

Review

RNA interference and its role in the regulation of eucaryotic gene expression^{*}

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Several years ago it was discovered that plant transformation with a transcribed sense transgene could shut down the expression of a homologous endogenous gene. Moreover, it was shown that the introduction into the cell of dsRNA (double-stranded RNA) containing nucleotide sequence complementary to an mRNA sequence causes selective degradation of the latter and thus silencing of a specific gene. This phenomenon, called RNA interference (RNAi) was demonstrated to be present in almost all eukaryotic organisms. RNAi is also capable of silencing transposons in germ line cells and fighting RNA virus infection. Enzymes involved in this process exhibit high homology across species. Some of these enzymes are involved in other cellular processes, for instance developmental timing, suggesting strong interconnections between RNAi and other metabolic pathways. RNAi is probably an ancient mechanism that evolved to protect eukaryotic cells against invasive forms of nucleic acids.

Regulation of eukaryotic gene expression occurs at different stages of protein biosynthesis: at the transcriptional, RNA processing and translational levels, and at the level of

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Abbreviations: NMD, nonsense mediated decay; PTGS, posttranscriptional gene silencing; RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; RISC, RNA-induced silencing complex; RdRp, RNA-dependent RNA polymerase; TGS, transcriptional gene silencing; VIGS, virus induced gene silencing.

protein maturation/degradation. The post-transcriptional level has recently attracted much attention because of the discovery of the phenomenon called RNA interference (RNAi) (Fire *et al.*, 1998). Soon after this discovery it became clear that earlier reports about post-transcriptional gene silencing (PTGS) in plants (Napoli *et al.*, 1990), gene quelling in fungi (Cogni *et al.*, 1996) and gene silencing with antisense RNA (Fire *et al.*, 1991) describe specific variants of RNAi observed in *Caenorhabditis elegans* (Fire *et al.*, 1998). Gene silencing was first described in plants. Already twelve years ago Napoli *et al.* (1990) discovered that the introduction of transcribed sense transgenes could shut down the expression of homologous endogenous genes. At that time PTGS was named co-suppression since it silenced both the transgene and the endogenous gene. In 1996 Cogni *et al.* (1996) described transgene-induced gene silencing (quelling) in *Neurospora crassa* using the carotenoid biosynthesis gene *albino-1* as a visual marker.

Since the discovery of RNAi an immense amount of data concerning its occurrence in different eukaryotic organisms has been accumulated: it has been found in trypanosomes (Ngo *et al.*, 1998), hydra (Lohmann *et al.*, 1999), fruit fly (Kennerdell & Carthew, 1998), zebrafish (Wargelius *et al.*, 1999), frog (Oelgeschlager *et al.*, 2000), mammals (Wianny & Zernicka-Goetz, 2000), fungi (Cogni *et al.*, 1996) and different plants (Waterhouse *et al.*, 1998).

This review describes three similar phenomena: RNAi, quelling and PTGS. It also presents recent opinions on RNAi mechanism.

RNAi

Over the last decade exogenous antisense RNA has been widely used to silence eukaryotic endogenous genes. The most widely accepted interpretation of these phe-

nomena was that antisense RNA is able to hybridise with the mRNA and to inhibit translation (Fire *et al.*, 1991). However, it was also discovered that injection of sense RNA into the cell leads to a similar effect (Guo & Kemphues, 1995). It was a big surprise when it was demonstrated that injection of double-stranded RNA had the most profound impact on gene silencing. The above described phenomenon, discovered in *Caenorhabditis elegans* by Craig C. Mello and his co-workers (Fire *et al.*, 1998), was called RNA interference (RNAi).

The most important observations in this work were as follows:

- 1) RNAi can spread within the individual and be transmitted to offspring

- 2) only a few molecules of dsRNA are sufficient to trigger RNAi suggesting the presence of catalytic and amplification components in the interference process

- 3) RNAi occurs at the posttranscriptional level since dsRNA fragments corresponding to promoter and intron sequences do not activate the RNAi pathway

- 4) RNAi is a highly specific process: the injection of dsRNA segments homologous to particular gene exons eliminates or decreases only the level of the corresponding mRNA

Further experiments carried out on *C. elegans* showed that the triggering of RNAi can be achieved *via* different ways: microinjection of dsRNA into the cytoplasm of the intestine (Fire *et al.*, 1998), feeding the worms on engineered *Escherichia coli* producing dsRNA against the target gene (Timmons & Fire, 1998) and even simply soaking the worms in a solution containing dsRNA (Tabara *et al.*, 1999).

Additional experiments conducted to clarify the genetic requirements for inheritance of RNAi in *C. elegans* revealed that the target locus is not needed for transmission of the interference effect to the offspring, suggesting a role in the transmission of an extragenic sequence-specific silencing factor (Grishok *et al.*, 2000).

Further experiments using classical genetic tools and the establishment of *in vitro* systems in *Drosophila* and human cells have greatly broadened our knowledge about RNAi mechanism and enzymes involved in this process.

HOW DOES IT WORK?

RNAi can be divided into four stages: (i) double-stranded RNA cleavage, (ii) silencing complex formation, (iii) silencing complex activation, and (iv) mRNA degradation. The first one includes ATP-dependent, processive dsRNA cleavage into double-stranded fragments 21 to 25 nucleotides long. They contain 5' phosphate and 3' hydroxyl termini, and two additional overhanging nucleotides on their 3' ends (Hamilton & Baulcombe, 1999; Elbashir *et al.*, 2001) (see Fig. 1). The gener-

ated fragments were called small interfering RNAs (siRNAs). It was shown that the already mentioned structural features of siRNAs are crucial for the next stages of the RNAi pathway, e.g. modification of the 5' end of the antisense strand inhibits siRNA activity, whereas blunt-ended siRNAs are very inefficient intermediates in further steps (Martinez *et al.*, 2002; Sijen *et al.*, 2001). Genetical experiments revealed that RNase III-like nuclease named Dicer is involved in the production of siRNAs (Ketting *et al.*, 2001). In the second step siRNAs are incorporated into a protein complex called RISC (RNA-induced silencing complex) which is inactive in this form to conduct RNAi (Nykänen *et al.* 2001). The third step involves unwinding of the siRNA duplex and remodelling of the complex to create an active form of RISC (Nykänen *et al.* 2001; Hammond *et al.*, 2000). The final step in-

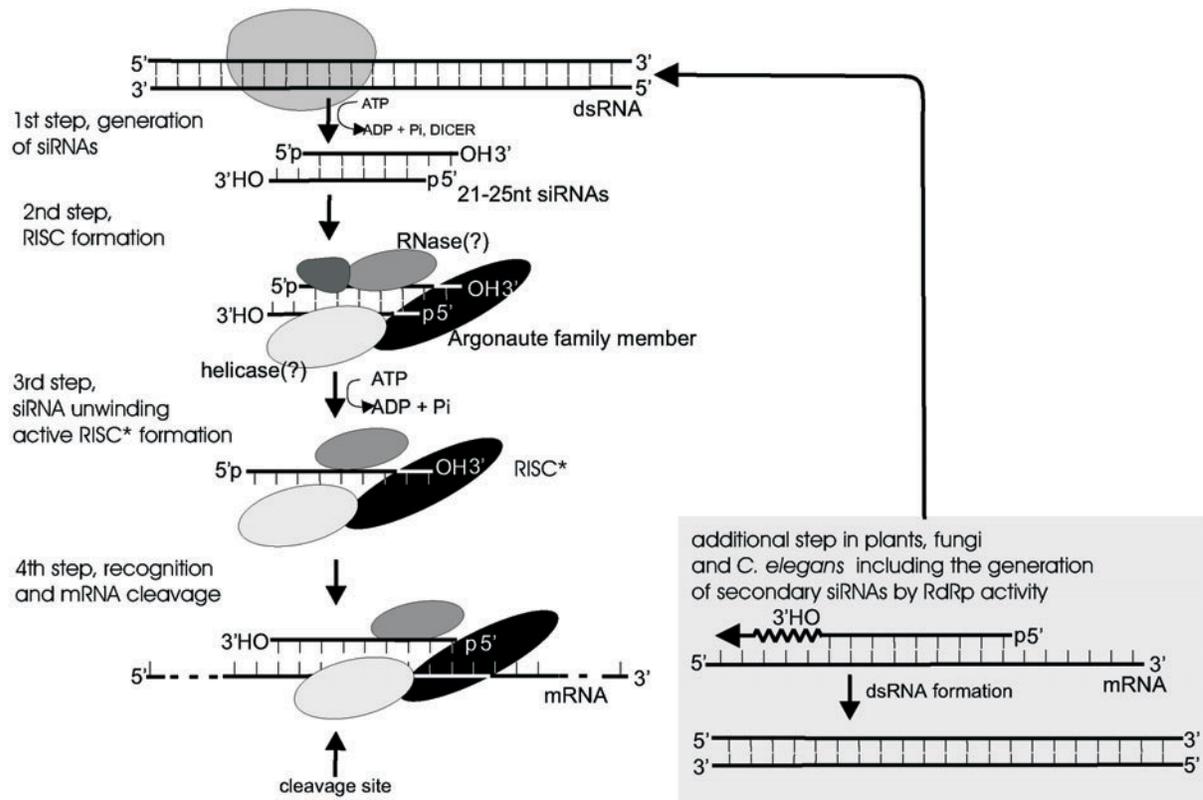


Figure 1. Schematic representation of four-step gene silencing pathway.

dsRNA, double-stranded RNA; siRNA, small interfering RNA; RISC, RNAi-induced silencing complex; RdRp, RNA-dependent RNA polymerase.

cludes the recognition and cleavage of mRNA complementary to the siRNA strand present in RISC (guide strand of siRNA) (Nykänen *et al.* 2001; Hutvagner & Zamore, 2002). In some organisms (*C. elegans*, *Arabidopsis thaliana*) an additional step in the RNAi pathway has been described involving a population of secondary siRNAs derived from the action of a cellular RNA-dependent RNA polymerase (RdRp). They are most likely generated during cyclic amplification in which RdRp is primed on the target mRNA template by existing siRNAs (Sijen *et al.*, 2001). Figure 1 shows the four stages of RNAi.

Screening for the genes encoding enzymes involved in PTGS, quelling and RNAi revealed strong homology between the proteins crucial in all three phenomena.

ENZYMES INVOLVED IN RNAi/PTGS/QUELLING

A large number of genes whose products are somehow implicated in RNA silencing have been identified in *C. elegans*, *D. melanogaster*, *Homo sapiens*, *Dictyostelium discoideum*, *N. crassa*, *Chlamydomonas reinhardtii* and *A. thaliana*. The identified genes encode dsRNases, RNA-dependent RNA polymerases, RNA-dependent helicases and proteins of unknown function.

The enzyme first discovered in *Drosophila* and called Dicer has dsRNase activity and is involved in the first step of RNA silencing – the production of siRNAs. It belongs to the RNase III-family. Dicer is ATP-dependent and contains several characteristic domains: an N-terminal helicase domain, a PAZ domain (a domain conservative throughout evolution found in Piwi/Argonaute/Zwille proteins in *Drosophila* and *Arabidopsis* and involved in developmental control), dual RNase III domains, and a double stranded RNA-binding domain (Bernstein *et al.*, 2001a). A homologue of this gene was found and characterised in *C. elegans-dcr-1* (Ketting *et al.*, 2001).

Evolutionarily conserved homologues of Dicer were also identified and tested in plants, fungi and mammals (Bernstein *et al.*, 2001 b). *dcr-1* mutants show a defect in RNAi and, additionally, phenotypic abnormalities connected with a role for Dcr-1 in a regulatory pathway in which small temporal RNA (stRNA let-7) and its target (lin-41) are involved (Ketting *et al.*, 2001).

During studies on the biochemistry of RNAi several proteins engaged in RISC formation were characterised. Chromatographic purification of crude extracts from *Drosophila* embryo lysate and human HeLa cells exhibiting RNAi activity revealed the presence of a specific RNAi nuclease, distinct from Dicer, that degrades target mRNAs. Active fractions also contain 25 nucleotide long RNA species homologous to the target mRNA (Hammond *et al.*, 2000). During further studies on *Drosophila* embryo lysate the formation of a protein complex on siRNAs was shown. A 360 kDa siRNP complex (RISC) was purified. It did not require ATP for the assemblage, contained ds-siRNAs but was not competent to cleave a target RNA. Another complex of 232 kDa was isolated from *Drosophila* embryo lysate that contained unwound siRNAs and all of the factors required for efficient, sequence-specific mRNA cleavage. This active complex has been termed RISC* (Nykänen *et al.*, 2001). These results suggest that an RNA helicase can participate in the transformation of RISC into RISC*. Hannon and co-workers have reported the isolation of a 500 kDa complex from *Drosophila* S2 cells that displays the target mRNA cleavage activity (Hammond *et al.*, 2000). Moreover, one protein was identified as a component of RISC – Argonaute 2 belonging to the Argonaute protein family. The authors give two possible explanations for the discrepancies between the estimated molecular sizes of RISC*: (1) the different chromatographic procedures used enable the isolation of larger precursor complexes and smaller active complexes, and (2) a dissociable cofactor is present in the larger RISC*, such

as an ATP-dependent RNA helicase (Nykänen *et al.*, 2001). As a result of the analysis of different protein fractions from HeLa cells extract, active human RISC was isolated. Its molecular size was estimated to be between 90 and 160 kDa. When the same methods of purification and analysis of RISC were used for *D. melanogaster* S2 cells cytoplasmic extract, the isolated complex was of similar molecular size as the human RISC. Two proteins of approximately 100 kDa each, both members of the Argonaute family, namely eIF2C1 and eIF2C2/GERp95, were identified in human RISC (Martinez *et al.*, 2002). Argonaute proteins form a distinct class of proteins containing PAZ and PIWI domains and, as earlier reports suggested, they take part in posttranscriptional gene silencing. Recently it has been shown that PPW-1 protein, a PAZ/PIWI protein is required for efficient RNAi in *C. elegans* germ line (Tijsterman *et al.*, 2002). The rat ortholog GERp95 of human eIF2C was identified as a component of the Golgi apparatus or endoplasmic reticulum (Cikaluk *et al.*, 1999). eIF2C was found as a component of SMN complex (Survival of Motor Neurons complex), which is a regulator of ribonucleoprotein assembly (Mourelatos *et al.*, 2002). The exact role of these proteins in RISC is still unclear. In human RISC it is still not possible to point to the candidate for the specific ribonuclease, probably present in limited amounts and thus escaping detection. The identification of other RISC components is still to come.

An intensive search for mutants defective in RNAi revealed an additional protein family involved in this process: RNA helicases. The *A. thaliana* mutant SDE3, defective in the production of an RNA helicase, is unable to carry out RNAi response mediated by a transgene. The *sde3* locus encodes a protein that was classified as RNA helicase from the Upf1p-like family. However, two subfamilies seem to emerge from this protein family: one including SDE3 and proteins from mouse, human and *Drosophila* and the other subfamily that

contains Upf1p and its homologue in *C. elegans*—SMG-2. The latter contains a conserved cysteine-rich region and multiple SQ doublets near the C-terminus that are absent in the SDE3 RNA helicase. From these data it seems unlikely that SDE3 is a functional homologue of Upf1p and SMG-2 (Dalmay *et al.*, 2001). The SMG-2 protein from *C. elegans* exhibits strong homology to the Upf1p RNA helicase from yeast and is involved in NMD (nonsense mediated decay) and in the persistence of silencing by RNAi (Domeier *et al.*, 2000). Despite the similarities in the sequences of SMG-2 and Upf1p, expression of Upf1p in *C. elegans* does not rescue *smg-2* mutants which can be explained by the fact that there is no RNAi pathway in *Saccharomyces cerevisiae* and some functions of Upf1 could be lost (Page *et al.*, 1999; Aravind *et al.*, 2000). The exact biochemical role of the SMG-2 helicase in RNAi is not clear. Another protein identified in *C. reinhardtii* called MUT 6 is a DEAH-box helicase family member and mutations in its gene activate transposons (Wu-Scharf *et al.*, 2000). It is speculated that MUT 6 unwinds dsRNA in some steps of RNAi. Since Dicer contains a helicase domain that is probably involved in early step(s) of dsRNAs formation, it can be assumed that the above described RNA helicases operate at a downstream step(s) of proper RISC formation containing antisense siRNA.

As a result of screening for genes involved in RNAi a family of proteins that exhibit the activity of RNA-dependent RNA polymerase was also identified. The *C. elegans* nuclear genome contains four members of this gene family: *ego-1*, *rrf-1*, *rrf-2* and *rrf-3*. Among these *rrf-1* was found as the gene coding for RdRp involved in RNAi: this enzyme is necessary to enrich the initial pool of siRNA directed against the target mRNA with the fraction of secondary siRNAs that cannot derive directly from the input dsRNA. The secondary siRNAs exhibited distinct polarity (the antisense strands were complementary to an upstream part of the initially targeted mRNA fragment)

suggesting a cyclic amplification process in which the 3' hydroxyl group of the antisense RNA serves as a primer (Sijen *et al.*, 2001) (see Fig. 1). Clear experiments confirm the role of RdRp proteins in RNAi in *A. thaliana* (SDE1/SGS2) (Dalmay *et al.*, 2000) and *N. crassa* (*qde-1*) (Cogni & Macino, 1999). However, no RdRp has been identified by homology in the genomes of either flies or humans. Two contradictory reports on RdRp activity have been published, one showing RdRp activity in *Drosophila* embryo lysates (Lipardi *et al.*, 2001) and the other showing that, for each dsRNA, the positions at which the target mRNA sequence was cleaved were precisely confined to the region spanned by the sequence of the dsRNA, with no cleavages observed upstream from the sequence present in the dsRNA (Zamore *et al.*, 2000). Also, a recent report concerning the mechanism of RNAi in human cells, using an *in vitro* system, convincingly contradicts the involvement of RdRp activity in this process: it was shown that modification of the 3' ends of antisense siRNA did not interfere with the reconstitution of RNA silencing (thus siRNA priming is not necessary to trigger gene silencing) while in an identical experiment in *C. elegans* such siRNAs were not functional (Martinez *et al.*, 2002). Obviously more experiments have to be carried out to solve this problem but it should be kept in mind that both experiments contradicting the role of RdRp in RNAi in *Drosophila* and human were done using *in vitro* systems. Table 1 summarizes the functions of different proteins involved in RNAi/PTGS and quelling.

QUELLING

Much earlier than RNAi discovery, transgene-induced gene silencing was observed in the filamentous fungus *N. crassa* (Pandit & Russo, 1992; Romano & Macino, 1992). The phenomenon was called quelling. It affected both transgenes and endogenous genes. As in

plants, methylation of DNA was observed during posttranscriptional gene silencing. However, it was not obligatory for this process, since mutants of *N. crassa* defective in DNA cytosine methylation still promoted quelling (Cogni *et al.*, 1996). Several genes coding for proteins involved in quelling were identified, namely *QDE-1*, *QDE-2* and *QDE-3*. *QDE-1* encodes a protein with homology to tomato RNA-dependent RNA polymerase (Cogni & Macino, 1999a), *QDE-3* encodes a DNA helicase whose role in quelling is unclear (Cogni & Macino, 2002). Mutants of *N. crassa* in this gene are not active in gene silencing. Catalanotto *et al.* (2002) reported that, like in RNAi, small sense and antisense RNAs are also involved in quelling. While the accumulation of these RNAs depends on the presence of functional *qde-1* and *qde-3* genes, the *qde-2* product is involved in a downstream step of quelling. Accordingly it was found that QDE-2 protein can participate in RISC-like complex formation. The QDE-2 protein (as AGO1 in plants and RDE-1 in *C. elegans*) contains PAZ and PIWI domains thus belongs to the Argonaute protein family. The finding that RdRps are involved in posttranscriptional gene silencing in *N. crassa*, *C. elegans* and plants suggests that these phenomena are mechanistically related.

POSTTRANSCRIPTIONAL GENE SILENCING (PTGS)

PTGS is regarded as a mechanistic variant of RNAi. This term defines both the co-suppression of an endogenous gene by the introduction of a transgene into the nuclear plant genome and the cellular defence mechanism induced by RNA virus infection.

As in the case of quelling in fungi and RNAi in animals, PTGS operates *via* siRNAs and their accumulation requires either transgene sense or antisense transcription or RNA virus replication (Hamilton & Baulcombe, 1999). PTGS induced locally spreads systemically to

Table 1. Proteins implicated in RNAi/PTGS/quelling phenomena

Protein	Organism	Function in RNAi/PTGS/quelling
Dicer	<i>Drosophila melanogaster</i>	Processing of long dsRNAs into siRNAs and stRNAs
Dicer	<i>Homo sapiens</i>	Processing of long dsRNAs into siRNAs
DCR-1	<i>Caenorhabditis elegans</i>	Processing of long dsRNAs into siRNAs and stRNAs
CARPEL FACTORY (CAF/Sin-1)	<i>Arabidopsis thaliana</i>	Processing of long dsRNAs into siRNAs
RDE-1	<i>Caenorhabditis elegans</i>	RNAi initiation
Argonaute-1 (Ago-1)	<i>Arabidopsis thaliana</i>	PTGS initiation
Argonaute-2 (Ago-2)	<i>Arabidopsis thaliana</i>	Component of RISC
eIF2C1	<i>Homo sapiens</i>	Component of RISC, translational initiation
eIF2C2/GERp95	<i>Homo sapiens</i>	Component of RISC, translational initiation, component of Golgi apparatus or endoplasmic reticulum, component of SMN complex
QDE-2	<i>Neurospora crassa</i>	Probable participation in RISC-like formation
SDE-3	<i>Arabidopsis thaliana</i>	RNA helicase, RNA unwinding
QDE-3	<i>Neurospora crassa</i>	DNA helicase, unclear role in quelling
SMG-2	<i>Caenorhabditis elegans</i>	Homologue of yeast Upf1p RNA helicase, persistence of silencing by RNAi
MUT-6	<i>Chlamydomonas reinhardtii</i>	DEAH-box helicase, RNA unwinding
EGO-1	<i>Caenorhabditis elegans</i>	RNA-dependent RNA polymerase, involved in germline RNAi
RRF-1	<i>Caenorhabditis elegans</i>	RNA-dependent RNA polymerase, involved in somatic RNAi
SDE-1/SGS-2	<i>Arabidopsis thaliana</i>	RNA-dependent RNA polymerase involved in PTGS
QDE-1	<i>Neurospora crassa</i>	RNA-dependent RNA polymerase involved in quelling
SGS-3	<i>Arabidopsis thaliana</i>	Not known

other tissues of the plant. Such a process requires the presence of a signal molecule transporting information about PTGS triggering. siRNAs were found in both the site of silencing initiation and in distal tissues. Although the nature of the signalling molecule remains unknown, siRNAs are small enough to move through plasmodesmata and/or via the vascular system. Consequently, at present siRNAs are the best candidates for signal molecules ensuring systemic spreading and specificity of PTGS. Systemic gene silencing in plants was investigated using grafting procedure. It

was shown that silencing was transmitted from silenced stocks to non-silenced scions. Transmission was unidirectional, always from stock to scion, transgene specific, locus independent and required a transcriptionally active transgene in the target scion (Palauqui *et al.*, 1997).

Interestingly, it was observed that PTGS in transgenic tobacco plants correlates with site-specific DNA methylation (Ingelbrecht *et al.*, 1994), which points to the link between PTGS and TGS (transcriptional gene silencing). In a brilliant work Jones *et al.* (1999)

demonstrated sequence-specific methylation of a transgene when PTGS was induced using RNA virus-based vectors carrying different parts of the transgene coding sequence or transgene promoter. If vectors containing the transcribed region were used, PTGS affected both the transgene and viral RNA levels. The methylation profile of the transgene was similar irrespective of the part of the transgene coding sequence used in the viral vector. However, when the promoter region was inserted into the viral vector, the transgene RNA level was reduced but the level of viral RNA was unchanged. This supports the idea that, in plants, dsRNA homologous to the promoter does not induce PTGS but rather TGS, which reduces transcription efficiency due to promoter methylation. Taking together the described experiments it can be concluded that gene specific methylation is tightly connected to PTGS and occurs directly *via* RNA–DNA interaction (RNA-directed DNA methylation – RdDm). In the case of promoter methylation the only interaction leading to it would be between RNA and the promoter DNA as described above. Although PTGS and TGS have been long considered as separate pathways, now it is clear that both could form alternative, nonexclusive ways to regulate gene expression (Vaucheret & Fagard, 2001).

Many papers have been devoted to virus induced gene silencing (VIGS) which can be classified as a specific variant of PTGS. It was proposed that the mechanism of virus related PTGS involves two separate stages: initiation and maintenance. The initiation of PTGS is dependent on the virus presence but maintenance is virus independent. The VIGS process, once initiated persists in the absence of the inducing virus (Ruiz *et al.*, 1998). In addition, it was found that some plant RNA viruses (potyviruses and cucumoviruses) encode proteins that suppress PTGS. This discovery confirmed earlier suggestions that plants use PTGS as an important part of virus protection system. One of them, Hc-protease (HcPro), encoded in the potato virus Y (PVY)

genome, is responsible for the lack of siRNAs. Moreover, grafting experiments showed that HcPro prevents the plant from responding to the mobile silencing signal but does not inhibit its production or sending of the signal. These data contradict the assumption of siRNAs as PTGS signal molecules: the mobile signal is produced at a step preceding accumulation of siRNAs (Mallory *et al.*, 2001). Another suppressor of gene silencing called 2b is encoded by cucumber mosaic virus (CMV) (Brigneti *et al.*, 1998). Protein 2b inhibits PTGS at an earlier step than HcPro: it affects silencing initiation. The third identified silencing suppressor was found in potato virus X (PVX). It is the p25 protein responsible for the movement of PVX within a plant. This suppressor inhibits the generation of the systemic silencing signal and prevents spreading of PTGS in the plant (Vionnet *et al.*, 2000). It is clear that plant RNA viruses developed different strategies to evade plant defence system. If a plant uses PTGS as the defence mechanism against RNA viruses, mutants defective in some proteins involved in gene silencing should be hypersensitive to viral infection. Indeed, this is the case: *A. thaliana* mutants *sgs3* (impaired in PTGS) display enhanced susceptibility to viruses, showing clearly that PTGS represents an antiviral defence mechanism (Mourrain *et al.*, 2000). Another *A. thaliana* mutant defective in PTGS (*sde1/sgs2* mutant impaired in cellular RdRp activity) was unable to evoke transgene mediated gene silencing but VIGS was efficiently induced. This result can be explained by the fact that viruses encode their own RNA-dependent RNA polymerase responsible for the generation of double-stranded RNA and do not require the cellular enzyme (Dalmay *et al.*, 2000).

RNAi/PTGS/QUELLING: SIMILARITIES AND DIFFERENCES

There are several lines of evidence that RNAi/PTGS/quelling are closely related phe-

nomena which share several common features. All of them are induced by dsRNA, occur posttranscriptionally in the cytoplasm and spread in the whole organism. In each case siRNAs are generated and then employed in specific mRNA degradation. As a result the expression of a target gene is inhibited.

As mentioned in the previous chapter, plant RNA virus replication can be strongly inhibited by PTGS. On the other hand, RNA virus protects itself by producing PTGC suppressors. Recent data show that an insect RNA virus also induces RNAi in *Drosophila* cells. Additionally it encodes an RNA silencing inhibitor (Li *et al.*, 2002). Since RNAi has also been observed in the case of mammalian cells it is possible that mammalian viruses can be also affected by RNAi (Alquist, 2002).

Enzymes crucial for posttranscriptional gene silencing with high sequence homology have been identified in the case of RNAi, quelling and PTGS (see the chapter in this review devoted to enzymes), suggesting strong similarities in the biochemical mechanisms of these processes. These enzymes have been proposed to function at the steps in the silencing pathway that are common to all three phenomena. However, the role of RdRp in all these processes seems to be unclear: its role in the signal amplification has been proved in the case of PTGS, VIGS, quelling and in some cases of RNAi – namely in *C. elegans*. However, RdRp is most likely redundant in the case of RNAi in *Drosophila* and mammals. In plants PTGS seems to be tightly connected with TGS that occurs *via de novo* DNA methylation. On the other hand, RNAi does not lead to methylation of silenced genes. For years it was thought that *Drosophila* and *C. elegans* nuclear DNA is not methylated. Although this is probably true in the case of *C. elegans* (Wassenegger, 2002), recent reports present data that nuclear DNA of *D. melanogaster* is methylated, however to a lesser extent than human and plant DNA (Lyko, 2002). It remains to be established

whether DNA methylation in the fruit fly is connected to RNAi or not. PTGS/quelling/RNAi can be induced in plants, fungi and *C. elegans* by the delivery of long dsRNAs that function as substrates for Dicer. Interestingly such a procedure can not be applied for mammalian cells. The delivery of long dsRNAs into mammalian cells results in a general blockage of protein synthesis because of the interferon response and apoptosis. The only example of successful silencing by delivery of long dsRNAs was observed for early embryos suggesting that the cells from this stage of development are incapable of interferon response (Wianny & Zernicka-Goetz, 2000). To overcome these difficulties, short dsRNAs are used to trigger RNAi in mammalian cells (Martinez *et al.*, 2002).

The discovery of RNAi/PTGS and quelling opens up completely new possibilities of research of the functioning of particular animal, plant and fungi genes without the necessity of altering the genome structure (Fjose *et al.*, 2001; Wojtkowiak *et al.*, 2002).

BIOLOGICAL ROLE OF RNAi/PTGS/QUELLING

The data collected until now suggest that RNAi/PTGS/quelling derive from an ancestral mechanism that controlled nucleic acids invasions. In different eukaryotic kingdoms RNAi/PTGS/quelling are responsible for silencing of transposons, viruses and/or transgenes. These assumptions were supported by experiments showing that silent transposons are reactivated in PTGS or RNAi impaired *Arabidopsis* (Hirochika *et al.*, 2000), *C. elegans* (Ketting *et al.*, 1999) and *Chlamydomonas* mutants (Wu-Scharf *et al.*, 2000).

Additionally, the same enzymes as in RNAi/PTGS are used in several other cellular processes. In *Arabidopsis* mutations in the Dicer-like gene have dramatic developmental consequences suggesting an important, yet

undiscovered role of small RNAs in developmental processes (Hutvagner *et al.*, 2001). In the case of *C. elegans* Grishok *et al.* (2001) have found that Dicer is required for both *lin-4* and *lin-7* maturation and thus functions in developmental timing. Also in *C. elegans* seven genes *smg 1-7* are responsible for non-sense-mediated mRNA decay (NMD). Three of these genes are required for persistence of RNAi (Mango, 2001). Although the interconnections between gene silencing and other cellular processes seem to be well proved, it still remains to be clarified exactly how all of these metabolic pathways interplay and influence each other.

RNAi/PTGS/quelling represent processes whose biochemistry was completely unknown until late 90-ties. In all of these processes small RNAs (siRNAs) are involved. Generally, small untranslated RNAs exhibit different functions in the eukaryotic cell (tRNAs, snRNAs, snoRNAs, stRNAs – small temporal RNAs). It seems now that a large number of additional micro-RNAs (miRNAs) exist in eukaryotes performing a multitude of functions (Grosshans & Slack, 2002). Challenges concerning the still undiscovered functions of small RNAs, beside their functions in gene silencing and developmental timing, are ahead of us and assure fascinating scientific adventure in the future.

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