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REVIEWS

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# Gene Silencing in Plants

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**Abstract**—The review considers the cytoplasmic silencing of viral RNAs by short RNAs and the silencing of endogenous mRNAs by specific short double-stranded microRNAs. The role of some cell factors such as Dicer, Argonaute, RNA-dependent RNA polymerase, RNA polymerase IV, and pectin methylesterase is described in detail. The role of viral proteins and nucleic acids in silencing suppression and possible biotechnological applications of this mechanism are discussed.

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*Key words*: Argonaute, Dicer, pectin methylesterase, RNA polymerase IV, RNA-dependent RNA polymerase, transcriptome, gene silencing

## INTRODUCTION

Gene silencing (GS) has been the subject of wide discussion in recent years. GS is a nucleotide-specific mechanism regulating the eukaryotic transcriptome. Keen interest in GS is emphasized by the fact that, in 2006, Andrew Z. Fire and Craig C. Mello were awarded the Nobel Prize in medicine for the discovery of RNA interference in nematode *Caenorhabditis elegans*.

Yet the story began far earlier. As Baulcombe [1] noted, it was observed as early as in 1928 that, in some tobacco plants infected with the tobacco ring spot virus, upper noninoculated leaves did not contain the virus and were resistant to infection. This is a typical manifestation of GS. Such patterns of virus infection were observed in many works and described in detail in classic Mathews' monograph [2]. Systematic studies of GS did not start until construction of transgenic plants became available. Unexpectedly, an undesirable phenomenon was observed. Transgene mRNAs were undetectable in some plant cell lines carrying an alien or homologous gene integrated in the genome. In 1990, Jorgensen and colleagues [3] reported that integration of an additional endogenous nitrate reductase gene (*Nia*) reduced the *Nia* mRNA content in the cell by a factor of 50, rather than increasing the transcription level as was expected for a higher gene dosage. The terms cosuppression and GS were proposed at that time. In 1992, Dougherty and colleagues showed that introduction of a nontranslated potivirus fragment into the tobacco genome not only results in plant resistance against the virus [4] but is also accompanied by degradation of virus RNA [5]. An important discovery was made several years after: RNA degradation in GS leads to the accumulation of specific short (21–25 nt) double-stranded RNAs (dsRNAs) [6], which are now known as short/small interfering RNAs (siRNAs). This finding marked the start of systematic studies of GS in plants. GS includes transcriptional suppression, destabilization of mRNA, and inhibition of its translation [7]. In each of these processes, dsRNA or hairpin mRNA is generated, long dsRNA or hairpin mRNA is cleaved into short dsRNAs by Dicer, and target mRNA or viral RNA is degraded or its translation suppressed by a specific RNA-induced silencing complex (RISC) (Tables 1, 2).

This review considers the main mechanisms of GS and the cell and viral factors involved.

## GS AND THE PLANT TRANSCRIPTOME

### Short dsRNAs (siRNA, miRNA, and ta-siRNA) As Main Participants in GS

At least three mechanisms of GS are known for plants: cytoplasmic RNA silencing accompanied by siRNA production, endogenous mRNA silencing associated with specific short double-stranded microRNA (miRNA), and DNA methylation and transcriptional suppression.

**Cytoplasmic RNA silencing** functions mostly in transgenic and virus-infected plants and is associated with siRNA production. It is this mechanism that is responsible for transgene cosuppression (see above). The scenario of viral RNA silencing is best known (Fig. 1). The cell infected with viral genomic RNA

**Table 1.** GS glossary

| | |
|---|---|
| **Argonaute (Ago)** is a member of a group of proteins containing the PAZ (RNA-binding) and PIWI domains and is similar to RNase H. | **RNA-dependent RNA polymerase (RdRp, or RDR)** is a cytoplasmic or viral RNA polymerase that amplifies the GS signal. Six putative RDRs, including best-studied RDR6, are known for *A. thaliana*. |
| **Dicer (Dicer, DCR, in animals or Dicer-like, DCL, in plants)** is a multidomain enzyme that belongs to the RNase III family and processes dsRNA or structured hairpin precursor RNA into siRNA and miRNA, respectively. | **RNA-dependent DNA methylation** is a GS event that occurs in the nucleus and consists in cytosine methylation in the region of RNA/DNA complementarity. |
| **Transgene-induced GS** is GS caused by a nonreplicating nucleic acid. | **HEN1 RNA methyltransferase** modifies the 3'-terminal nucleotide of miRNA and siRNA in *A. thaliana* cells. |
| **RNA-induced silencing complex (RISC)** is an Ago-containing RNP complex wherein one siRNA or miRNA strand interacts with the mRNA target to cause its degradation or inhibit its translation. | **RNA polymerase IV** is a cell polymerase that is responsible for siRNA amplification and nuclear DNA methylation. |
| **Virus-induced GS** is GS induced by a replicating nucleic acid. | **Systemic and local GS** is GS spreading from the induction site over the affected leaf (local GS) or to upper intact leaves (systemic GS). |
| **Small/short interfering RNAs (siRNAs)** are 21- to 25-bp dsRNAs with a 2-nt 3'-overhang that result from cleavage of long dsRNAs or structured ssRNAs. | **Slicer** is an activity of the Ago PIWI domain that cleaves mRNA in the region of its complementary interaction with siRNA or miRNA in the RISC. |
| **MicroRNAs** are 21- to 25-bp dsRNAs with a 2-nt 3'-overhang that result from cleavage of cell hairpin precursor RNAs (pre-miRNAs). | **GS suppressor** is a cell or viral protein that blocks induction, amplification, or spreading of GS. |
| **Pectin methylesterase** is a plant cell-wall stress protein involved in GS. | **Spreading of RNA targeting (SRT)** is a local expansion of target RNA degradation to the flanking regions, noncomplementary to the inducing nucleic acid. |
| **Posttranscriptional GS** includes the silencing of a sense transcript (S-PTGS) and an inverted repeat-containing transgene (IR-PTGS). | ***Trans*-acting siRNAs (ta-siRNAs)** are siRNAs that complementarily interact with the target mRNAs, as miRNAs do, but their targets originate from different loci. |
| | **Gene silencing (GS)** is a term accepted in plant biotechnology for the nucleotide-specific mechanism regulating the eukaryotic transcriptome. |

produces viral RNA-dependent RNA polymerase (RdRp), which synthesizes replicative dsRNA. This RNA provides a substrate for RNase III known as Dicer, which cleaves (choppes) dsRNA. At the next step, the so-called guide strand of the resulting siRNA is incorporated in the RISC and, as its component, complementarily interacts with the target viral RNA. The other (passenger) strand is removed from the RISC and is degraded. The specific protein Argonaute (Ago), which is the main and, possibly, only protein component of the RISC [8], cleaves the target RNA

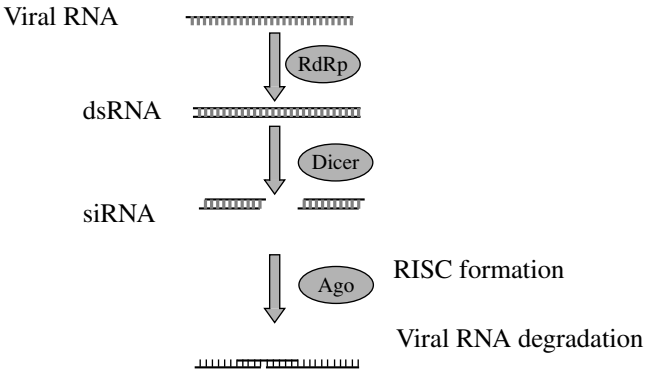


**Fig. 1.** Simplified scheme of viral RNA silencing.

(Fig. 1). It was believed until recently that the main source of siRNA is viral replicative dsRNA, which is synthesized by viral RdRp on the template of the single-stranded viral genome. Surprisingly, it was found that only 20% of siRNA is produced from dsRNA, while the other 80% originate from hairpin regions of viral genomic single-stranded RNA (ssRNA) [9, 10], suggesting alternative mechanisms for siRNA generation.

**Endogenous mRNA silencing caused by miRNA.** In this case, translation of cell mRNA is suppressed by its complementary pairing with miRNA. This interaction induces degradation of the target mRNA or directly blocks translation. It is known that miRNAs are produced from cell untranslated transcripts of 120–150 nt. Like siRNAs, miRNAs are dsRNAs of 21–24 nt and are produced by Dicer from a miRNA precursor. Short RNAs of this type were first identified in *C. elegans* [11].

**DNA methylation and transcriptional suppression.** Evidence for this mechanism was first obtained with transgenic plants allowing transcription of viroid cDNA. Elevated transcription triggers methylation of viroid cDNA. Methylation of transgenic cDNA and a transcription promoter was shown to involve specific siRNAs and a modified histone [12]. It is thought that

**Table 2.** Cell factors involved in GS

| Protein | Domain, motif | Biochemical activity | Metabolic strand |
|---|---|---|---|
| Ago1 | PAZ, PIWI | Cleavage of mRNA in the region of its complementary interaction with siRNA or miRNA in the RISC | miRNA, PTGS, ta-siRNA, Chromatin* |
| Ago4 | PAZ, PIWI | | Chromatin |
| DCL1 | RNase III, dsRNA-binding, chelicase with the DEAD box, PAZ, DUF283 | miRNA synthesis | miRNA, siRNA |
| DCL2 | RNase III, dsRNA-binding | Synthesis of 21-nt siRNA | Viral dsRNA, siRNA |
| DCL3 | RNase III, dsRNA-binding | Synthesis of 24-nt siRNA | Chromatin |
| DCL4 | RNase III, chelicase, dsRNA-binding, PAZ | Synthesis of 21-nt siRNA | ta-siRNA, PTGS |
| HEN1 | dsRNA-binding, S-adenosyl-binding | RNA methyltransferase | All levels of silencing |
| HST (HASTY) | RanGTP-binding | Homolog of animal exportin | miRNA |
| HYL1 | dsRNA-binding | dsRNA binding | miRNA |
| MET1 | Cytosine-DNA methyltransferase | Cytosine-DNA methyltransferase | Chromatin |
| NRPD1a | RNA polymerase | RNA polymerase | Chromatin, siRNA |
| NRPD1b | RNA polymerase | RNA polymerase | Chromatin |
| NRPD2 | RNA polymerase | RNA polymerase | Chromatin |
| RDR6 | RdRp | RdRp | PTGS |
| SGS3 | Coiled-coil function, Zn binding | | SRT, ta-siRNA, siRNA, PTGS |

* Chromatin modification.

RNA silencing at the chromatin level protects the cell genome against transposons.

## Dicer

Dicer was first identified in *Drosophila* cells [13]. A similar plant enzyme is known as Dicer-like (DCL). The Dicer gene occurs in one copy in human, mouse, and nematode cells and in two copies in insect (*Drosophila melanogaster*) and fungal cells. Plant DCL genes are more numerous: there are four DCL genes in the *Arabidopsis thaliana* genome (Fig. 2a), five in the poplar *Populus trichocarpa* genome, and six in rice *Oryza sativa* genome [14]. The DCL function was determined with *A. thaliana.* All DCLs are divided into four types: DCL1 is involved in miRNA biogenesis, DCL2 is involved in siRNA production from viral dsRNA, DCL3 is responsible for chromatin modification, and DCL4 plays a role in producing *trans*-acting siRNA (ta-siRNA). *DCL1, DCL2, DCL3*, and *DCL4* each occur in a single copy in the genome. *DCL1* mutations are lethal, while inactivation of *DCL2–DCL4* only slightly affects the viability in *A. thaliana* [15]. The difference in Dicer gene set between mammals and plants presumably reflects different strategies utilized to maintain the cell transcriptome. One Dicer gene is sufficient for the purpose in mammals, which have humoral and cell immunity and produce interferons, while at least four Dicer genes are necessary in plants.

DCL includes a nuclear localization signal (NLS), two-domain helicase (with domains DExD and C), the PAZ (**P**IWI, **A**rgonaute, **Z**wille) domain, two-domain RNase III (domains a and b), and two-domain dsRNA-binding sequence (Fig. 2b). In addition, Dicer has the Duf283 domain with an unknown function in all plants, animals, and fungi examined. DCL2 initially contacts dsRNA via its dsRNA-binding sequence. The PAZ domain ensures specific interactions with two protruding (nonpaired) 3'-terminal nucleotides, while the two RNase III domains form a kind of a dimer. The resulting "dimeric" RNase III cleaves each strand of dsRNA at two sites located 2 nt apart to yield siRNA with a 2-nt overhang at the 3' end and monophosphate at the 5' end.

In animals, miRNAs and siRNAs are produced by different RNase III enzymes: Drosha and Dicer, respectively. Drosha acts together with dsRNA-binding proteins (DGCR8 or Pasha) to process the precursor miRNA primary transcript (pri-miRNA). Drosha shortens pri-miRNA to produce pre-miRNA. Expor-

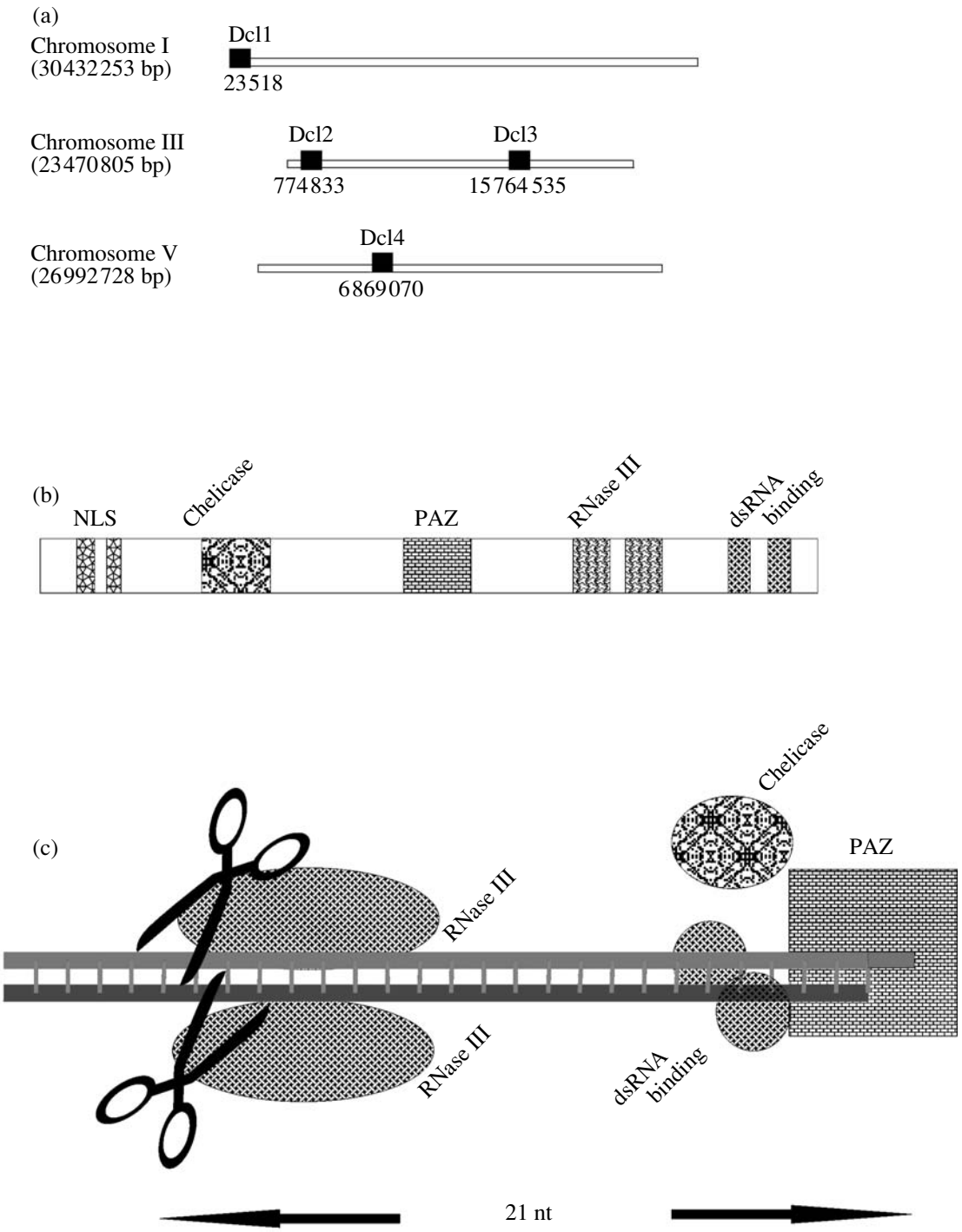


**Fig. 2.** Plant Dicer: (a) positions of the DCL1, DCL2, DCL3, and DCL4 genes on *A. thaliana* chromosomes; (b) domain structure of Dicer; and (c) model of siRNA generation.

tin-5 transfers pre-miRNA into the cytoplasm, where Dicer completes miRNA maturation.

In plants, miRNA generation by DCL1 proceeds via three steps [16]. First, pri-miRNA is synthesized by RNA polymerase II and converted into pre-miRNA. The second step is generation of a shorter precursor (short pre-miRNA). The third step yields mature miRNA (Fig. 3).

In animals, the transfer of mature miRNA or the miRNA/DCL1 complex is controlled by exportin-5, which is involved in nucleocytoplasmic transport of pre-miRNA and tRNA. HASTY (HST), an exportin-5

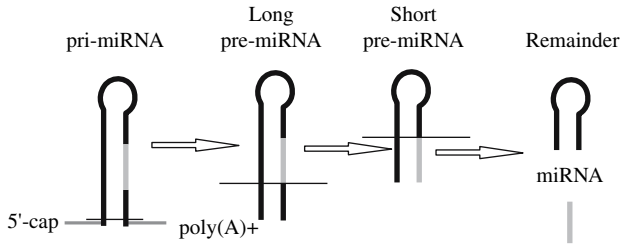


**Fig. 3.** Steps of miRNA maturation in plants.

homolog, was identified in *A. thaliana* [17]. HASTY inactivation suppresses miRNA accumulation but has no effect on tRNA or endogenous siRNA transport [18]. All maturation steps involve the dsRNA-binding protein HYL1, which is capable of complexation with DCL1 and Ago [19]. HYL1 harbors two domains with the dsRNA-binding sequence.

Methylases play an important role in the miRNA and siRNA functions. Short RNAs are subject to 3'-tailing with 1–30 uridine residues, which act as a degradation signal. In *A. thaliana*, methyltransferase HEN1 (**H**UA **EN**HANCER 1) methylates 2'-OH of the 3'-terminal nucleotide in miRNA and siRNA [20]. The *hen1* mutant has a lower miRNA content. It is thought that HEN1 protects short RNAs from tailing and subsequent degradation. Interestingly, the most effective viral suppressors of GS interact with short RNAs to block their methylation (see below) [21].

Recent studies identified another class of short RNAs that possess properties of both siRNA and miRNA [22]. These endogenous ta-siRNAs are encoded by genes transcribed by RNA polymerase II, are functionally similar to miRNA, and direct RISC-dependent degradation of the target mRNAs originating from loci differing from the ta-siRNA loci. Generation of pre-ta-siRNA requires DCL1, RDR6, and a cell suppressor of GS (SGS3). The target RNA is degraded by the RISC with the involvement of DCL4 and Ago (Fig. 4).

The identification of miRNA and ta-siRNA was followed by a search for their targets. The search is relatively simple in plants because, in contrast to animal analogs, most plant miRNAs are highly homologous to their target mRNAs. The list of mRNAs with known miRNA partners is continuously increasing (Table 3). It should be noted that miRNA-dependent regulation of genome expression controls, in particular, the production of its key enzyme, DCL1. It was found that miR162 complementarily interacts with the DCL1 mRNA, causing its degradation. Thus, DCL1 synthesis is regulated via a negative feedback [23].

Another class of short RNAs includes natural antisense transcript siRNAs (nat-siRNAs). Such RNAs are found when two neighboring genes occur in different DNA strands (*cis*-antisense genes) in a plant genome and code for overlapping transcripts. The overlap is processed by DCL2, RDR6, SGS3, and NRPD1a to yield 21-nt nat-siRNAs, which direct degradation of one of the parental transcripts [24]. Since

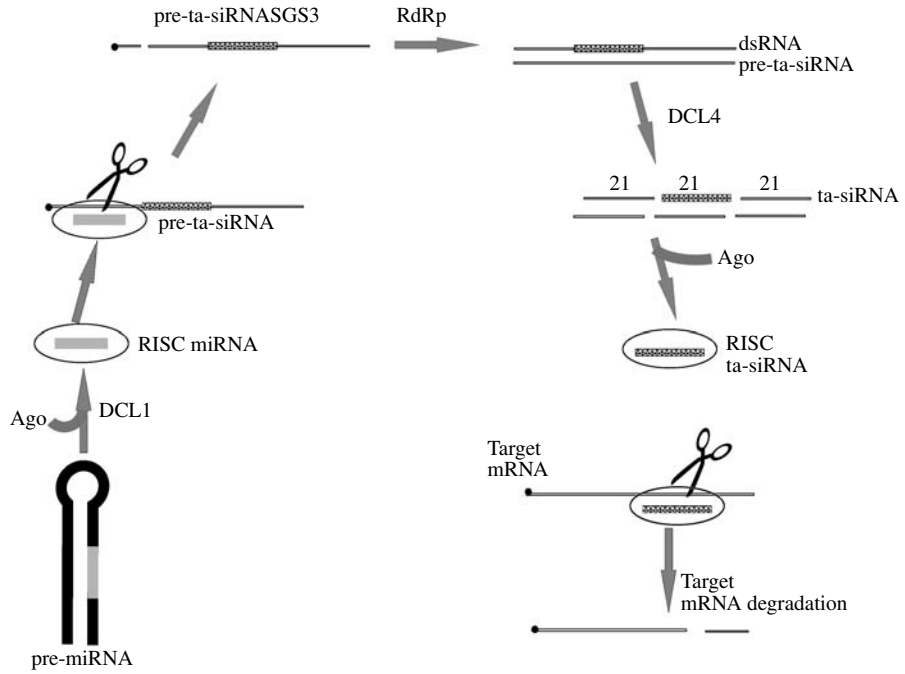


**Fig. 4.** Model of ta-siRNA function in *A. thaliana.*

**Table 3.** Short RNAs (miRNAs and ta-siRNAs) and their targets in *A. thaliana*

| Short RNA | Target mRNA | Target function |
|---|---|---|
| miRNA | | |
| miR156 | SBP | Transcription factor |
| miR159 | MYB | ″ |
| miR319 | TCP | ″ |
| miR160 | ARF | ″ |
| miR161 | PPR | Unknown |
| miR162 | DCL | miRNA metabolism |
| miR163 | SAMT | Metabolism |
| miR164 | NAC | Transcription factor |
| miR166 | HD-ZIPIII | ″ |
| miR167 | ARF | ″ |
| miR168 | AGO1 | miRNA metabolism |
| miR169 | HAP | Transcription factor |
| miR171 | SCR | ″ |
| miR172 | AP2 | ″ |
| miR173 | TAS1, TAS2 | ta-siRNA biogenesis |
| miR390 | TAS3 | ta-siRNA biogenesis |
| miR393 | TIRI/F box | Hormonal signal |
| | bHLH | Transcription factor |
| miR394 | F box | Hormonal signal |
| miR395 | ATPS | Metabolism |
| | AST | Metabolism |
| miR396 | GRF | Transcription factor |
| miR397 | Laccase/Cu-oxidase | Metabolism |
| miR398 | CSD | Stress response |
| | CytC oxidase | Metabolism |
| miR399 | E2-UBC | Ubiquitin conjugation |
| miR447 | 2PGK | Metabolism |
| miR403 | AGO2 | miRNA metabolism |
| ta-siRNA | | |
| TAS1 | Unidentified | Unknown |
| TAS2 | PPR | Unknown |
| TAS3 | ARF | Transcription factor |

numerous *cis*-antisense genes have been revealed in plants and animals, GS of this type will be studied in detail in the nearest future.

## Argonaute and the RISC

Short RNAs produced by Dicer are incorporated in the RISC, whose main component is Ago. There are several Ago homologs in most eukaryotes: 8 in human, 10 in *A. thaliana*, 5 in *D. melanogaster*, and 27 in *C. elegans*, while yeasts have only a single copy of the Ago gene. It is thought that different Ago proteins determine different RISC functions. The PIWI and PAZ domains are essential components of all Ago proteins (Fig. 5).

Only one Ago, a slicer cleaving mRNA, is known for animals. The slicer activity is determined by the Asp-Asp-His (DDH) motif of the PIWI domain; similar motifs occur in two nucleases, RNase H and transposase. While animal miRNAs are responsible for translational suppression of mRNA, most plant miRNAs complementarily (usually, without a mismatch) interact with mRNA in the RISC, which eventually leads to mRNA cleavage. Of the ten *A. thaliana* Ago proteins, Ago1 is the best candidate for a slicer [25]. Recent experiments confirmed, indeed, that the PIWI domain of *A. thaliana* Ago1 acts as a slicer and is capable of cleaving model mRNA in the presence of miRNA without other protein factors [8].

As is the case with DCL1, Ago1 production is regulated via a feedback by another short miRNA, miR168 (Table 3) [26]. Ago1 regulation is thought to occur at two levels: concerted transcription of the Ago1 and miR168 genes and stabilization of miR168 accumulation via Ago1 synthesis.

## RNA-Dependent RNA Polymerase

RdRp, which synthesizes RNA on an RNA template, was identified in phages more than 40 years ago. Further studies revealed RdRp in RNA phytoviruses. The plant genome also codes for RdRp, although the initial finding was left unexplained. It is clear, however, that RdRp is a stress protein whose activity greatly increases in leaf wounding and virus infection. The RdRp gene was cloned and sequenced from tomato. It was initially believed that RdRp is involved in phytovirus replication, but low specificity of the enzyme was discouraging. The discovery of GS revived interest in RdRp. It was assumed that, in eukaryotic cells, RdRp is important not only for suppressing an exogenous nucleic acid but also for the regulation of the cell genome. The role of cell RdRp in virus infection is unclear, since siRNAs are produced mostly of viral ssRNA in this case [9, 10]. Yet RdRp is certainly involved in three GS events: DNA methylation with the subsequent formation of heterochromatin, systemic GS, and the so-called spreading of RNA targeting (SRT) [27]. SRT consists in local expansion of target RNA degradation to the flanking regions, noncomplementary to the inducing nucleic acid. This process is impossible without RdRp and, consequently, is regarded as indicative of RdRp. It was found with the green fluorescent protein (GFP) mRNA used as a target that SRT extends over 332 nt in the 5' or 3' direction and requires active transcription of the transgene and DNA methylation. SRT was observed in animals (*C. elegans* and *D. melanogaster*)

but, in contrast to plant SRT, extends over shorter distances and only in the 3'–5' direction.

### Pectin Methylesterase As a GS Inductor in Plants

Another stress protein playing an important role in plant GS is pectin methylesterase, a cell wall enzyme that is involved in growth and morphogenesis [29]. The primary cell wall of plants contains cellulose, hemicellulose, and pectin, which consists of branched polygalacturonic acid. Pectin components are synthesized in the Golgi system, and up to 80% of polygalacturonic acid is methylated prior to incorporation in the cell wall, where pectin methylesterase demethylates polygalacturonic acid to produce methanol. Interestingly, methanol is one of the main volatile organic components of the atmosphere, where its content is about $10^9$ tons.

The plant genome harbors several pectin methylesterase genes. The enzyme is synthesized as a precursor. When a leaf is mechanically wounded, transcription of the pectin methylesterase genes dramatically increases, the mature enzyme accumulates in the cell wall, and its enzymatic activity grows higher. The plant pectin methylesterase precursor has a leader region, which varies in size from several tens to several hundreds of amino acid residues [30]. Secretion of pectin methylesterase into the cell wall is controlled by the transmembrane domain of the proenzyme leader; deletion of this domain completely abolishes the transport of intracellular pectin methylesterase to the cell surface. Recent studies implicated pectin methylesterase in the cell control of virus infection. The enzyme proved to interact with the transport protein of the tobacco mosaic virus [31] and plays a role in the spreading of virus infection in the plant [31–33]. Yet the role of pectin methylesterase is not restricted to these functions. Activation of pectin methylesterase in the cell wall silences the tobacco mosaic virus RNA. Intense degradation of viral RNA leads to a considerable accumulation of 21-nt siRNA. Moreover, GS induced by pectin methylesterase affects transgenic RNA as well, which is accompanied by suppression of DCL1 transfer into the nucleus. Eventually, this leads to a rapid accumulation of siRNA in the cytoplasm. The significance of DCL1 for the plant cell was verified experimentally. DCL1 is involved not only in miRNA generation, but also in the regulation of cytoplasmic RNA accumulation and cannot be completely substituted by DCL2, DCL3, and DCL4 [15]. Both pri-miRNAs and long cytoplasmic RNAs (viral or cell) contain hairpins, which serve as a substrate for DCL1. Introduced in the cell, pri-miR171 sequesters DCL1 and dramatically increases the accumulation of viral RNA [34].

Located at the cell boundary and capable of perceiving external signals, pectin methyltransferase acts as a cell transcriptome guardian that generates a signal (possibly, methanol) about foreign nucleic acid entering the cell and, consequently, mobilizes the cell and induces GS.

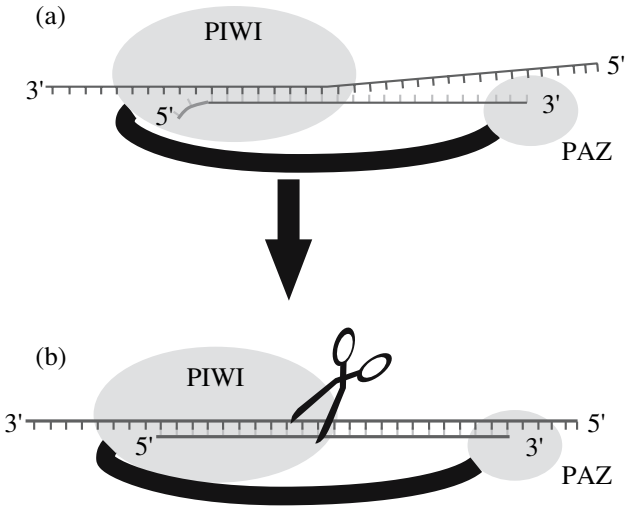


**Fig. 5.** Model of RISC functioning: (a) initial complementary interactions of miRNA with the target mRNA and (b) complete pairing and cleavage of the target mRNA.

### Systemic GS

Studies of cosuppression of the nitrate reductase gene (*Nia*) showed that *Nia* silencing, causing chlorosis (discoloration) of leaves, can spread from the induction site over the affected leaf (local GS) and to upper intact leaves (systemic GS) [35]. To identify the signal ensuring systemic GS (Table 4), a chimeric plant was constructed so that its lower part (stock) was from a transgenic plant with silenced *Nia* and the upper part (graft) was from an intact plant [35–38]. Chlorosis usually spread over the graft two weeks after grafting. This effect was also observed with other transgenes (e.g., *GFP*) and other experimental systems, based on agrobacterial infection or ballistic transformation. A similar phenomenon was observed in *C. elegans.* The highest efficiency of GS induction was reported for transcribed cDNA, dsRNA, and siRNA. There is still no explanation for the experimental finding that specific siRNA causes the production of nonhomologous siRNAs corresponding to the 3'- and 5'-flanking regions of the target RNA. Yet GS can arise spontaneously, without introduction of an exogenous nucleic acid (Table 4). Surprisingly, transgenic plants are capable of spontaneous GS, which depends, to a great extent, on the level of transgene transcription. On direct evidence, GS is induced and spreads over the plant when the transcription level reaches a certain threshold [39]. It is even more surprising that promoterless cDNA, which is incapable of ensuring mRNA synthesis, also induces GS and is

**Table 4.** Inductors of systemic GS

| Systemic signal inductor in transgenic plants | Relative strength* | Comment | Reference |
|---|---|---|---|
| Spontaneous induction | +(+) | Systemic GS of *Nia* in 5–45% of plants after grafting | 36 |
| Transcribed cDNA (35S–Nia2) | +++ | Systemic GS of *Nia* after ballistic transformation | 37 |
| Nonntranscribed cDNA (Nia2) | +(+) | | |
| cDNA (35S–TET$^R$) | +++ | Systemic GS of the tetracycline resistance gene (TET$^R$) after ballistic transformation | 38 |
| ssRNA | + | | |
| dsRNA | +++ | | |
| siRNA | +++ | | |

* Systemic GS was induced in (+++) 50% or more, (++) 20–50%, or (+) less than 20% of transgenic plants.

more effective than ssRNA. Obviously, any nucleic acid homologous to the transgene can induce systemic GS. The situation is similar to that in oncology at the time of Paul Erlich, who said that everything, even a lick of a stick, can induce cancer.

As viruses, local GS spreads through plasmadesmata over the distance of 10–15 cells from the induction site [40]. The spreading involves RdRp, whose best substrates are aberrant uncapped RNAs [41]. Systemic GS also requires RdRp, but the enzyme plays a role in its induction but not in signal spreading through phloem.

The GS signal is as yet unidentified. An appealing idea is that siRNA acts as a transport form at least in local GS, which is supported by experiments with viral proteins suppressing GS [42]. This property seems to be inherent in 21-nt, but not 25-nt, siRNAs. DCL4 is involved in generating transported 21-nt siRNA. A model advanced on this basis postulates that DCL4 cleaves dsRNA synthesized by RdRp into transported 21-nt siRNAs, which are then transferred from one cell to another via plasmodesmata and induce GS [42]. The scenario of systemic GS in phloem is far more complex. It seems that any GS-inducing nucleic acid can mediate signal transduction through the plant. In addition, the process involves PSRP1, which has been recently identified in cucumber phloem juice and is capable of binding siRNAs and of facilitating their cell-to-cell transfer [43]. A role of pectin methylesterase in GS cannot be excluded. Methanol produced in the apoplast can easily be transferred through the plant to induce GS. Data on the role of cadmium ions in GS are also noteworthy. Cadmium induces synthesis of a glycine-rich protein (cdiGRP), which affects the phloem transport of the systemic signal.

Systemic GS is known for animals. This was observed with *C. elegans* fed on dsRNA-producing *Escherichia coli.* The GS signals spread throughout the nematode body with the obligatory participation of RdRp. In addition, systemic signal transduction involves SID1, a membrane protein that forms a channel for intercellular dsRNA transport.

## RNA Polymerase IV

In plants, one of the GS mechanisms is associated with genomic DNA methylation [44]. The methyl group is added to cytosine not only in CG dinucleotides, as in animals, but also in CNG or CNN trinucleotides, where N is A, T, or C. This facilitates transcriptional GS, which involves three groups of factors in *A. thaliana*: DNA methyltransferases; histone-modifying enzymes; and factors DRD1, DRD2, and DRD3 (defective in RNA-directed DNA methylation). DRD1 is involved in chromatin remodeling, while DRD2 and DRD3 are subunits of RNA polymerase IV. This enzyme includes subunits NRPD1a, NRPD2a (DRD2), and NRPD1b (DRD3). The function of RNA polymerase IV depends on its subunit composition. RNA polymerase IVa consists of NRPD1a and NRPD2a and is involved in siRNA amplification. RNA polymerase IVb (NRPD1b and NRPD2a) acts together with DRD1 to ensure siRNA-mediated DNA methylation and chromatin remodeling. This duet utilizes intergenic regions and euchromatin retrotransposons as targets in *A. thaliana* nuclei [44].

## Viral GS Suppressors

During evolution, viruses acquired specific tools to evade the cell defense systems. Every mechanism protecting viral RNA from degradation can be regarded, in principle, as a mechanism of protection from GS. Two such mechanisms were known long before the discovery of GS. These are virion assembly and compartmentalization. Virion assembly is the most efficient way to protect the viral genetic material from external factors. For instance, rod-shaped virions of the tobacco mosaic virus can persist for many years both within and beyond cells. Compartmentalization

**Table 5.** Viral GS suppressors

| Family | Virus | Gene | Function |
|---|---|---|---|
| Plant (+)RNA viruses | | | |
| Carmoviruses | Turnip crinkle virus | P38 | Coat protein |
| Cucumoviruses | Cucumber mosaic virus | 2b | Virus transport |
| Closteroviruses | Beet yellow virus | P21 | Replication enhancer |
| Comoviruses | Cowpea mosaic virus | S protein | Small coat protein |
| Hordeiviruses | Barley stripe mosaic virus | γb | Replication enhancer, virus transport, seed transmission, pathogenicity factor |
| Pecluviruses | Peanut clump virus | P15 | Virus transport |
| Poleroviruses | Beet western yellow virus | P0 | Pathogenicity factor |
| Potexviruses | Potato virus X | P25 | Virus transport |
| Potyviruses | Potato virus Y | HcPro | Virus transport, protease |
| Sobemoviruses | Rice yellow mottle virus | P1 | Virus transport, pathogenicity factor |
| Tombusviruses | Tomato bushy stunt virus | P19 | Pathogenicity factor |
| Tobamoviruses | Tobacco mosaic virus | 130K | Replicase component |
| | | 30K | Virus transport |
| Tymoviruses | Turnip yellow mosaic virus | P69 | Virus transport |
| Plant (–)RNA viruses | | | |
| Tospoviruses | Tomato spotted wilt virus | NSs | Pathogenicity factor |
| Plant DNA viruses | | | |
| Geminiviruses | African cassava mosaic virus | AC2 | Transcriptional activator |

is localization of the viral genetic material in specific cell sites (compartments) and is also quite efficient. For instance, the turnip yellow mosaic virus utilizes membrane vesicles formed on the chloroplast surface to replicate its genomic RNA.

To suppress GS, viruses utilize various proteins, including proteases, coat proteins, replicative enhancers, and, most commonly, transport proteins and pathogenicity factors (Table 5). Transport proteins dilate plasmodesmata to facilitate cell-to-cell transport of viral genetic material. The term pathogenicity factor is rather vague. It was proposed before the discovery of GS to designate the proteins that induce or enhance the signs of virus infection (deformity and mosaicism of leaves, necroses, growth retardation, dwarfishness, etc.). It is clear now that GS suppression disarms the plant and allows the infection signs to develop in full measure. Potyvirus HcPro was the first to be identified as a GS suppressor. HcPro synthesis in plants increases their susceptibility to virus infection. On the other hand, HcPro stabilizes any mRNA and dramatically increases the accumulation of the target protein in plants.

Viral suppressors prevent the accumulation of short RNAs and abolish both local and systemic GS [45–47]. To perform these functions, most of the known suppressors bind with siRNA or miRNA (Fig. 6).

First and foremost, this is true for best-studied tombusvirus P19, closterovirus P21, cucumovirus 2b, and potyvirus HcPro. P19 is most effective among all known viral GS suppressors. P19 has maximal affinity for 21-nt siRNA and miRNA regardless of the presence of a 3'-overhang. The efficiency of P19 binding is appreciably reduced as the siRNA or miRNA length increases even by 1 nt (to 22 nt). Such selectivity is unusual and unique to P19 among all suppressors. Suppressor binding inhibits the interaction of siRNA and miRNA with Dicer and Ago, prevents their local and systemic spreading, blocks their methylation, and, consequently, stimulates their degradation [21].

GS suppressor genes were found in animal viruses: nodaviruses, retroviruses, orthomixoviruses, adenoviruses, and poxviruses. GS suppressors proved to be universal: suppressors of animal viruses are active in plant cells and suppressors of plant viruses are active in animal cells. This is probably explained by the fact that suppressors bind short dsRNAs regardless of the cell origin.

Viral RNA can also suppress GS. For instance, adenovirus VA1 RNA blocks GS in animal cells. Untranslated RNA of the red clover necrotic mosaic virus blocs GS in plant cells [48]. Experiments performed in my lab explained this phenomenon and revealed a competition between viral RNA and pre-miRNA for DCL1. Introduced in plant cells, noncod-

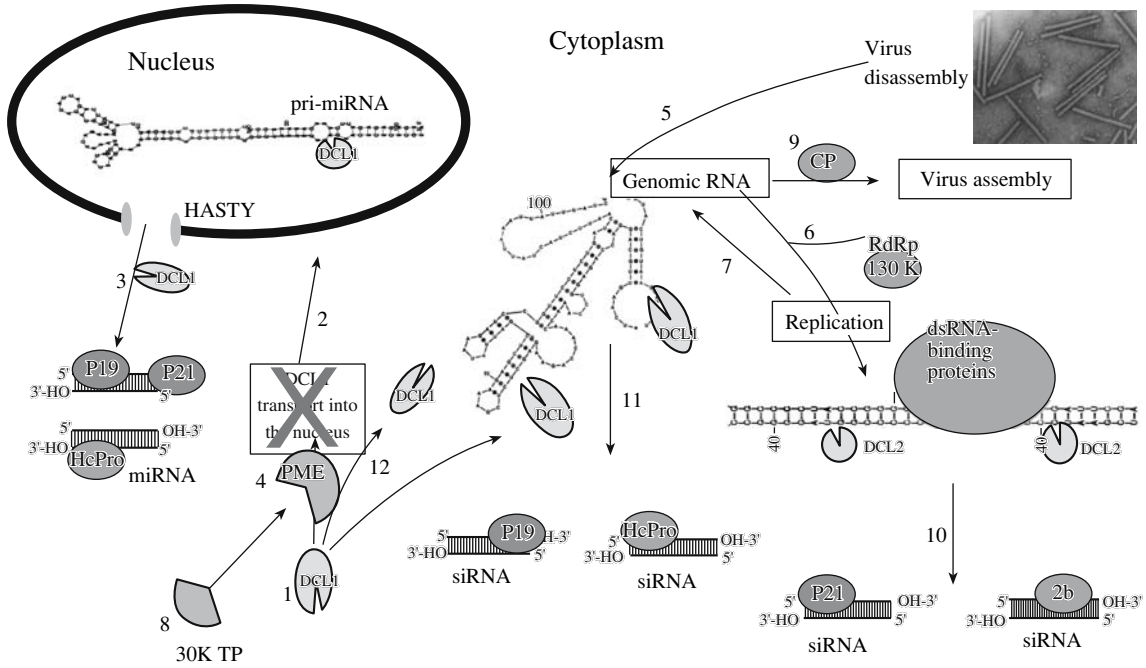


**Fig. 6.** Role of DCL1 in virus aggression and cell defense. CP, coat protein; PME, pectin methylesterase; TP, transport protein.

ing RNA suppresses tobacco mosaic virus RNA silencing caused by pectin methylesterase [34].

A model of the association between viral RNA silencing and miRNA biogenesis is shown in Fig. 6. DCL1 is synthesized in the cytoplasm (1), transferred into the nucleus (2), and plays a role in the first step of miRNA generation from pri-miRNA. HASTY mediates the transfer of pro-miRNA and DCL1 into the cytoplasm, where miRNA is produced (3). Cell infection with a virus induces pectin methylesterase, which blocks DCL1 transport into the nucleus (4). This leads to the accumulation of DCL1 in the cytoplasm and, on the other hand, blocks miRNA maturation. Synthesis of miR162, which regulates the DCL1 mRNA stability, is also suppressed, which promotes further accumulation of DCL1 in the cytoplasm [14]. After entering the cell, the virus is uncoated (5) and its genomic RNA is released. Cap-dependent translation of genomic RNA leads to the formation of a viral protein–RdRp complex, which includes the 130K and 183K proteins [12]. Then, dsRNA and, eventually, a new generation of genomic ssRNA are produced. Viral RNA can direct synthesis of a small amount of the early transport and coat proteins via cap-independent internal translation initiation [49, 50]. These proteins not only perform their main functions but also block GS. The transport protein binds with pectin methylesterase to inhibit DCL1 accumulation in the cytoplasm [14], while the coat protein packs viral ssRNA into RNP, inaccessible for DCL2 [15, 16]. Genomic ssRNA usually has a distinct secondary structure, forming stem–loops, which can be degraded by DCL1 [17]. Their amount in the cytoplasm increases upon pectin methylesterase induction [18]. It is possible that the efficiency of the DCL1 interaction with genomic RNA is low, far lower than with pro-miRNA. However, a single nick can already suffice to abolish infectivity of viral genomic RNA.

Some viruses utilize not only protein and nucleic acid suppressors but also use other mechanisms to evade viral RNA silencing. For instance, virus reproduction and transport to neighbor cells is quicker than local and systemic GS spreading. This mechanism seems to play a major role in GS evasion by the tobacco mosaic virus [51]. Among all phytoviruses, the tobacco mosaic virus holds the lead in spreading rate and virus progeny content (4–5 g/kg green weight).

## GS and Biotechnology

Studies of GS are of a great applied significance. As factories producing foreign proteins (including therapeutic proteins, antibodies, and recombinant vaccines), plants have several advantages over bacterial, yeast, and mammalian cells. Plant cells are biologically safe, as they are free from viruses and prions pathogenic for humans. Synthesis of target proteins in plants does not require expensive equipment (fermenters), culture media, and sterility systems. Growing experimental plants is incomparably cheaper than culturing bacterial, yeast, or animal cells. As a result, the cost of proteins overproduced in plants is 10–30 times

lower than that of similar products obtained with bacteria.

Yet the use of transgenic plants to produce target proteins is hindered by the fact that the level of their production upon stable transformation is usually extremely low (0.01–0.2% of the total soluble protein). On the other hand, construction of transgenic plants is rather time-consuming. The use of transgenic plants is limited by state regulations and safety requirements. Hence, transient expression of target proteins in plants seems more promising. A necessary gene or a virus vector genome is inserted in a binary vector and introduced in plant cells via arobacterial injection or infiltration. With monocistronic constructs, production of the target protein in 5–10 days reaches 5–10% of the total soluble protein. A higher accumulation of the target protein is achieved via agrobacterial infiltration with self-replicating recombinant virus vectors, which is due to the high efficiency of initial plant infection and subsequent accumulation of virus vector RNA as a result of replication. These techniques are even more efficient when GS is suppressed: the accumulation of the target protein can reach 80% of the total soluble protein (4–5 g/kg green weight). A desktop technology of producing a target protein in plants is already available and allows the target protein to be isolated in gram amounts from a few plants under laboratory conditions.

To conclude, another possible application of GS should be noted. Plants have come to be used as a source of cheap specific siRNA preparations. Recent experiments yielded plants producing siRNAs against the influenza virus, and the siRNAs proved to be active in mammalian cells [52].

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