. 3C) and the flowers were moderately malformed, with some stamens transformed into petals (Fig. 3D). The other *AC2*-expressing *N. tabacum* line showed less leaf malformation; however, in this line, the flowers were severely malformed, having all stamens transformed into petals (Fig. 3D through G). The seed set was strongly reduced in both *AC2* lines. In the *AC2*-expressing *N. benthamiana*, only a mildly altered phenotype with some blistering of the leaf surfaces was observed in one line; however, the mRNA-negative line also displayed occasional rolling of the leaf blades into a funnel- or cup-shaped form (Fig. 2F). Cup-shaped leaves also were observed in the *AC2*-expressing *N. benthamiana* R1 generation (data not shown), and seed set was severely reduced.

Distinct phenotypes also were observed in plants expressing the other transgenes. *P1*-RYMV caused high lethality in *N. benthamiana* in the R0 generation, and only 3 of the 10 regenerated lines produced seed in this generation. It also caused malformations in the R1 generation (data not shown) and stunting in the R2 generation (Fig. 2G), although the transgene mRNA did not accumulate to detectable level in the surviving R2 progeny lines (Table 1). Occasionally, severe leaf rolling or cup-shaped leaves occurred in the R2 plants (Fig. 2H). In one of the lines, flower stalks were bent and flowers occasionally malformed (Fig. 2I). The seed set of this line and the seedling growth were poor. In *N. tabacum*, this transgene caused no specific leaf or growth phenotype, although the gene was expressed in these plants at high levels (Fig. 1B). In contrast to *P1*-RYMV, the *P1*-CfMV caused no specific phenotype, except that some flowers of one of the *N. benthamiana* lines had bending stalks.

P19-expressing *N. benthamiana* plants had blistered leaf epidermis, hairy and serrated leaves in one of the lines, and

Table 1. Most pronounced phenotypes observed in different transgenic lines of *Nicotiana benthamiana*

Lines[a]	Leaf	Flower	Stunting	Others
Wild type (WT)	Normal	Normal	No	…
pBin61	Normal	Normal	No	…
HcPro	Severe curling, changed vein pattern, no petioles, hairy	Short petals and stamens, protruding pistils, no petioles	Moderate, quantification difficult due to the creeping growth	Hairy creeping stems
AC2	Occasionally cup-shaped, mild blistering	Normal	Moderate, final height approximately 75% of the WT plants	…
P1-RYMV	Occasionally cup-shaped	Few flowers, sterile, bending stalks, malformed petals	Severe, final height 30–40 % of the WT plants	Poor seedling growth (50% impaired)
P1-CfMV	Normal	Bending stalks	No	…
P19	Mild blistering, mildly serrated, hairy	Bending stalks	No	…
P25	Normal	Smaller, not fully opened	Mild, final height 80–90% of the WT plants	Early senescence
2b	Normal	Normal	No	…

[a] RYMV = *Rice yellow mottle virus* and CfMV = *Cocksfoot mottle virus*.

Table 2. Most pronounced phenotypes observed in different transgenic lines of *Nicotiana tabacum*

Lines[a]	Leaf	Flower	Stunting	Others
Wild type (WT)	Normal	Normal	Normal	…
pBin61	Normal	Normal	No	…
HcPro	Thick and hairy, short internodes, blistering	Normal	Severe, final height approximately 50% of the WT plants	Late flowering (2 months later)
AC2	Thick and hairy, short internodes, blistering, cup-shaped	Moderate malformation, stamens transformed into petals, some sepals transformed to petals, less stamens (4)	Severe, final height approximately 50% of the WT plants	Late flowering (1 month later)
P1-RYMV	Normal	Normal	No	…
P1-CfMV	Normal	Normal	No	…
P19	Normal	Severe malformation	No	…
P25	Normal	Normal	No	…
2b	Normal	Normal	No	Late flowering (1 month later)

[a] RYMV = *Rice yellow mottle virus* and CfMV = *Cocksfoot mottle virus*.

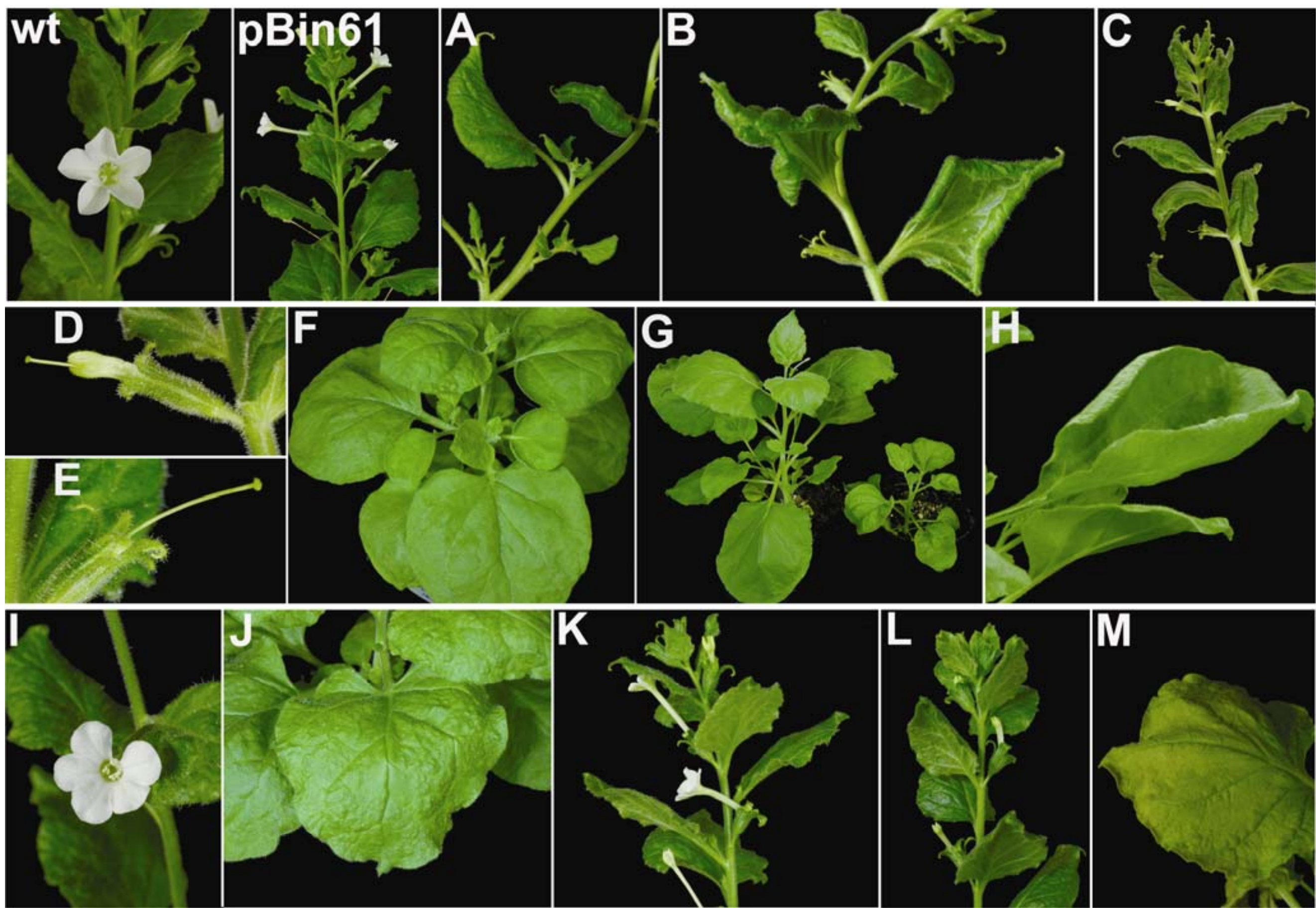

Fig. 2. Phenotypes observed in *Nicotiana benthamiana* plants expressing different transgenes. wt, Wild-type, nontransgenic *N. benthamiana*; pBin61, *N. benthamiana* transformed with empty pBin61 vector; **A** through **E,** *HcPro*-transgenic plants with **A,** strongly curled leaves; **B,** emergence of flowers and leaves without petioles; and **C** through **E,** malformed flowers with short petals and stamens and protruding pistils. **F,** *AC2* transgenic plant showing rolling of the leaf blades into mild cup-shaped form. **G** through **I,** *P1-Rice yellow mottle virus* transgenic plants showing **G,** stunting compared with wild-type plant on the left; **H,** cup-shaped leaf; and **I,** a malformed flower. **J** and **K,** *P19* transgenic plants showing **J,** blistered leaf epidermis and **K,** occasional bending of the flower stalks and mild serration. **L** and **M,** *P25* transgenic plants, showing **L,** small, malformed flowers and **M,** early senescence.

Fig. 3. Phenotypes observed in *Nicotiana tabacum* plants expressing different transgenes. **A,** *N. tabacum* plants transformed with *HcPro* gene compared with a wild type plant on the right. **B,** *AC2* gene-expressing plant, showing the dark and hairy leaves and stem with short and thick internodes, a phenotype very similar to the *HcPro* transgenic plants. **C,** Leaves of *AC2*-expressing plants occasionally turned into funnel- or cup-shaped forms. **D** through **I,** Malformed flowers with stamens converted to petals, occasional bending of the flower base, or with petals fused or reduced in number, observed frequently in transgenic plants expressing AC2 (D through G) and P19 (H and I). Healthy flower is shown in G, on the left.

occasional bending of the flower stalks (Fig. 2J and K). In *N. tabacum*, P19 caused occasional malformation of flowers (Fig. 3H and I).

One of the *P25*-expressing *N. benthamiana* lines had very small, not fully opened flowers (Fig. 2L) with reduced seed set. This line also was moderately stunted and senescenced early (Fig. 2M). The other line showed longitudinally rolled leaves.

None of the *2b*-expressing transgenic lines in either of the *Nicotiana* spp. showed any specific phenotypes, although both selected lines expressed transgene mRNA on fairly high levels.

Effects of the HcPro and AC2 silencing suppressors on the structure of the leaf tissues.

The most severe leaf malformations occurred in both *Nicotiana* spp. transformed either with the *HcPro* or the *AC2* genes; therefore, thin sections of these leaves were prepared to investigate how the tissue structures were altered in these transgenic plants. Microscopic analysis of the thin sections indicated that, in both *Nicotiana* spp., *HcPro* transgene caused a significant increase in the numbers of the palisade and spongy mesophyll cells, leading to reduction of the air space between the cells (Fig. 4B and E, *N. benthamiana* sections; H and K, *N. tabacum* sections). Particularly in the *HcPro*-expressing *N. benthamiana* plants, the lower epidermis of the leaves appeared expanded, leading to frequent bulging-out of the epidermis (Fig. 4B and E). Particularly in the thin sections of one of the *HcPro*-expressing lines, the guard cells were frequently observed from the top view (Fig. 4E) in contrast to the control samples, where guard cells typically are seen as cross-sections of the cells, indicating again that the epidermal cells were twisted and crowded. In contrast to the *HcPro*-expressing plants, the mesophyll cells of the *AC2* transgenic plants of both *Nicotiana* spp. were significantly larger than the mesophyll cells of comparable wild-type plants (Fig. 4C and F, *N. benthamiana* sections; I and L, *N. tabacum* sections). Particularly in *N. tabacum*, the cell walls appeared thinner than in the wild-type cells and the cells appeared to be distorted or wrinkled, with disturbed and clumped chloroplast distribution, suggesting that the cell wall structures were thinner or weaker in these tissues compared with the control samples (Fig. 4I and L).

The thin sections of the *HcPro* and *AC2* transgenic *N. benthamiana* and *N. tabacum* plants indicated that, at the cellular level, the malformations caused by each transgene were similar in both *Nicotiana* spp. Whereas, between the two transgenes, they were associated with different effects (i.e., strong increase of cell number, with distinctly small cells [hyperplasia] in the *HcPro* plants, and very large cells [hypoplasia] in the *AC2* plants).

Interference of the silencing suppressors with the crTMV-GFP spread and accumulation.

The infectious virus derived from the crTMV cDNA, with a GFP gene replacing the coat protein gene, readily caused a local infection in *N. benthamiana* and also slowly spread in this host. This construct has been used successfully to analyze RNA-silencing activity in *N. benthamiana* (Dorokhov et al. 2006) and, therefore, we used it here for further testing the expression of different silencing suppressors in the transgenic plants, as well as to test the suppressor effects on a heterologous virus infection. Two leaves from each of the three plants from each transgenic *N. benthamiana* line were infiltrated with an *Agrobacterium* suspension carrying 35S-controlled infectious crTMV-GFP clone. However, this analysis was not carried out in *N. tabacum* because the virus construct is barely infectious in this host.

The crTMV-GFP lesions spread slightly differently in the sibling plants of different transgenic *N. benthamiana* lines; however, the type of GFP-lesion spread and degree of luminosity still varied clearly between the different transgenic plants (Fig. 5). *AC2* transgene clearly enhanced both the spread and the mean surface brightness (average flux per pixel) of the GFP lesions (Fig. 5). The *2b* transgene and also both of the *P1*

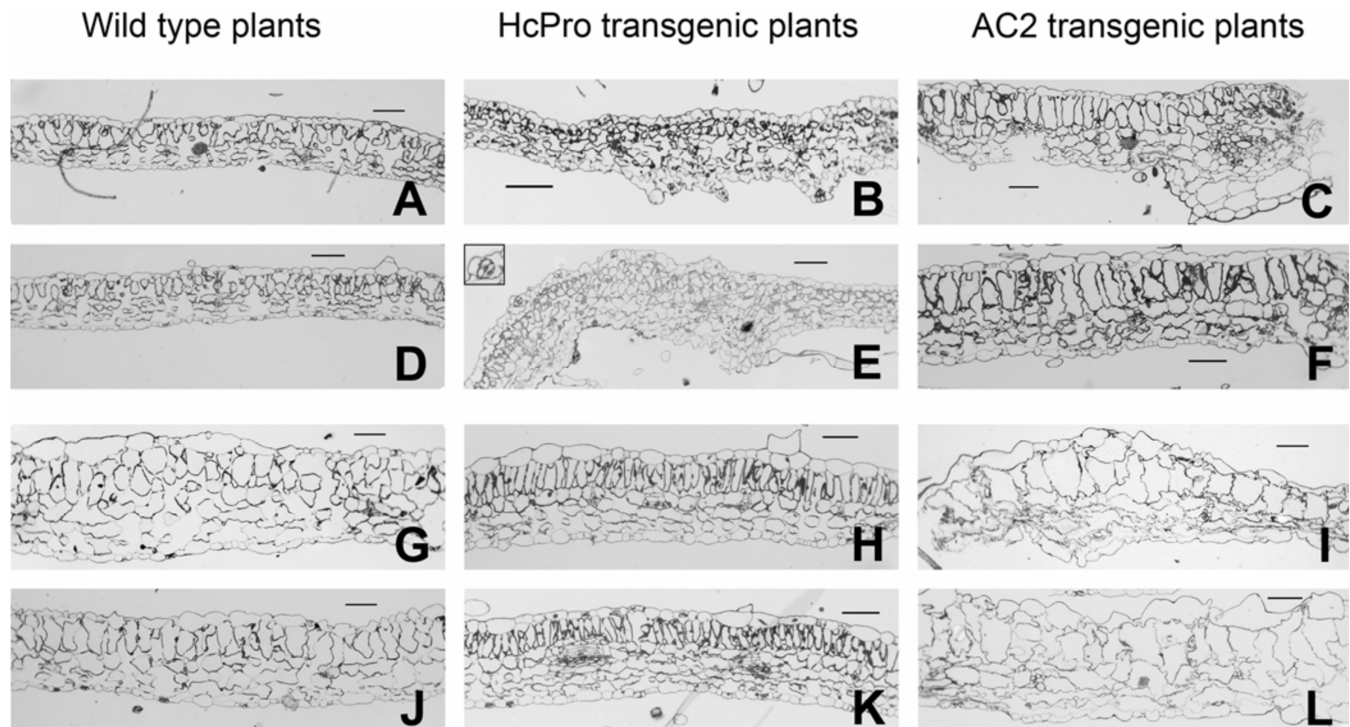

Fig. 4. Microscopic analysis of the leaf structures of the wild-type, *HcPro*, and *AC2* transgenic **A** through **F,** *Nicotiana benthamiana* and **G** through **L,** *N. tabacum* plants. The size bar in each frame corresponds to 100 µm. Two typical sites of each leaf type are portrayed. The enlarged detail in E shows one of the guard cells seen from the top view, observed frequently in the *HcPro* samples.

transgenes (Fig. 5) increased the spread of the lesions but did not significantly affect the brightness of the lesions. *P19* transgene did not affect the total infected area but increased its brightness, and also repeatedly caused spread of the infection into new infection foci (Fig. 5). Surprisingly, *HcPro* transgene, and even more strongly the *P25* transgene, reduced the spread of the crTMV-GFP lesions (Fig. 5). The pictures and measured GFP values shown in Figure 5 are derived from one experiment, 15 days after the infiltration of the plants. Very similar results were observed in the other experiments.

DISCUSSION

Different viral silencing suppressors are being studied intensively in various laboratories around the world. With these research efforts, a very complex view is emerging, indicating that suppressors encoded by various viruses interfere in different ways with the silencing pathways, affecting the degradation of the target RNAs and the spread of the silencing signal from cell to cell or over long distance (Voinnet 2005). To clarify the various silencing suppressor mechanisms, comparisons are needed to see how various suppressors function in different plant species.

In this work, we have analyzed for the first time in a comparative way the effects of seven different viral RNA silencing suppressors in *N. benthamiana* and *N. tabacum*. Up to now, similar comparative works have been published only in *Arabidopsis thaliana* (Chapman et al. 2004; Dunoyer et al. 2004). Some of the transgenes used in this study caused different effects in the two *Nicotiana* spp.. For instance, *P1*-RYMV caused high lethality in *N. benthamiana* in the R0 generation and malformations and stunting in the R1 and R2 generations, although the gene was expressed at a very low level in the surviving R2 progeny lines. In *N. tabacum*, this gene did not cause any (observable) adverse effect, even though it was expressed at high levels in the R2 generation. The *N. benthamiana* lines expressing *HcPro* gene had a strongly disturbed growth habit, with creeping stems and strongly malformed flowers as well as leaves. Plants expressing the *AC2* gene showed funnel- or cup-shaped leaf malformations and reduced seed set in both *Nicotiana* spp. In *N. tabacum* plants, both *HcPro* and *AC2* genes caused a similar, rigid growth habit with

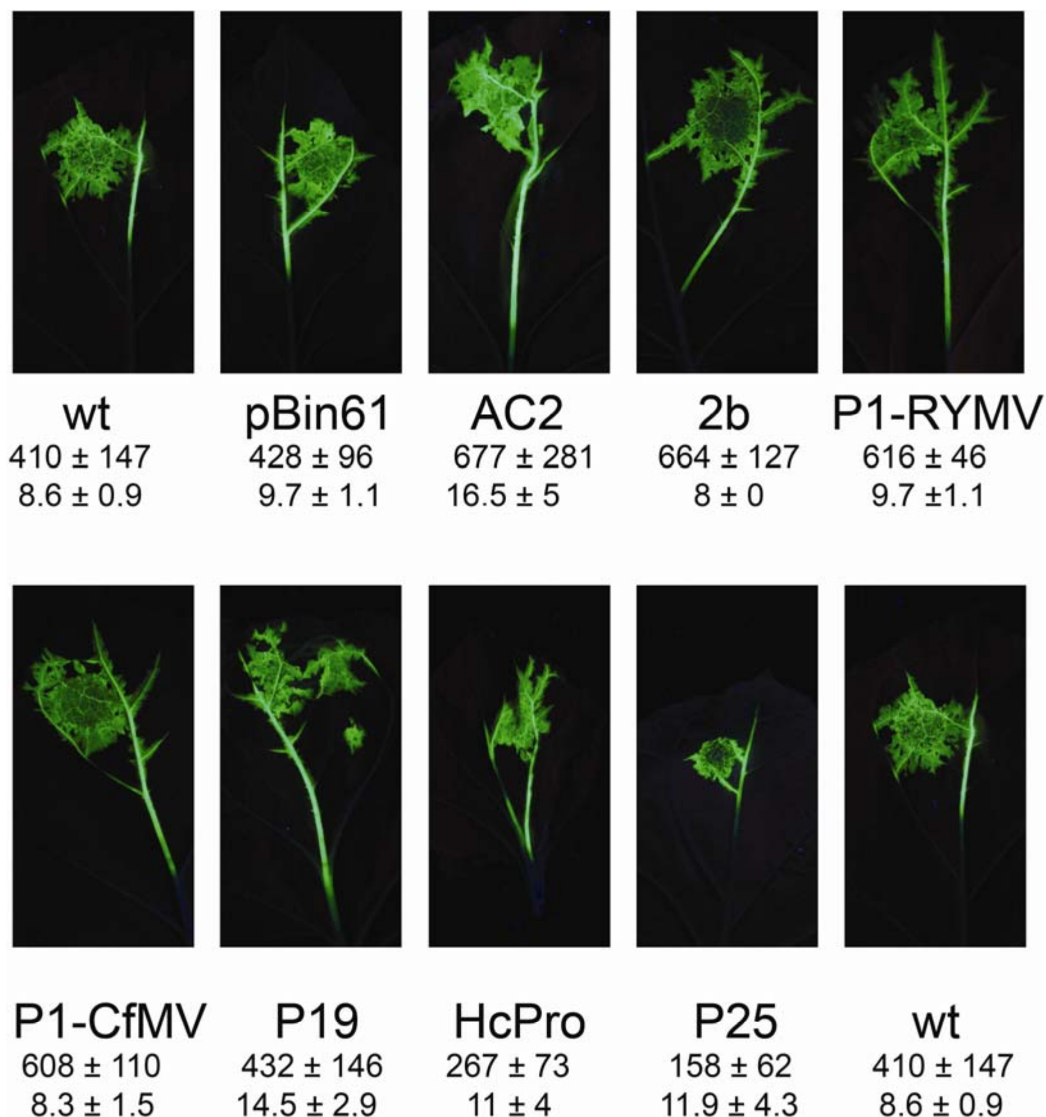

Fig. 5. Lesions of the crucifer-infecting *Tobacco mosaic virus*-green fluorescent protein infection in different transgenic and control *Nicotiana benthamiana* plants, viewed in 366-nm UV light 15 days after infiltration. The upper number under each frame indicates the mean area of three lesions and the lower number indicates the mean of the average surface brightness of three lesions, each from three separate plants, with the indication of the standard deviations. Each of the lesions shown in the figure is the medium representative of the three measured lesions. Each of the frames corresponds to real size of 45 mm in width and 90 mm in height. RYMV = *Rice yellow mottle virus* and CfMV = *Cocksfoot mottle virus*.

short internodes and thick, large, hairy leaves. However, at the cellular level, the malformations caused by these transgenes were similar in both species, whereas the effects of the two transgenes were clearly different from each other and were associated with hyperplasia in the *HcPro* transgenic plants, and with hypoplasia in the *AC2* transgenic plants. The most striking flower malformations in *N. tabacum* were caused by the *AC2* transgene; however, in *N. benthamiana*, this gene did not visibly affect the flower morphology, though seed set was severely reduced.

Some of the silencing suppressor transgenes caused very specific phenotypic effects. Particularly, *HcPro* gene caused a very severely distorted growth habit in both *Nicotiana* spp., and the *P25* gene, for example, caused small, not fully opened flowers in *N. benthamiana*. On the other hand, several of the genes caused similar effects in the transgenic plants within the same species. For instance, *P1*-RYMV, *HcPro*, *AC2*, and *P25* genes caused stunting in *N. benthamiana*. In addition, *P1*-RYMV, *P1*-CfMV, and *P19* genes caused bending of the flower base and *P19* and *AC2* genes caused blistering of leaves in *N. benthamiana*. These variable phenotypes suggest that the used transgenes interfere with different steps of the endogenous silencing processes. On the other hand, some of the different effects also may be related to the different expression levels of the transgenes.

It is of interest to compare these observed phenotypes with other silencing suppressor-transgenic plants described in the literature. *HcPro* gene has been extensively studied, and transgenic plants harboring either *HcPro* gene alone or together with P1 gene from different potyviruses have been produced in *A. thaliana*, *N. benthamiana*, and *N. tabacum* species (Anandalakshmi et al. 2000; Carrington et al. 1990; Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mallory et al. 2001, 2002; Mlotshwa et al. 2002, 2005; Pruss et al. 2004; Savenkov and Valkonen 2002; Shams-Bakhsh et al. 2007). Here, we studied HcPro protein (without P1) from PVY-N which, similarly to the other potyviral suppressors in *Arabidopsis,* caused severe malformations in the two transgenic *Nicotiana* spp. However, Mlotshwa and associates (2002), Shams-Bakhsh and associates (2007), and Savenkov and Valkonen (2002) reported no phenotypic alterations in transgenic *N. benthamiana* and *N. tabacum* cv. Samsun NN plants expressing HcPro protein of PVY-N or PVA, respectively. Also, no phenotype data was reported by Carrington and associates (1990) or Mallory and associates (2001, 2002) for *N. tabacum* expressing the HcPro derived from TEV, while severe malformations were reported in such transgenic tobacco plants by Anandalakshmi and associates (2000) and by Pruss and associates (2004). Therefore, the *N. benthamiana* transformed in this work with the *HcPro* gene of PVY-N is the first one reported to display a clear phenotype, including curled leaves with changed vein patterns, soft stems, and strongly malformed flowers as well as moderate stunting. Interestingly, some features described for TuMV *P1/HcPro*-transgenic *Arabidopsis* are similar with these observed phenotypic features (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mlotshwa et al. 2005). Different phenotypes, or lack of any phenotype observed in various studies in the *HcPro*-expressing transgenic plants, may be due to the high variation in the *HcPro*-coding region between different potyviruses (Flasinski and Cassidy 1998) or different expression levels of the transgenes in the different plants.

There is only one report describing the expression of tombusviral *P19* transgene in *N. benthamiana*. The *P19* transgene expressed in those plants was from *Artichoke mottled crinkle virus* and the described phenotype differed completely from the one observed in this work for TBSV *P19* (Silhavy et al. 2002). Intriguingly, the phenotypes reported for transgenic *Arabidopsis* expressing TBSV *P19* are similar to the ones observed here in *N. benthamiana*: serrated leaves and additional trichomes (Chapman et al. 2004; Dunoyer et al. 2004). In both *N. benthamiana* and *N. tabacum*, the altered phenotypes were observed here in plants in which the transgene mRNA accumulation remained below detection level, likewise with the reported transgenic *Arabidopsis*, harboring the *P19* gene of TBSV and showing altered phenotype (Dunoyer et al. 2004).

P25 protein of PVX caused a specific flower malformation and an early senescence phenotype in the *N. benthamiana* plants. *P25* transgenic *N. tabacum* plants showed no altered phenotype; however, this may have been due to the low level of transgene expression in these plants. This transgene has not caused any phenotype in *Arabidopsis* (Dunoyer et al. 2004), whereas the P25 protein from *Clover mosaic virus* has caused severe abnormalities in *N. benthamiana* leaves (Foster et al. 2002).

The 2b protein from three different CMV isolates has been used in silencing studies to elucidate its function as a suppressor. It has been shown that the *2b* gene from a severe strain causes more severe developmental abnormalities in *Arabidopsis* (Lewsey et al. 2007; Zhang et al. 2006). The *2b* gene used in this study was derived from a mild strain of CMV (Kin) and caused no developmental abnormalities in the two transgenic *Nicotiana* spp., as also was reported earlier when the corresponding gene of strain CMV-Q (another mild strain) was transformed into either *N. tabacum* (Ji and Ding 2001) or *Arabidopsis* (Lewsey et al. 2007; Zhang et al. 2006).

Up to now, no data about *AC2* gene expression in transgenic plants has been published. In this study, both *N. benthamiana* and *N. tabacum* harboring the *AC2* transgene exhibited altered phenotypes which appeared different on the intact plant level but similar on the cellular structure level. No transgenic plants have been produced from either *P1*-CfMV or *P1*-RYMV; therefore, the observed phenotypes caused by these two viral suppressors cannot be compared with previous reports. *P1*-RYMV caused severe stunting and leaf and flower malformations in the transgenic *N. benthamiana* plants but no abnormalities in the *N. tabacum* plants. On the other hand, *P1*-CfMV did not cause any observable effects on growth, except for some bending of flower stalks occurring in the *N. benthamiana* transgenic plants. The fact that the phenotypic alterations are more pronounced in the case of *P1*-RYMV is in correlation with the results published for the suppression efficiency of both sobemoviral proteins in these two *Nicotiana* spp.; P1-RYMV was the stronger of the two suppressors in agroinfiltration experiments (Sarmiento et al. 2007).

The disturbed phenotypes in the silencing suppressor-expressing transgenic plants likely are due to the interference of these suppressors with the endogenous RNA silencing pathways. Previously, 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; Mallory et al. 2002, 2004; Millar and Gubler 2005; Park et al. 2002; Ray et al. 1996; Vazquez et al. 2004). Such interference, targeted at an early step in the silencing pathways, would impair the regulation of multiple miRNA-regulated target genes, such as *SCL6,* targeted by miR171; NAC-domain proteins (*CUC1* and *CUC2*), targeted by miR164 (Mallory et al. 2004; Rhoades et al. 2002); *AP2*, and *ARF8* and *ARF 10*, coding for transcription factors, expressed specifically in inflorescence and leaves, respectively; and regulating their differentiation (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mallory et al. 2002; Park et al. 2002).

In addition to the abovementioned genes, other miRNA-regulated transcription factors also have similar effects, as observed in our transgenic plants. These include the *GAMYB*-like genes (e.g., *MYB33*) which malfunction in *Arabidopsis*, causing stunting, spindly growth, sterility, and reduced petiole lengths (Millar and Gubler 2005), and the *PHABULOSA* gene, which malfunction in *Arabidopsis*, causing leaves with upward curling (Mallory et al. 2004). It is not clear which specific steps or target genes of the endogenous silencing pathways are targeted in the two *Nicotiana* spp. by the silencing suppressors used in this study. The different phenotypes observed in these two species indicate that the effects of these silencing suppressors are, at least to some extent, species specific. In general, more severe developmental disturbances were observed in transgenic *N. benthamiana* than in *N. tabacum* plants. All the transgenes, except for the *2b*, caused various developmental defects in *N. benthamiana*; however, in *N. tabacum*, defects occurred only in the *HcPro*- and *AC2*-expressing plants, and in one line of the *P19* transgenic plants (even though the mRNA level of this line remained below detection level). The high lethality of *P1*-RYMV-transformed *N. benthamiana* lines in the R0 generation and the very low expression level of this transgene in surviving plant lines also were in contrast with the *N. tabacum* lines, where P1-RYMV was expressed on high levels without any adverse effect.

In addition to their effects on the endogenous silencing pathways, the viral silencing suppressors also interfere with the plant defense reactions with different viruses. The crTMV-GFP construct is very suitable for quantitating silencing suppression effects because, according to Kurihara and Watanabe (2004), crTMV itself does not suppress silencing, at least in *Arabidopsis* (which is a natural host of crTMV). In this work, we observed that the spread and accumulation of the crTMV-GFP construct was very differently affected in the different transgenic *N. benthamiana* plants. Both the crTMV-GFP lesion spread and brightness were strongly enhanced in the *N. benthamiana* lines expressing the *AC2* gene. This spread also was increased in the *2b*, *P1*-RYMV, and *P1*-CfMV transgenic lines. *P19* transgene specifically caused the infection to spread into new foci within the infiltrated leaves. Surprisingly, both the *HcPro* and *P25* transgenes reduced the spread of the crTMV-GFP construct. These different reactions suggest that the used suppressors affect differently the silencing functions targeted against the accumulation of this virus in cells, or against the cell-to-cell spread of the silencing signal. Some of these results, particularly those related to *HcPro* and *P25* transgenes, contradict the original hypothesis, which assumes that the silencing suppressors should enhance viral infections. However, these results seem to be in good accordance with previous results of Pruss and associates (2004), showing that expression of the HcPro of TEV in transgenic tobacco plants enhances their resistance against TMV and against *Tomato black ring virus*. Understanding the mechanisms of this resistance enhancement needs further investigation.

MATERIALS AND METHODS

Viral silencing suppressor constructs and their transformation to *Nicotiana* spp.

P19 of TBSV, *P25* of PVX, *HcPro* of PVY (strain N), *2b* of CMV (strain Kin), *AC2* of ACMV (*Geminivirus*), and *P1* of RYMV (*Sobemovirus*) in pBin61 vector, obtained from the laboratory of D. Baulcombe (through Plant Bioscience Ltd.), and *P1* of CfMV (*Sobemovirus*) in pBin61 vector (Sarmiento et al. 2007) were used for transformation. These constructs and empty vectors were introduced into *Agrobacterium tumefaciens* through electroporation, and transformed into leaf disks of *N. benthamiana* and *N. tabacum* cv. Xanthi (nn) L. by standard procedures (Smith et al. 1994). The transformants were regenerated on Murashige-Skoog (MS) medium using Km selection (Km at 100 μg/ml, cefotaxime at 250 μg/ml, and vancomycin at 100 μg/ml). Rooted plantlets were transferred to pots and grown to maturity in the greenhouse at 25°C with a 16-h photoperiod. In all, 10 lines with each silencing suppressor construct in both *Nicotiana* spp. were regenerated. All lines produced adequate amounts of seed for propagation, except *P1*-RYMV lines, where only three of the regenerated *N. benthamiana* plants produced seeds.

Propagation of the R1 and R2 generations.

Seed of each of the 10 selected R0 transgenic 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 seed. Five independent R1 lines with altered phenotype were selected and their seed were germinated on Km-containing MS medium. Two or three independent lines showing 100% germination, indicating homozygote transgene status, were selected for further analysis. For any further experiments, the seed were germinated in soil and the plants propagated in controlled greenhouse conditions as described above.

PCR and Northern blot analysis.

For PCR amplification of the transgene sequences from the genomic DNA of the selected plant lines, the plant DNAs were extracted with cetyltrimethylammonium bromide procedure (Siddiqui et al. 2007), and amplified using either primers annealing to the 35S promoter and terminator sequences (for *P1*-RYMV gene) or specific primers annealing to the coding regions of the transgenes. Primers used were as follows: for detection of *P1*-RYMV, 5′-ACTCAGCAGACTGTGGCAAAA-3′ (forward) and 5′-TACCGGAGTGCTCGGGGATAT-3′ (reverse); for detection of *P19*, 5′-AGGGAACAAGCTAACAGTGAAC-3′ (forward) and 5′-TACCTTCAGGGCATCCTCTTG-3′ (reverse); for detection of *P25*, 5′-GTTTAGGTTATTCTAGGACTTC-3′ (forward) and 5′-TCCCTTTGACCTGGTGATAGCG-3′ (reverse); for detection of *AC2*, 5′-ACTCTACTCAGGTTCCAATCAAAG-3′ (forward), and 5′-TCTGAGGCTGTAAGGTTGTCC-3′ (reverse); for detection of *HcPro*, 5′-GTTGCATCCGAATGGGAC-3′ (forward), and 5′-TGTGAGCCAAACGAGTCAACTAC-3′ (reverse); and for detection of *2b*, 5′-TGACAGTAGTGGTGTCGACC-3′ (forward), and 5′-ACGACCCTTCGGCCCATTCGTTAC-3′. For the detection of *P1*-CfMV 35S primer and terminator, specific forward and reverse primers were used (i.e., 5′-GTGGATTGATGTGACATCTCC-3′ and 5′-GCTCAACACATGAGCGAAACC-3′, respectively).

For Northern blot analysis, total RNA was extracted according to Sijen and associates (1996). Briefly, leaves (0.5 g) were ground in liquid nitrogen and powder was extracted in hot phenol and RNA extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM LiCl; 10 mM EDTA; and 1% sodium dodecyl sulfate) (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°C, followed by centrifugation (13,000 rpm for 20 min at 4°C). Pellets were resuspended in double-distilled water and RNA was recovered by ethanol precipitation. RNA (5 μg) was separated on 1% agarose denaturing gel 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°C for 2 h. PCR-amplified digoxigenin-labeled probes were generated for each suppressor from original construct by using PCR primers, 5′-GTGGATTGATGTGACATCTCC-3′ (35S promoter region) and 5′-GCTCA

ACACATGAGCGAAACC-3′ (35S-terminator region). Northern blots were probed and developed according to the manufacturer's instructions (Roche, Mannheim, Germany).

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 postfixed 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 a Canon EOS 20D digital camera.

***Agrobacterium* infiltration.**

Fresh overnight cultures of *Agrobacterium* cells, carrying a 35S-controlled infectious clone of the crTMV cDNA with a GFP gene replacing the coat protein gene (a gift from Y. Dorokhov), adjusted to an optical density at 600 nm = 1.0 as final density, were induced with acetosyringone as described by Hamilton and associates (2002). Equal volumes (approximately 100 μl) of the cell suspension were infiltrated to the two uppermost, fully expanded leaves of three plants of each transgenic *N. benthamiana* line. In two of three repeating experiments, the infiltrated plants were incubated for 15 days and, in one experiment, for 7 days before detaching the leaves for photography**.** The infiltrated plants were maintained in the greenhouse under the conditions mentioned above.

GFP imaging.

The GFP was visualized by using a hand-held 366-nm wavelength UV lamp (BLACK RAY, UVL-21, Ultra-Violet Products, Inc., Upland, CA, U.S.A.). Photographs were taken at 15 days postinfiltration with a Canon EOS 20D digital SLR camera. Equal exposures of each leaf sample were obtained by fixing lamps, camera, and sample positions. The pictures were analyzed using Photoshop CS3. Only the green channel images were used. The brightness values of each pixel in every image were checked to ensure that none were saturated. This is important because information about the true brightness is lost if saturation levels are reached. No image had saturated pixels.

The raw format images were transferred to linear tiff format (note: jpeg format images, normal gamma-corrected tiff, and most other formats are not linear). The linearity of this transformation has been verified by astronomical measurements of stars of different magnitudes covering a factor of approximately 100 in brightness. The linearity was better than 3%, which is sufficient for these measurements (H. Lehto, *unpublished results*). The total brightness of the GFP spots was measured with an aperture photometry technique, in which the background level brightness was subtracted automatically. The units in the total brightness are in an arbitrary but linear scale. The surface area was measured using jpg images because, here, the linearity is not critical. The edges of the spots were typically sharp. A suitable cut-off level for the surface area was selected by eye. The result is not sensitive to small differences at this level. 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. This accuracy is sufficient for detecting significant differences between samples.

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