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
32p   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         |
ORIGI NAL PUBLICATION S

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


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* DCL1 with 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 length, 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 length (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 (Mg2+) 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-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.,
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 (Cioco 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 unwinding step. In Drosophila cells, AGO2 cleaves not only the target mRNA but also the “passenger” strand (antiguide 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 seems that insects and vertebrates do not possess these enzymes (Wassenegger and Krčzal, 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 Krčzal, 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-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 Der2 to channel siRNA into RISC. In a similar way, Der1 interacts with Loquacious to process miRNAs (Forstemann 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 C2H2 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 and 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*

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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 temporarily 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). Specific 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 where 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 PPRs 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, HSN1, 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 RRM (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 crosstalk 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/HePro 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, *NtRdRP1*, *NbRdRP1m*) 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 phosphodiester 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 innate 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)

<table>
<thead>
<tr>
<th>Virus genus</th>
<th>Virus name</th>
<th>Suppressor</th>
<th>Implicated motif</th>
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<td>P23</td>
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<td>AC2/AC4</td>
<td>DNA binding, NLS/ miRNA binding</td>
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<td>Mungbean yellow mosaic virus</td>
<td>AC2</td>
<td>DNA binding, NLS</td>
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<tr>
<td>Caulimovirus</td>
<td>Cauliflower mosaic virus</td>
<td>P6</td>
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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
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 capacity 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 proteolitic 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 demonstrated that this motif is required for sRNA binding and therefore for the suppressor activity of HcPro of some potyviruses (Shibolet 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 HePro 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 32P-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 32P-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-RL1 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 HePro 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

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

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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).
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 i-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/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

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

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

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

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 Leever, 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).
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., 62).
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 agroinfiltration 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, P1 of 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.
REFERENCES


Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, M. and Matzke, A. J. (2002). HD-A6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. EMBO J. 21, 6832-6841.


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AtRL12 is an endogenous suppressor of RNA silencing

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


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Cockfoot 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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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


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Teadustöö põhisuunad: RNAi ja tema supressioon taimedes
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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 SalI. 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). 32P-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'-cgggtgccggaaactacag-g-3') and 3'UTR3' (5'-cgggtgccgtaaaatccaaagtagta-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. 32P-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 Eco105I and KpnI. 35S promoter of Cauliflower mosaic virus (CaMV) was cut out from pANU5 (Mäkinen et al., 1995) with HindIII and KpnI. pCAMBIA1301 was linearized with
HindIII and EcoI36II. 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 EcoI and HindIII and ligated to SmaI and HindIII sites of pTZ-RLI-P giving the construct pRLI2prom1. In order to obtain pRLI2prom, pRLI2prom1 was cut with PstI and ligated into empty vector pTZ57R/T, previously linearized with PstI. This same fragment from pRLI2prom was also cloned into pCAMBIA1300 PstI 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 OD600. 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 electrophoresis 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. 32P-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’-ctcggtaccgctatggccatgcccc-3’) and RLIexpr3’ (5’-cggggtacctaaggaaccagcggag-3’) and cloned into pTZ57R/T vector. The coding sequence was then cut out with SmaI and XhoI and cloned into the same sites of pGEX6P-2 (Amersham). Transformed E. coli strain BL21(DE3)pLysS cells were grown from overnight cultures in 2xYTF medium in the presence of ampicillin (100 µg/ml) at 30 °C to the optical density of A600 = 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 NaH2PO4, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 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

$5 \times 10^{-3}$ 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-RL1 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).

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...
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%,
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 32P-labeled GFP-anti-sense riboprobe. Ethidium bromide staining of rRNA as a loading control for each Northern analysis (A and B).](image1)

![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.](image2)
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., 1998; Kasschau and Carrington, 1998), 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 (Martinanda et al., 1998) and HIV (Martinanda 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 AtRLI2 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 (Martinanda et al., 1998; Zimmerman et al., 2002; Karcher et al., 2005; Yarunin et al., 2005) and with RLI conformational changes, which enable its mecanochemical 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 ein2) 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 determined 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.

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References


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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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; Palaquii 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).
CfMV-NO ORF1 was amplified with primers 5′-CCTAGATCTAGCTAGCTAGCTAGGTCCGGAACCTCC-3′ and 5′-GAGCTGCAGAACCACATCCGTGACAC-3′ and inserted into pTZ57R/T (Fermentas) to generate pTZ:CfMVORF1. CfMV-NO ORF1 (nt 62–441) was excised with XbaI and BamHI 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 between the 35S promoter and Nos terminator to give pBIN61-P1. 35S-C_P1 refers to Agrobacterium tumefaciens (C58C1) containing pBIN61-P1.

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%.
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 CfMV-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 Potos 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 BamHI site) and 5′-ACGCGTCGACTACTCTGTCCTGCC-3′ (containing a SalI 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).

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 (Sadovy 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 auresivirus P14 (Merai et al., 2005) as well as for tospoviruses NS5 and tenuiviruses NS3 (Bucher et al., 2003).

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References


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 Km), 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-infected 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 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).

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, Peclovirus], 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 miRNAs (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 tobruviruses, 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 (P1 of Rice yellow mottle virus [RYMV] and Cocksfoot mottle virus [CMV], 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-CMV transgenes (Fig. 1A). Thus, at least one transgene-expressing N. tabacum line was

**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 [CMV], 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-CMV transgenes (Fig. 1A). Thus, at least one transgene-expressing N. tabacum line was
obtained for each of the transgenes except for the \textit{P1-CfMV} and \textit{P25}; however, these still were maintained for the analysis due to their effects in \textit{N. benthamiana}. One of the \textit{P25}-harboring \textit{N. tabacum} lines showed reduction of the accumulation of PVX RNA in PVX-inoculated plants (data not shown), suggesting that the \textit{P25} sequence was targeted by RNA silencing in this line. The presence of the transgene in all \textit{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 \textit{HcPro} in both \textit{Nicotiana} spp. In \textit{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 slow seed production also were observed in most of the \textit{HcPro} expression lines in the R0 and R1 generations (data not shown). Both \textit{HcPro} transgenic \textit{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 \textit{AC2} transgenic lines in \textit{N. tabacum} exhibited a phenotype very similar to the \textit{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 \textit{AC2}-expressing \textit{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 \textit{AC2} lines. In the \textit{AC2}-expressing \textit{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 \textit{AC2}-expressing \textit{N. benthamiana} R1 generation (data not shown), and seed set was severely reduced.

Distinct phenotypes also were observed in plants expressing the other transgenes. \textit{P1-RYMV} caused high lethality in \textit{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 \textit{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 \textit{P1-RYMV}, the \textit{P1-CfMV} caused no specific phenotype, except that some flowers of one of the \textit{N. benthamiana} lines had bending stalks.

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

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### Table 1. Most pronounced phenotypes observed in different transgenic lines of \textit{Nicotiana benthamiana}

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

\(^{a}\) \textit{RYMV} = \textit{Rice yellow mottle virus} and \textit{CfMV} = \textit{Cocksfoot mottle virus}.

### Table 2. Most pronounced phenotypes observed in different transgenic lines of \textit{Nicotiana tabacum}

<table>
<thead>
<tr>
<th>Lines(^a)</th>
<th>Leaf</th>
<th>Flower</th>
<th>Stunting</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WT)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>…</td>
</tr>
<tr>
<td>pBin61</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
<td>…</td>
</tr>
<tr>
<td>\textit{HcPro}</td>
<td>Thick and hairy, short internodes, blistering</td>
<td>Normal</td>
<td>Severe, final height approximately 50% of the WT plants</td>
<td>Late flowering (2 months later)</td>
</tr>
<tr>
<td>\textit{AC2}</td>
<td>Thick and hairy, short internodes, blistering, cup-shaped</td>
<td>Moderate malformation, stamens transformed into petals, some sepals transformed to petals, less stamens (4)</td>
<td>Severe, final height approximately 50% of the WT plants</td>
<td>Late flowering (1 month later)</td>
</tr>
<tr>
<td>\textit{P1-RYMV}</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
<td>…</td>
</tr>
<tr>
<td>\textit{P1-CfMV}</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
<td>…</td>
</tr>
<tr>
<td>\textit{P19}</td>
<td>Normal</td>
<td>Severe malformation</td>
<td>No</td>
<td>…</td>
</tr>
<tr>
<td>\textit{P25}</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
<td>Late flowering (1 month later)</td>
</tr>
<tr>
<td>2b</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
<td>…</td>
</tr>
</tbody>
</table>

\(^{a}\) \textit{RYMV} = \textit{Rice yellow mottle virus} and \textit{CfMV} = \textit{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 \textit{N. tabacum}, P19 caused occasional malformation of flowers (Fig. 3H and I).

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

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

Effects of the \textit{HcPro} and \textit{AC2} silencing suppressors on the structure of the leaf tissues.

The most severe leaf malformations occurred in both \textit{Nicotiana} spp. transformed either with the \textit{HcPro} or the \textit{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 \textit{Nicotiana} spp., \textit{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, \textit{N. benthamiana} sections; H and K, \textit{N. tabacum} sections). Particularly in the \textit{HcPro}-expressing \textit{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 \textit{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 \textit{HcPro}-expressing plants, the mesophyll cells of the \textit{AC2} transgenic plants of both \textit{Nicotiana} spp. were significantly larger than the mesophyll cells of comparable wild-type plants (Fig. 4C and F, \textit{N. benthamiana} sections; I and L, \textit{N. tabacum} sections). Particularly in \textit{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 \textit{HcPro} and \textit{AC2} transgenic \textit{N. benthamiana} and \textit{N. tabacum} plants indicated that, at the cellular level, the malformations caused by each transgene were similar in both \textit{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 \textit{HcPro} plants, and very large cells [hypoplasia] in the \textit{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 \textit{N. benthamiana} and also slowly spread in this host. This construct has been used successfully to analyze RNA-silencing activity in \textit{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 \textit{N. benthamiana} line were infiltrated with an \textit{Agrobacterium} suspension carrying 35S-controlled infectious crTMV-GFP clone. However, this analysis was not carried out in \textit{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 \textit{N. benthamiana} lines; however, the type of GFP-lesion spread and degree of luminosity still varied clearly between the different transgenic plants (Fig. 5). \textit{AC2} transgene clearly enhanced both the spread and the mean surface brightness (average flux per pixel) of the GFP lesions (Fig. 5). The \textit{2b} transgene and also both of the \textit{P1}

![Fig. 4. Microscopic analysis of the leaf structures of the wild-type, \textit{HcPro}, and \textit{AC2} transgenic plants.](image-url)
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

![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.](image-url)
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*-CMV, 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 and associates 2007). Here, we studied *HcPro* protein (without *P1*) from PVY-N which, similar 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*-CMV 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*-CMV 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 sobmoviral 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 biogenesis 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; Rhodeas et al. 2002); *AP2*, and *ARF8* and *ARF10*, 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 GLMYB-like genes (e.g., MYR33) 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 HePro- 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-CIMV transgenic lines. P19 transgene specifically caused the infection to spread into new foci within the infiltrated leaves. Surprisingly, both the HePro 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 HePro 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 HePro 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, HePro 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 CIMV (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′-AATCAGCAGACTCTGGCACAAG-3′ (forward) and 5′-TACCGAGTGCTGAGGAATAT-3′ (reverse); for detection of P19, 5′-AGGGAAACAGCATAACTATAAC-3′ (forward) and 5′-TACCCATGGCGCATCTCTCTG-3′ (reverse); for detection of P25, 5′-TTTGGATTATTTCTGAGACT-3′ (forward) and 5′-TCCCTTTGAGCCTTGAAGCG-3′ (reverse); for detection of AC2, 5′-ACTCTACTCTAATCCAAAG-3′ (forward), and 5′-TCTGAGCTGTAAAGGTTTGC-3′ (reverse); for detection of HcPro, 5′-GGTGCATCCGAATGGGAC-3′ (forward), and 5′-TGTGAGCCAACAGGAGTCAACT-3′ (reverse); and for detection of 2b, 5′-TGACGAGTGCTGAGGAATAT-3′ (forward), and 5′-TGTGAGCCAACAGGAGTCAACT-3′ (reverse); and for detection of N. tabacum 35S primer and terminator, specific forward and reverse primers were used (i.e., 5′-GTGATGGATTGTGACATCTCC-3′ and 5′-GCTCAACATGACGCAAAA-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
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 (approximate 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: tiff 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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LITERATURE CITED


