Chapter A3

RNA Silencing: A Natural Resistance Mechanism in Plants

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Introduction

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

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In this chapter we will describe the RNA silencing process with emphasis on the functioning of the mechanisms and its role in natural virus infection in plants. In addition, applications of RNA silencing in plants and implications of RNA silencing for research in other organisms will be discussed.

The discovery of RNA silencing

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

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

impaired RNA silencing revealed more details about the mechanisms involved in this process (Elmayan et al. 1998; Dalmay et al. 2000). Certain mutants affected in the silencing pathway showed enhanced susceptibility to virus infection, confirming their involvement in antiviral activity (Dalmay et al. 2000; Mourrain et al. 2000). Over recent years, many components of the plant silencing pathways (Fig. 1) have now been uncovered and will be further discussed later in this chapter.

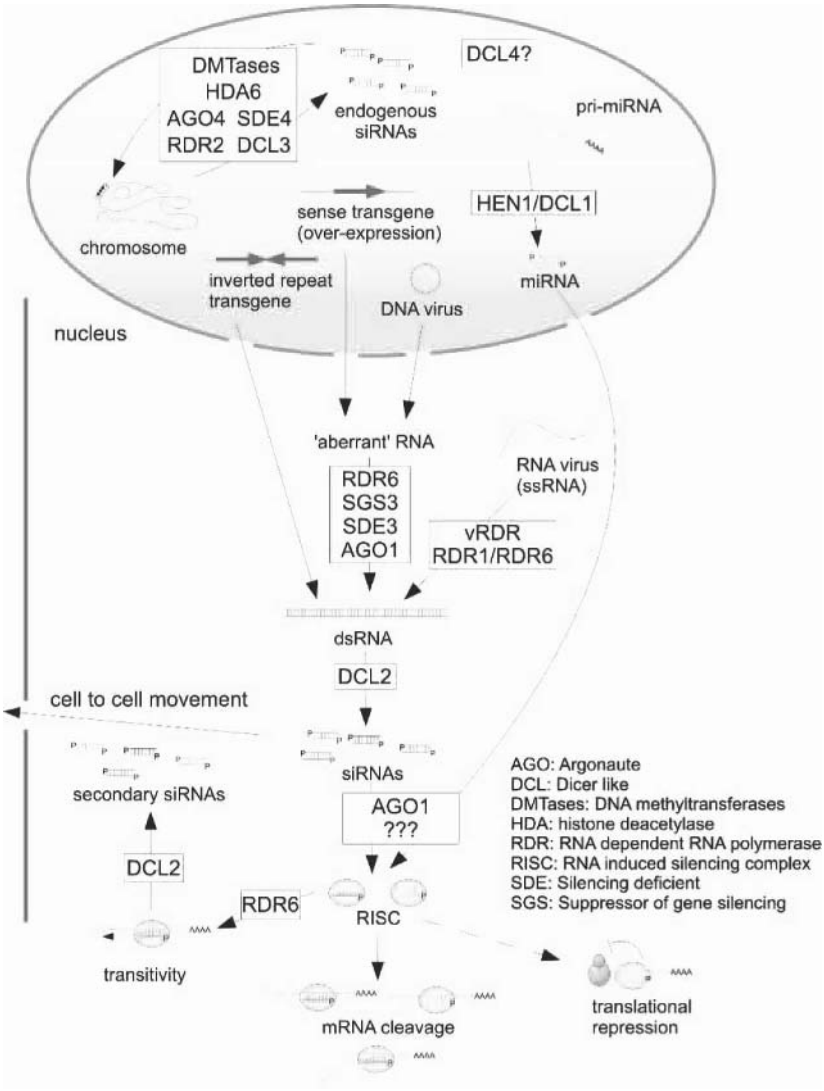

Fig. 1. A model of the RNA silencing pathways in plants. The squares indicate identified proteins or genes involved in the different silencing processes. ??? indicates the position of proteins associating with DICER such as R2D2 that have been identified in animals but not (yet) in plants.

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

To explain the extreme sequence specificity of the RNA silencing process, small RNA molecules had been envisaged in models throughout the second half of the nineties. However, it was not until 1999 that Hamilton and Baulcombe (1999) unequivocally proved that plants containing a silenced transgene indeed accumulated small (ds)RNA molecules whose sequence was identical to the transgene. They observed the same kind of approximately 25 bp sequence-specific small RNAs in PVX infected plants, suggesting a role of these molecules in a sequence specific antiviral defense mechanism. A further breakthrough pointing to the involvement of RNA silencing in antiviral defense was the discovery of virus specific RNA silencing suppressors (Anandalakshmi et al. 1998; Brigneti et al. 1998; Voinnet et al. 1999). This will be discussed in detail in subsequent paragraphs.

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

Mounting the plant antiviral defense

In plants, the control of virus replication is considered as one of the primary roles of RNA silencing. Although expressing viral transgene RNAs can precondition this response, the natural response is adaptive and requires recognition of ‘foreign’ molecules for initiation. This recognition is subsequently converted into ‘effector’, ‘memory’ and ‘warning’ signals to alert the systemic parts of the plant. DsRNA molecules have been shown to

be most potent initiators of RNA silencing (Smith et al. 2000). As most plant viruses are RNA viruses that replicate *via* double stranded replication intermediates, it is tempting to suggest that these molecules are a trigger for RNA silencing. This is, however, put too simply. Most, if not all plant RNA viruses may replicate via dsRNA. The chance that these RNAs appear as naked RNA in the cell is very small since replication complexes are protected by viral replication and/or capsid proteins. Viral replication often takes place inside specialized replication structures and dsRNA can immediately be unwound by viral and host RNA helicases (Ahlquist 2002). Though we do not dismiss the possibility of detection of these structures by RNA silencing, we think viral mRNAs, which might be recognized by the plant as being 'aberrant' (e.g. non-capped or non-polyadenylated mRNAs), are (also) an important target which can be converted into double stranded RNA by plant RNA-dependent RNA polymerases (RdRps). This would explain the generation of virus specific siRNAs in plants infected with geminiviruses (single-stranded DNA viruses) (Vanitharani et al. 2003).

The *Arabidopsis* genome encodes four Dicer-like enzymes that have the ability to process dsRNA into siRNA molecules (Schauer et al. 2002). In a normal virus infection, plants contain a significant amount of siRNAs originating from the virus (Hamilton and Baulcombe 1999). These siRNAs can subsequently be used in two ways: either they are unwound and one strand is incorporated into the RNA induced silencing complex (RISC) to target and degrade RNAs homologous to the siRNA, or a plant RdRp uses the siRNA as a primer on homologous mRNAs and synthesizes dsRNA that then is processed by Dicer into secondary siRNAs (a phenomenon called 'transitivity') (Vaistij et al. 2002). This latter step leads to the amplification of the intracellular silencing signal. In plants, RNA silencing generated secondary siRNAs can originate from 5' and 3' parts of the targeted site in the messenger, indicating that the transitivity is bidirectional. This is in contrast to *C. elegans* where secondary siRNAs only originate from the 5' side of the target mRNA in relation to the inducer molecule (Sijen et al. 2001). This may be related to the fact that both siRNA strands seem to be stable in plants (Hamilton and Baulcombe 1999), while in *C. elegans*, only the antisense strand is maintained. In mammals and insects, transitivity was reported not to be present. Indeed no endogenous RdRp, which would be required for this activity, has been identified (Schwarz et al. 2002). Next to the predominant 21 nt species of siRNAs observed in all eukaryotes, the plant silencing machinery has the unique ability to produce, a second size class of siRNAs, of around 24 nt (Hamilton et al. 2002). The longer class of siRNAs has been correlated with the long-distance spread of RNA silencing. This ability allows the viral siRNAs produced by the plant silencing

machinery to move to adjacent cells advancing the spread of the virus. RISC is thought to be pre-programmed with these siRNAs allowing an immediate recognition and elimination of incoming viruses. The shorter class of siRNAs is thought to operate in local RNA silencing (Hamilton et al. 2002). This size class has also been reported to be able to move from cell to cell, however, spreading no further than up to 15 cells (Himber et al. 2003).

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

Suppressor proteins: Viral counter measures against RNA silencing

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

It has long been known that certain proteins expressed by viruses played an important role in their virulence (Pruss et al. 1997). It was observed that co-infection of combinations of viruses could cause increased symptom severity compared to each of the viruses alone. These mixed infections indicated that at least one of the viruses possessed a character that could support the replication and spreading of the other virus. Potyviruses were reported early on to increase the virulence levels of another virus (see Chapter by Palukaitis and MacFarlane). The actual underlying mechanism started to become understood in studies of mixed infections of PVX with different potyviruses. Mutational analysis of the *Tobacco etch virus* (TEV) revealed that the helper component-proteinase (HC-Pro) was required for the synergistic activity of TEV (Shi et al. 1997). A first indication that HC-Pro could actually block a general plant antiviral pathway was found when transgenic plants constitutively expressing HC-Pro were produced. Heterologous viruses such as TMV and CMV showed enhanced accumulation and pathogenicity in these plants (Pruss et al. 1997). In the case of CMV, virulence could be linked to its 2b protein (Brigneti et al. 1998). These results were later confirmed by studies where the 2b gene of

different CMV subgroups were replaced (Shi et al. 2002). Indication that RNA silencing is indeed involved in virus resistance came with the reports that HC-Pro can enhance virulence of heterologous viruses by directly suppressing RNA silencing (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 2001).

Assays used to identify suppressors of RNA silencing

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

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

Another method makes use of GFP or GUS (beta-glucoronidase) silenced transgenic plants. Plants expressing a reporter gene are systemically silenced by the infiltration of *Agrobacterium* expressing (a fragment of) the RNA of that reporter gene, or plants are genetically silenced (e.g. using inverted repeats). Subsequently, these plants are infected with different viruses or virus constructs and the reporter gene expression is monitored. Restoration of reporter gene expression indicates that the tested virus encodes a suppressor of RNA silencing. PVX encodes a suppressor of RNA silencing that cannot restore the reporter gene expression in this assay and makes it a good vector to test other viral genes for their silencing suppression capability (Brigneti et al. 1998). Additionally it has been

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

Genome	Genus	Virus	Suppressor protein	Suppressed RNA silencing mechanism	Reference
DNA	Begomovirus	ACMV	AC2	-	Voinnet, et al. 1999; Vanitharani, et al. 2004
			AC4	local	Vanitharani, et al. 2004
		TGMV	AL2	-	Wang, et al. 2003
		TYLCV-C	C2	local and systemic	Dong, et al. 2003; Van Wezel. et al. 2003
(+) RNA	Curtovirus	BCTV	L2	-	Wang, et al. 2003
	Carmovirus	TCV	CP (P38)	local	Thomas, et al. 2003; Qu, et al. 2003
	Closterovirus	BYV	p21	local	Reed, et al. 2003
		CTV	p20	local	Lu, et al. 2004
			p23	local and systemic	Lu, et al. 2004
			CP	systemic	Lu, et al. 2004
		BYSV	p22	local	Reed, et al. 2003
	Comovirus	CPMV	S coat protein	local	Voinnet, et al. 1999; Canizares; et al. 2004; Liu, et al. 2004
	Cucumoviruss	CMV	2b	local* and systemic	Brigneti, et al. 1998; Lucy et al. 2000
	Furovirus	PCV	P15	local and systemic	Dunoyer, et al. 2002
	Hordeivirus	BSMV	γb	-	Yelina, et al. 2002
	Polerovirus	BWYV	P0	local and not	Pfeffer, et al. 2002
	Potexvirus	PVX	P25	systemic	Voinnet, et al. 2000
	Potyvirus	PVY	HC-Pro	local and systemic*	Brigneti, et al. 1998; Anandalakshmi, et al. 1998
	Sobemovirus	RYMV	P1	-	Voinnet, et al. 1999
	Tobamovirus	TMV	126-kDa protein	-	Voinnet, et al. 1999 ;Ding, et al. 2004
		ToMV	130-kDa protein	local	Kubota, et al. 2003
	Tombusvirus	TBSV	P19	local and systemic (binds siRNAs)	Voinnet, et al. 1999; Lakatos, et al. 2004
			P19	local and systemic	Silhavy, et al. 2002
	Tymovirus	TYMV	p69	local	Chen, et al. 2004
(-) RNA	Tenuivirus	RHBV	NS3	local	Bucher, et al. 2003
	Tospovirus	TSWV	NS_S	local	Bucher. et al. 2003; Takeda, et al. 2002

observed that if PVX expresses a heterologous suppressor of RNA silencing it causes more severe symptoms compared to the empty vector (Pruss et al. 1997; Brigneti et al. 1998).

Finally, one can produce transgenic plants that constantly express a suppressor of RNA silencing. A significant drawback with this method is that (high) expression of suppressors of RNA silencing often leads to developmental defects in the plants (Anandalakshmi et al. 2000). Nevertheless, some successes have been reported (Kasschau et al. 2003; Chapman et al. 2004; Dunoyer et al. 2004).

RNA silencing suppressor proteins

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

HC-Pro of potyviruses

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

being used by the different groups (*Agrobacterium* infiltration versus grafting). Additionally, there are conflicting reports on whether or not HC-Pro affects the methylation of a silenced transgene locus in the plant genome (Llave et al. 2000; Mallory et al. 2001).

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

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

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

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

2b of cucumoviruses

While HC-Pro had a direct and strong effect on the maintenance of RNA silencing, *Cucumber mosaic virus* (CMV) 2b was shown to affect the RNA silencing pathway differently. 2b cannot suppress RNA silencing in tissues where RNA silencing is already established. However, it was shown to be able to prevent the initiation of RNA silencing in newly emerging tissue (Beclin et al. 1998; Brigneti et al. 1998). This suggested that 2b might be involved in inhibiting the systemic spreading of the silencing signal. Further analysis revealed that CMV 2b carries a monopartite nuclear localization

signal (NLS) that is required for the 2b silencing suppression activity (Lucy et al. 2000). This was very surprising, since at that time components of RNA silencing were thought to operate in the cytoplasm only. How 2b prevents the silencing signal from spreading throughout the plant remains to be investigated.

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

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

P19 of tombusviruses

One of the most immediate suppressors of RNA silencing is P19 of the tombusviruses, such as *Cymbidium ringspot virus* (CymRSV). P19 was found to suppress RNA silencing by binding siRNAs in their double stranded form (Silhavy et al. 2002). P19 only very inefficiently binds single-stranded siRNAs, long dsRNAs, or blunted 21 nucleotide (nt) dsRNAs. However, a 2 nt overhang at the 3' end is sufficient for P19 to bind 21 nt RNA duplexes (Silhavy et al. 2002). The step of the RNA silencing pathway upon which P19 has an effect on was indicated by biochemical experiments

performed in *Drosophila* cell extracts. It was found that P19 activity prevents siRNAs from incorporating into RNA silencing effectors such as RISC (Lakatos et al. 2004). Furthermore, specific binding of siRNAs by P19 efficiently blocks the development of systemic spreading of silencing. This substantiates the previously suggested involvement of siRNAs in the spreading of RNA silencing. Either P19 suppresses systemic silencing by binding the siRNAs, preventing them from moving through the plant, or it inhibits the activity of an siRNA-primed RdRp which is thought to be involved in the formation of the systemic signal (Voinnet 2001).

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

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

RNA silencing suppressors of negative strand viruses

The first suppressors of RNA silencing of negative stranded RNA viruses to be found were NS_S of *Tomato spotted wilt virus* (TSWV) and NS3 of *Rice hoja blanca virus* (RHBV) (Takeda et al. 2002; Bucher et al. 2003). The accumulation of the NS_S protein had long since been shown to coincide with symptom severity of the virus (Kormelink et al. 1991), but it was not until 2002 that NS_S was proven to be an efficient suppressor of RNA silencing. For its identification the transient expression system using *Agrobacterium* and the viral infection GFP silenced plants were used. Although NS_S efficiently suppresses RNA silencing of sense transgenes by preventing the production of siRNAs it is not able to suppress RNA silencing mediated by dsRNA (Takeda et al. 2002). This indicates that NS_S acts upstream of the plant RdRp. While its activity appears to be similar to HC-Pro at the molecular level, only further analysis will reveal how NS_S exactly suppresses RNA silencing and whether it has any effect on systemic silencing or the miRNA pathway.

The NS3 of the distantly related *Tenuivirus* RHBV also efficiently suppresses RNA silencing but it is intriguing that even though it inhibits the mRNA degradation, it does not prevent the accumulation of siRNAs (Bucher et al. 2003). An interesting feature of negative stranded plant viruses is that insects do not only transmit them, but they also replicate in their insect vectors. This may suggest that NS_S and NS3 suppress RNA silencing in both hosts, possibly in a step of the pathway, which plants and insects have in common.

Consistent with the idea that silencing suppressors can function in both insect and plant cells, it has been shown for the insect-infecting positive-sense RNA virus *Flock house virus* (FHV) that it encodes a suppressor of RNA silencing that is active both in plants and in *Drosophila* cells (Li et al. 2002).

RNA silencing suppressors of DNA viruses

In addition to the silencing suppressors of RNA viruses described above, DNA viruses have also been shown to encode suppressors of RNA silencing. This is interesting considering the fact that these viruses replicate in the nucleus and their genomes consist of DNA. Hence geminivirus-derived dsRNA intermediates never occur during replication. It has, however, been reported that geminiviral mRNAs in the plant are targeted by RNA silencing in a plant RdRp (RDR6, previously named SGS2/SDE1) dependent manner (Muangsan et al. 2004). In GFP-silenced plants the bipartite geminivirus *African cassava mosaic virus* (ACMV) was shown to weakly suppress RNA silencing and AC2 was identified to be its suppressor of RNA silencing (Voinnet et al. 1999; Hamilton et al. 2002; Vanitharani et al. 2004). Further investigation revealed that AC4 of ACMV was a strong suppressor of RNA silencing (Vanitharani et al. 2004). However, for the *East African cassava mosaic Cameroon virus* (EACMCV) the unrelated AC2 encodes a strong suppressor of RNA silencing. Similar to the synergism observed for PVX and PVY, mixed infections of ACMV and EACMCV revealed enhanced virulence. AC2 and AC4 were shown to be involved in this synergism. AC4 of ACMV could enhance EACMCV DNA accumulation and reciprocally AC2 increased the accumulation of ACMV DNA (Vanitharani et al. 2004). Although RNA silencing was originally regarded as entirely cytoplasmic, there is evidence that elements of the mechanism also have effects in the nucleus (Fig. 1). The fact that AC2 requires a DNA-binding domain and an NLS for its activity as a suppressor of RNA silencing might fit this notion (Dong et al. 2003). Also the AL2 and L2 proteins of the bipartite *Tomato golden mosaic virus* and the monopartite *Beet curly top virus*, respectively, were reported to act as suppressors of RNA silencing (Wang et al. 2003).

The way these proteins exercise their function is unclear, although they have been shown to increase susceptibility to virus infection by inactivating the SNF1 and ADK kinases (Hao et al. 2003; Wang et al. 2003). Whether and how these inactivated endogenous proteins are involved in RNA silencing is not known.

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

The role of RNA silencing in antiviral defense in animals

Evidence that RNA silencing plays a role in antiviral defense in insect cells came from experimental infections of FHV in insect cells (Li et al. 2002). Replication of the virus in *Drosophila* cells, similar to the situation observed in plants, leads to the production of siRNAs originating from the virus. This strongly indicates that RNA silencing in insect cells actively targets the virus. Furthermore it was found that FHV encoded a suppressor of RNA silencing (B2) which was not only functional in insects but also plants. Recent reports show that the NS1 protein encoded by *Influenza A virus* acts as a suppressor of RNA silencing in plants and insects (Bucher et al. 2004; Delgadillo et al. 2004; Li et al. 2004). It was also shown that NS1 efficiently binds siRNAs and that the dsRNA binding domain that is involved in the siRNA binding is required for the suppression of RNA silencing (Bucher et al. 2004). Further work is required to show that NS1 suppresses RNA silencing in mammalian cells and that indeed RNA silencing is an antiviral mechanism used to counter influenza.

Other functions of RNA silencing

As important as it is, the antiviral activity of RNA silencing is certainly is not its only function in plants. By using components of the RNA silencing machinery several other processes are supported. These processes play an important role in plants and perhaps even more so in other multicellular organisms. Among these processes are transposon silencing, transcriptional gene silencing due to sequence specific DNA methylation, chromatin

condensation and (developmental) gene regulation by miRNAs. Perhaps more so than the other RNA silencing functions, the latter process, one of the most recent sapling of the RNA silencing tree, has turned out to be of major consequence for molecular biology as it influences gene expression in an unforeseen way and scale.

Transcriptional gene silencing

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

Chromatin modeling

A second role of methylation of perhaps a greater magnitude than TGS was recently discovered in *Schizosaccharomyces pombe*, where RNA silencing was shown to play a role in chromatin structure, centromeric cohesion and cell division. Mutational analyses showed that RNA silencing compounds were required for the pericentromere organization in *S. pombe* (Volpe et al. 2003). Three genes that encode key enzymes of the RNA silencing machinery, Argonaute ($ago1^+$), Dicer ($dcr1^+$) and an RdRp ($rdp1^+$), were shown to be essential for this process. The RdRp is required for the production of the dsRNA from transcripts originating from the pericentromeric heterochromatin composed of complex repeats. These RNA duplexes are rapidly processed by Dicer and incorporated into what was termed the RNAi-induced transcriptional gene silencing (RITS) complex, a complex with high biochemical similarity to RISC (Verdel et al. 2004). Ago1 of *S. pombe* is a key component of these complexes and binds the siRNA. RITS activity is exerted in the dividing cell leading to the recruitment of the chromodomain protein Swi6, sequence specific methylation of centromeric regions and ultimately to chromosome condensation (Noma et al. 2004). Though discovered in yeast, these features seem to be conserved among all eukaryotes including vertebrates (reviewed by White and Allshire 2004 and Dawe 2003).

Transposon and endogenous repeat associated gene silencing

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

transposon silencing (reviewed in Bender 2004). Similarly, a great body of work in *C. elegans* revealed that several factors involved in RNAi (mut-7 an RNaseD homolog, mut-14 an RNA helicase and mut-16) are required for transposon silencing (Sijen and Plasterk 2003).

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

Development: miRNAs regulating timing and patterning

One of the recent major discoveries in developmental biology was the finding that many higher organisms produce endogenous small RNAs that are essential for the regulation of genes, of which many are involved in development. The most notable of these are the miRNAs. miRNAs are characterized by their phylogenetic conservation across species and their involvement in basic biological processes of development, such as cell death and patterning. Typically, miRNAs are encoded by the genome as more or less imperfect inverted repeats as part of (much) larger processed transcripts, which are actively transported to the cytoplasm (Meister and Tuschl 2004). Depending on the degree of homology to the target mRNAs in the cytoplasm, these miRNAs guide the RISC complex for the cleavage or inhibition of translation of mRNAs homologous to the miRNA. Most miRNAs in plants studied so far have a (near) perfect match with their target mRNA in the open reading frame leading to mRNA cleavage (Rhoades et al. 2002). Translational inhibition by miRNA binding but not cleavage was so far only observed in one case {APETALA2, (Aukerman and Sakai 2003)}, while this is the main mode of action for miRNAs in animals (Ambros 2004).

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

By this mechanism miRNAs derived from the *lin-4* and *let-7* transcripts were shown to modulate the translation of their target genes *lin-14*, *lin-28* and *lin 41*, *hbl-1* respectively.

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

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

Plant viral RNA silencing suppressors interfere with miRNA action

As mentioned earlier, the expression of viral suppressors of RNA silencing in transgenic plants was shown to lead to strong developmental defects (Kasschau et al. 2003; Chapman et al. 2004; Dunoyer et al. 2004). Further research revealed that these proteins interfere with the action of miRNAs on the regulation of genes involved in plant development. For instance transgenic plants stably expressing HC-Pro over-accumulate miRNAs and show developmental defects (Mallory et al. 2002; Kasschau et al. 2003). Not only does HC-Pro change the accumulation levels of miRNAs, it also prevents their activity. It has been shown that HC-Pro could prevent the miRNA- guided cleavage of certain mRNAs and therefore cause a higher accumulation of these mRNAs. It appears that HC-Pro might affect the activity and the turnover of the miRNAs by interfering with one of the factors involved in their biogenesis or their cellular localisation.

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

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

The biochemistry of the RNA silencing machinery

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

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

Dicer

In plants, several molecular processes can generate small RNAs. Naturally occurring small RNAs can be: (1) miRNAs involved in gene regulation; (2) endogenous siRNAs (also known as repeat associated siRNAs); (3) transposon-derived, and (4) virus-derived siRNAs. DsRNAs can also be produced artificially by the expression of constructs arranged as inverted repeats which will result in the production of siRNAs processed from long dsRNA precursors and destruction of mRNAs with a homologous sequence (Smith et al. 2000). All siRNAs are products of cleavage of dsRNA by members of an RNase III-like enzyme family, first discovered in

Drosophila (Bernstein et al. 2001) and termed Dicer in animals or Dicer-like (DCL) in plants. Dicers are multi-domain proteins that typically contain one or more dsRNA binding domain(s), a DExH RNA helicase, a PIWI/ARGONAUTE/ZWILLE (PAZ) domain and two neighbouring RNase III-like domains. It has been reported that human Dicer works as an intra-molecular dimer of its two RNase III domains (Zhang et al. 2004). The products of the endonucleic cleavage by Dicer enzymes are RNA duplexes that have 5' phosphates and 2 nt 3' overhangs, mostly around 21 nt in size. It is interesting to note that while many animals only encode a single Dicer, *Drosophila* encodes two (Lee et al. 2004), and *Arabidopsis* has evolved four Dicer homologues (DCL1, DCL2, DCL3 and DCL4) (Schauer et al. 2002). It would appear that the multiple roles Dicer plays in the different branches of the RNA silencing in animals are divided over the different homologues in plants.

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

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

RISC

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

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

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

Concluding remarks

Taking into account all the information discussed in this chapter it is possible to conclude that RNA silencing has evolved as an efficient, general

way of counteracting the deleterious influence of foreign nucleic acids. However, it is very interesting that RNA silencing is not only involved in this defensive process, but also in very basic biological processes such as gene regulation and development. That is why this research has reached great momentum. Certainly, more surprising discoveries will be revealed.

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