Post Transcriptional Gene Silencing in Plants
PTGS
siRNA
POST TRANSCRIPTIONAL GENE SILENCING - PTGS

Plants: **Cosuppression** or **Epigenetic gene silencing**: 1990: found that transgenes could suppress the expression of similar endogenous genes, which resulted in loss of function in 10-40% of the transgenic plants. Exp: Chalcone synthase (anthocyanin synthese pathway) in Petunia

Both, endogenous and transgene were transcribed but almost no mRNA was detectable, so silencing is on the RNA level → **RNA silencing** (not gene silencing), coding region was methylated.

CaMV: Cauliflower Mosaic Virus Promotor
NOS-Ter: Nopalinsynthase Terminator
GENE SILENCING

**PTGS**: Post transcriptional gene silencing

**TGS**: Transcriptional gene silencing

**HDGS**: Homology dependent

PTGS: - transcribed, but no full transcript found
- coding region methylated
- not dependent on ongoing translation
- fully reversible during meioses

TGS: - promoter inactive
- promoter methylated
- chromatin remodelling
- frequently meiotically heritable
- both transgene and endogenous genes are methylated
- non symmetrical methylation (non CG or CNG) → hallmark of RNA directed DNA methylation.
- X inactivation: Xist – Tsix: dsRNA? and methylation
PTGS can also be induced by homologous DNA, with no promoter sequence (minimal length 300 bp) ➔ „ectopic pairing“ ➔ aberrant RNA


Figure 1. Model of Three Phases of Posttranscriptional Gene Silencing
(a) Initiation. Cells carrying a transgene or a transgene and sequence-related endogenous genes express transcripts correctly. Introduced DNA pairs with sequence-homologous DNA in the plant nucleus. Similar aberrant RNA could be produced spontaneously in the absence of incoming DNA if transgenes and endogenous genes ectopically pair in somatic cells.
(b) Spread. Homologous pairing interferes with correct transcription and leads to transcription of aberrant RNA from the transgene. The aberrant RNA enters the cytoplasm where it initiates a sequence-specific RNA degradation process (shown in [c]). In addition, a signal is produced that moves to neighboring cells through cell–cell contacts and eventually is transported through the vascular system. The aberrant RNA may be at least a part of the systemically spreading signal. The aberrant RNA is drawn here as a double-stranded RNA. Hatching indicates antisense sequences.
(c) Maintenance. The signal moves systemically through vascular tissues. When it enters a new cell it stimulates the initiation of more aberrant RNA and more signal molecules, perhaps through ectopic pairing with homologous DNA in the nucleus. Aberrant RNA activates an RNA-directed RNA polymerase (RdRp). RdRp, which can make short antisense copies of transcripts, would specifically copy the aberrant RNA. The antisense fragments bind to functional transcripts from the endogenous gene and the transgenes, making them targets for degradation by double-strand-specific RNAses.
This figure is based on Voinnet et al. (1998) and Palauqui and Balzergue (1999).
N. clevelandii - TMV: systemic infection with strong symptoms
Virus induced PTGS

Plants use PTGS as a defence against viruses even if there is no homologous region present. Virus infection ➔ replication ➔ begin to spread ➔ plant starts degrading RNA ➔ recovery ➔ these plants are virus resistant: cross protection from viral strains that are closely related.

N. glutinosa - TMV:
resistence:
hypersensitive response
Cross protection: for several viruses an RNA mediated process: Model system: Potato virus X (PVX)

**PVX: a vector for gene expression in plants**

*Inoculation of plants with infectious DNA*

Additional:
recombinant PVX virus: with exon sequences of an endogenous gene: gene expression was suppressed if the virus was replication competent (virus replication: ds RNA): decline of viral RNA and host mRNA: **VIGS**: virus induced gene silencing
GFP-Nicotiana benthamiana – model plant for PTGS

Transgenic Tobacco GFP plant infected with Agrobacteria containing a GFP gene

Construct with a GFP gene for Agrobacteria transformation

C: GFP expressing plant
D: silenced plant

Brigneti et al., 1998, EMBO J. 17, 6739-6746
**BOX 1. A lexicon of gene-silencing effects**

**Antisense interference**
Blocking the activity of genes by artificially providing complementary single-strand antisense nucleic acid corresponding to the target gene.

**Cosuppression**
The ability of some transgenes to silence themselves and homologous chromosomal loci simultaneously.

**dsRNA-triggered interference**
Blocking the activity of genes by artificially providing sense and antisense RNA corresponding to a target gene.

**Homology-dependent trans silencing**
The ability of an RNA or DNA trigger to silence a corresponding chromosomal locus in *trans* (i.e. without any genetic linkage to the target locus).

**Post-transcriptional gene silencing (PTGS)**
The ability of some viruses, transgenes or RNAs to trigger the post-transcriptional degradation of homologous cellular RNAs.

**Quelling**
A cosuppression phenomenon in *Neurospora crassa*.

**Repeat-induced gene silencing (RIGS)**
(Not discussed in this paper but included in this box for clarity.) In general, RIGS refers to a localized (*cis*-acting) effect, in which regions of tandemly repeated sequence are silenced, frequently without silencing homologous genes at other sites in the genome.

**RNAi**
The ability to block activity of a cellular gene by injection of homologous RNA (generally used in *Caenorhabditis elegans*).
"RNA interference" ⇒ **RNAi**, a phenomenon in *C. elegans* and *Drosophila* for silencing of gene expression through a signal induced by double stranded RNA (**dsRNA**)

*C. elegans*: injection of dsRNA: corresponding gene product disappears in the somatic cells, also in F1 generation. However, F2 normal phenotyp, no mutation, no sequence difference: is post-transcriptional because only exon sequences show RNAi. In polycistronic pre-mRNAs only the sequences of the initial dsRNA are silenced. RNA degradation is at the level of nucleus and cytoplasm.

WHAT HAPPENS? WHY is DEGRADATION transmissible through the whole organism??
PTGS: siRNAs

Plants: 1999: small RNAs of 21-25 nt were detected which corresponded to the silenced gene, but only detectable in plants which were silenced. Also for viral sequences (Hamilton and Baulcombe, Science, 286, 950).

Drosophila: induced silencing of a gene with a dsRNA, cell extract had nuclease activity which was sequence specific: partial purified nuclease contained sequence specific 21-25 mers.

Cell extract could be induced with dsRNA to process the dsRNA into 21-23 nt RNA (dsRNA nuclease). Addition of mRNA resulted in degradation of sequences corresponding to the original dsRNA sequence:

⇒ siRNA: small interfering RNAs: hallmark in RNA silencing
A Model for the RNAi Pathway

Zamore, P.D., Nature structural biology, 8 (2001), 746

RISC: RNA induced silencing complex
An integrated model for RNAi and PTGS

In this model, the sequential action of Dicer (to generate siRNAs) and 'Slicer' (to cleave the target RNA) are considered the primary route for target destruction. Amplification of the siRNAs is postulated to occur by either (or both) 'random degradative PCR' or production of siRNAs from aberrant RNA - that is, the copying of the target RNA or a cleavage product of the target RNA by an RNA-dependent RNA polymerase to generate a dsRNA substrate for Dicer, thereby creating new siRNAs. In the random degradative PCR scheme, the polymerase is envisioned to be primed by an siRNA guide strand. Conversion of aberrant RNA to dsRNA is drawn here unprimed.

Hutvagner, G. and Zamore, P.D., Current Opinion in Genetics & Development, 12 (2002), 225
Virus induced gene silencing (VIGS)

RNA of a virus + a gene of interest → RdRp → dsRNA → Dicer-like dsRNase → 21-25nt siRNA → Endogenous mRNA → mRNA degradation

RISC-like nuclease complex → degradation of viral RNA
Systemic silencing: mobile signal

silencing signal moves both cell-to-cell and through phloem mimicking pattern of viral movement

Mlotshwa et al., The Plant Cell, Supp. 2002, S289
Viral cell-to-cell movement proceeds through plasmodesmata

plasmodesmata provide an intercellular transport pathway for small molecules
Intercellular transport of TMV RNA

Modified from Lazarowicz and Beachy, Plant Cell, 1999
Tobacco Mosaic virus movement protein (TMV-MP) opens plasmodesmata

10 kDa fluores. Dextran  + TMV-MP
Are siRNAs the Mobile Silencing Signal?

Evidence for
- siRNAs are always associated with RNA silencing
- small enough to move easily, yet large enough to provide sequence specificity
- sufficient to induce silencing in animal systems

Evidence against
- HC-Pro suppression of sense-transgene silencing eliminates siRNAs, but not systemic silencing
- rde-4 mutants in C. elegans are defective for siRNA production but not for systemic silencing

Other candidates
- long dsRNA - the precursor of siRNAs
- aberrant RNA - transcripts or derivatives thereof
- "tagged" messages - a mobile mRNA/protein complex
Viruses fight back: suppressors of RNA silencing

Plant viruses all have RNA silencing suppressors:

**PVX-p25** movement protein: affects systemic silencing,

**HcPro**-Potyviruses: suppress production of small RNAs, important for virus infectivity
Viral strategies for suppression and evasion of RNA-silencing

a | Direct interference with silencing-effector molecules is illustrated by the tombusviral P19 protein. The head-to-tail organization of the tombusviral P19 homodimers (blue and green) allows binding to small interfering RNA (siRNA) duplexes (yellow). Two sets of tryptophan residues (yellow) bind to the last set of base pairs on either end of the siRNA, leading to effective measurement of the duplex length, such that P19 selects siRNAs of 21nt for binding. The sequestered siRNA is prevented from entering the RNA induced silencing complex (RISC) and is therefore inactivated.

b | Recruitment of endogenous negative regulators of RNA silencing is illustrated by the potyviral helper component proteinase (HcPro). HcPro interacts with the calmodulin-like protein rgsCaM (regulator of gene silencing CaM) to inactivate the RNA-silencing pathway through an unknown mechanism at an intermediate step that involves both RISC and Dicer.
RNA-based silencing strategies in plants

A model for RNA-based transcriptional and posttranscriptional silencing. Steps involving dsRNA and steps that are affected by viral suppressors of PTGS and in various PTGS mutants are shown. TGS may be triggered directly by transcription of inverted repeat sequences in the nucleus and methylation of homologous promoter regions in the genome. In addition, dsRNA and other aberrant RNAs formed in the nucleus may be transported to the cytoplasm and enter the PTGS pathway. Two modes of dsRNA production lead to PTGS in the cytoplasm: first, virus-induced gene silencing mediated by the viral RdRP, and second, transgene-induced gene silencing mediated by the cellular RdRP. The dsRNA from either of these sources can be targeted by a putative dsRNA specific ribonuclease which generates 21–25 nucleotide RNAs of both polarities (small RNAs). These small RNAs are incorporated into a ribonuclease and act as guides for sequence-specific degradation of homologous RNAs. dsRNA from the cytoplasm may trigger methylation of homologous genomic sequences presumably by transfer of a signal molecule into the nucleus. Similarly, PTGS can be induced locally and then spread throughout the organism via production and transport of a mobile silencing signal. The identity of the signaling molecule(s) that induces methylation and systemic PTGS is unknown but is likely to incorporate an RNA component, possibly dsRNA as shown here or processed forms of dsRNA, such as the small RNAs. HC-Pro suppresses gene silencing at a step upstream of the accumulation of the small RNAs but downstream of the mobile silencing signal, probably via activation of an endogenous cellular suppressor of PTGS, rgs-CaM. The PVX p25 suppressor of PTGS prevents the accumulation and/or transport of the mobile silencing signal, probably by interfering with the cellular RdRP branch of the pathway.
More Dicer proteins in plants

Plants: short siRNA: 21-22nt, correlate with sequence specific degradation; long siRNA 24-26 nt involved in triggering systemic silencing

General pathway of RNAi in vitro

RNAi: RNA interference
siRNA: small interfering RNA
RISC: RNA-induced silencing complex

Nature Reviews | Molecular Cell Biology

Sontheimer, NRMCB, 2005, p127
Slicer: enzyme which cuts the target strand, strong evidence that Ago proteins possess this activity. Crystal structure show that the PIWI domain of Ago proteins are very similar to RNaseH and harbour endonuclease activity. Like RNase H it is dependent on divalent metal ions, produces 3’OH and 5’P and only cleaves one strand of the duplex RNA.
siRNA asymmetry, dsRNA processing and the implications for RISC assembly.

a | When 21-nt short interfering (si)RNAs are used to initiate RNA interference (RNAi), the strand with its 5′ terminus at the thermodynamically less stable end of the duplex is preferentially incorporated into the RNA-induced silencing complex (RISC). The guide strand is shown in red and the passenger strand in blue. Less stably base-paired regions of the siRNA are depicted in grey and the more stable regions are shown in black.

b | Dicer (Dcr) generates siRNAs from the ends of double-stranded (ds)RNAs. The multiple domains that are found in most Dcrs (the RNA helicase, DUF283, PAZ, RNase IIIa (RIIIa), RNase IIIb (RIIIb), and dsRNA-binding (dsRBD) domains) are shown. Current models indicate that Dcr interacts with nascent siRNAs asymmetrically, with its PAZ domain bound to the pre-existing dsRNA end and its dsRBD bound closer to the dsRNA-cleavage sites (arrows). The dual roles of Dcr in dsRNA cleavage and RISC assembly indicate that Dcr might remain bound to the siRNA after it is processed from the dsRNA precursor. Combined dsRNA-processing and target-cleavage assays suggest that the strand that has its 3′ end bound to the PAZ domain preferentially assembles into RISC.
dsRNA processing by dicer and RISC formation

RISC: RNA induced silencing complex.

thermodynamic asymmetry rule: for synthetic siRNA strand selection: The strand which has the its 5' end at the less stably base-paired region becomes the guide strand.
Table 2 | Biochemically documented RISC-assembly factors and RISC-associated proteins*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Domains</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dcr1</td>
<td><em>D. melanogaster</em></td>
<td>DUFS283, PAZ, RNase III, dsRBD</td>
<td>20</td>
</tr>
<tr>
<td>Dcr2</td>
<td><em>D. melanogaster</em></td>
<td>Helicase, DUFS283, FNase III, dsRBD</td>
<td>20,32</td>
</tr>
<tr>
<td>R2D2</td>
<td><em>D. melanogaster</em></td>
<td>dsRBD</td>
<td>20,24,32</td>
</tr>
<tr>
<td>Ago2</td>
<td><em>D. melanogaster</em></td>
<td>PAZ, PIWI</td>
<td>16,20,48,58</td>
</tr>
<tr>
<td>Rnr1/Fxr</td>
<td><em>D. melanogaster</em></td>
<td>RGG, KH</td>
<td>20,48,59</td>
</tr>
<tr>
<td>Vlg</td>
<td><em>D. melanogaster</em></td>
<td>RGG</td>
<td>20,59</td>
</tr>
<tr>
<td>Tsn</td>
<td><em>D. melanogaster</em></td>
<td>Tudor, SN</td>
<td>20,60</td>
</tr>
<tr>
<td>Dmp68</td>
<td><em>D. melanogaster</em></td>
<td>Helicase</td>
<td>40</td>
</tr>
<tr>
<td>Polysomes</td>
<td><em>D. melanogaster, T. brucel</em></td>
<td>Numerous</td>
<td>7,16,20,48,69</td>
</tr>
</tbody>
</table>

*| elf2C1(AGO1) | *H. sapiens* | PAZ/PIWI               | 9          |
| elf2C2(AGO2) | *H. sapiens* | PAZ/PIWI               | 9,16,29,75 |
| Gemin3     | *H. sapiens* | Helicase               | 10,19      |
| Gemin4     | *H. sapiens* | None known             | 10,19      |

*Some other proteins involved in RISC in a range of species have been discovered by genetic or bioinformatic means. In a few cases, for example, Agol, Aubergine and Armitage in *Drosophila* melanogaster, the biochemical roles of the proteins have been investigated by examining the activities of extracts from mutant embryos. However, these proteins have not been physically detected in association with active RISC. Agol, Aubergine, Dcr, Dicer, Dmp68, the D. melanogaster orthologue of the mammalian p68 RNA unwinding dsRBD, double-stranded-RNA-binding domain DUFS283, domain of unknown function 283; Rnr1/Fxr, the D. melanogaster orthologue of the fragile-X mental retardation protein (FMRP); KH, heterogeneous nuclear ribonucleoprotein (hnRNP) K homology; PAZ, piwi argonaute/zwille; RGG, arginine-glycine-glycine RNA binding; RNase, ribonuclease; SN, staphylococcal nuclease; Vlg, vasa intronic gene.*