HOST DEFENSE AGAINST VIRUS INFECTION OF PLANTS

- Post transcriptional gene silencing (PTGS) or RNA interference (RNAi) represents a sequence specific antiviral defense mechanism in plants.

- has parallels with the immune system of mammals.

- was hailed by Science as the breakthrough of the year in 2002.

“Small RNAs literally shape genomes, carving out chunks to keep and discarding others. They can direct genes to turn on or off during development. Science hails these electrifying discoveries, which are prompting biologists to overhaul their vision of the cell.”

- First discovered in plants during virus resistance studies, later found to be present in other higher eukaryotes.
POSTTRANSCRIPTIONAL GENE SILENCING

READING:


CO-SUPPRESSION:

- A phenomenon that results in the silencing of both a transgene and its homologous endogenous gene
- Uncovered during attempts to overexpress chalcone synthase (1990 Napoli et al., Plant Cell 7: 599-609)
- Chalcone synthase (CS) encodes a flower pigment biosynthetic enzyme
- When CS was introduced in sense orientation into transgenic plants with purple flowers, the following were observed:
  - plants with purple flowers
  - plants with white flowers
  - flowers with white and purple sectors
CO-SUPPRESSION of CS

Figure 1: Phenotypes of Chimeric CHS Transgenotes and Variations among Flowers on Single Plants.
Heritability of co-suppression - The introduced T-DNA correlated with the novel color phenotype.
CO-SUPPRESSION of CS:

- The CS transgene in white tissue cells fail to not only increase the flower pigment, but also represses the floral pigment biosynthetic pathway by co-suppressing the endogenous gene.
- The transcription rates of the CS transgene and the endogenous CS gene in white tissues were no different than in purple tissues.
- Expression of both the introduced CS gene and the endogenous gene were suppressed in white flowers.
- Many plants with white or white sectored flowers contained multiple methylated transgene copies.
- PTGS and co-suppression are very sequence specific, they silence viruses or genes with >75% sequence homology to the transgene.
POST-TRANSCRIPTIONAL GENE SILENCING:

- PTGS was first observed with tobacco etch virus (TEV) (1993 Lindbo et al., Plant Cell 5:1749-1759)
- Transgenic tobacco plants expressing either the full length TEV CP or an N-terminal truncated form were initially susceptible to infection, but recovered afterwards
- The resistance in the recovered tissue was virus specific
- Steady state transgene mRNA levels in the recovered tissue were 12-22-fold less than transgene mRNA levels in uninoculated tissue
- Nuclear run-off studies indicated that transgene transcription rates in recovered and uninoculated plants were similar
TEV symptoms in recovered transgenic plants:
Resistant state and reduced transcript accumulation were mediated by a post-transcriptional event - a cytoplasmic activity that targets specific RNA sequences for inactivation.
PROPOSED MECHANISM OF PTGS:

• Plant cells are able to sense elevated or aberrant RNA levels

• These sequences are then targeted and inactivated by a cellular factor that may be a protein or a nucleic acid

• The complex formed between the target RNA sequence and the cellular factor will direct the cellular enzymes to degrade the RNA
Virus-induced gene silencing (VIGS):

- A powerful tool for reverse and forward genetics in plants allowing rapid identification of gene function

- Example below illustrates potato virus X (PVX), which is used as a vector to induce gene silencing by cloning the target gene into the PVX vector.

![Diagram of virus replication and movement]
**VIRUS INDUCED GENE SILENCING**

b-c. VIGS of cellulose synthase

d-e. VIGS of phytoene desaturase

f-g. VIGS of GFP
Systemic gene silencing:

- Ectopically introduced DNA triggers systemic silencing of a stably integrated transgene
- DNA is introduced biolistically in a few cells, silencing spreads within 2-3 days via plasmodesmata and phloem
- The whole plant exhibits silencing phenotype
Systemic spread of silencing in GFP expressing plants:

Voinnet and Baulcombe
MECHANISM OF RNA SILENCING

- Transgene constructs engineered to produce double stranded RNA as opposed to single stranded RNA cause high level of RNA silencing.

- Replication (during which ds RNA intermediates are formed) rather than accumulation of the viral genome is required for silencing.
Induction of PTGS by double-stranded RNA:

• Because PTGS does not always involve high level of transcription of the inducing transgenes, it has been proposed that a qualitative feature of the transgene mRNA triggers silencing.

• Plants expressing sense mRNA of a virus derived transgene have been crossed with plants expressing the antisense mRNA of the transgene.

• All progeny that inherit both the sense and antisense transgenes are resistant to the virus.

• Progeny inheriting the sense, antisense or neither transgene are susceptible.
Fig. 4. Experiment to investigate the role of dsRNA in RNA-mediated resistance (RMVR). A plant expressing a sense virus-derived transgene was crossed with a plant expressing an antisense virus-derived transgene. Both plants are hemizygous for the transgene and susceptible to virus infection. The progeny have four different genotypes. Those progeny inheriting both a sense and an antisense gene are resistant to the virus (plants depicted with white leaves). Plants of the other three genotypes are susceptible to the virus (plants depicted with grey leaves). This experiment suggests that the sense and antisense transgene mRNA form a duplex that induces RMVR. Abbreviations: S, sense allele; A, antisense allele; –, no allele.
Discovery of small RNAs in PTGS:

• Since co-suppression is triggered by sense transcripts and targets sense mRNAs, it was likely that the nucleic acid involved was an antisense RNA.

• The work by Hamilton and Baulcombe (1999 Science 286:950-952) demonstrated that discrete species of 21-24 nt long RNAs with antisense sequence of the silenced genes accumulated in co-suppressed plants.

• Subsequently, the studies in *Drosophila* and *C.elegans* demonstrated the involvement of similar molecules in the RNAi process.

• These RNAs included both sense and antisense strands corresponding to the transgene, suggesting that they could be derived from the dsRNA.
Synthesis of small RNAs:


- Introduced dsRNA is recognized by proteins, which start from the dsRNA termini and cleave it into 22 nt fragments.

- The strands of the short dsRNA are separated and one strand is used as a guide to recognize single stranded RNA with complementary sequences.

- Each complex cleaves the ssRNA at a position around the middle of the guide sequence.
**Synthesis of small RNAs:**

- The 21-24 nt long RNAs are generated by a RISC complex (RNA induced silencing complex) first identified by Hammond et al., 2000 Nature 404:293-296 in *Drosophila* cells.

- The 21-24nt RNA duplexes have 5’ terminal phosphate and 2 nt long overhanging 3’ ends that are characteristic of RNase III like enzymes.

- Analysis of the *Drosophila* genome identified 3 genes that could encode such proteins.

- Only one of these, Dicer was able to produce 21-24 nt RNAs when incubated with long dsRNA, but not ssRNA in embryo extracts.

- Genes encoding Dicer-like enzymes have been identified in other organisms.
**Generation of small RNAs:**

- Silencing related small RNAs are called small interfering RNAs (siRNAs).
- They are processed by Dicer.
- They associate with the RISC complex.
Generation of small RNAs:

• Fractionation experiments in Drosophila embryo extracts indicated that Dicer activity could be separated from the activity that degrades homologous mRNAs.

• This activity belongs to a multisubunit complex, referred to as the ‘RNA induced silencing complex’ (RISC) which copurifies with siRNAs.

• Biochemical characterization of the Drosophila RISC revealed that it contains several proteins, including Ago2-a homolog of the translation initiation factor eIF2C.

• The two siRNA strands are unwound in an ATP-dependent reaction and only the strand of the antisense orientation is present in the active RISC.

• Endonucleolytic cleavage occurs at the center of the siRNA/target hybrid.
Mapping of RNA cleavage sites in *Drosophila* embryo extracts:
MECHANISM OF RNA SILENCING:

• Endogenously synthesized or exogenously applied dsRNA is processed by a ribonuclease III-like nuclease, Dicer into 21 to 25 nt dsRNAs with 2 nt 3’ overhangs.

• These dsRNA fragments called short interfering RNAs (siRNAs) associate with a 250 to 500 kD nuclease complex called RISC (RNA induced silencing complex).

• siRNA is unwound and the antisense strand guides RISC to mRNA that has complementary sequence.

• RISC then cleaves the targeted mRNA opposite the complementary siRNA.

• RNA silencing can be induced by a few molecules of dsRNA per cell, spreads systemically in animals and plants and persists from parents to untreated progeny.
a

5' p
3' HO
p 5'

b
dsRNA

ATP
ADP + P_i

siRNA duplex

siRNA-protein complex (siRNP)

RISC

ATP
ADP + P_i

siRNA-mediated target recognition

mRNA
m7G

(A)_n

mRNA cleavage
m7G
(A)_n
Spread of the silencing signal:

• In plants dsRNA is processed into 21 and 24nt primary siRNAs

• The 24nt siRNA was shown to be dispensable for local movement, while the 21 nt siRNA may spread over 10-15 adjacent cells

• Perception of the 21nt primary siRNA in recipient cells triggers de novo dsRNA synthesis by the action of SDE1 and SDE3 through the process of transitivity.

• The resulting dsRNA is then processed by a Dicer like enzyme into secondary siRNAs that are mainly of 21nt class.

• These secondary siRNAs spread over 10-15 cells.
Several genes were identified to be required for this “sense transgene PTGS”

- SDE1/SGS2 encodes a putative RNA dependent RNA polymerase
- SDE3 encodes a putative RNA helicase
- Current model is that the combined action of SDE1 and SDE3 produces dsRNA using transgene derived, single-stranded transcripts as templates.
  - The dsRNA then triggers PTGS.
  - The dsRNA can also promote DNA methylation and heterochromatin formation resulting in transcriptional gene silencing.
• Although RdRps are encoded by viruses, RdRp activities have been reported in uninfected cells.

• The best characterized one is from tomato, a 127 kD protein, which produces RNA products at least 100 nt long by extending primers on RNA templates or by initiating RNA synthesis without a primer opposite the 3’ end of the RNA.

• Homologs of the tomato RdRp gene have been found in Arabidopsis and in other plants, in C. elegans, Neurospora crassa and the fission yeast, Schizosaccharomyces pombe.

• Homologs of the tomato RdRp gene are required for gene silencing in Neurospora, C. elegans and Arabidopsis.
Proposed mechanism of PTGS in plants:

Proteins and domains involved in RNAi:

<table>
<thead>
<tr>
<th>Protein(s) or protein family</th>
<th>Domains</th>
<th>Domain function</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>Dicer family</td>
<td>RNA helicase, PAZ</td>
<td>RNA unwinding, Putative protein–protein interaction</td>
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<td>Argonaute family</td>
<td>RNaseIII, dsRNA binding, PAZ</td>
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<td>PIWI, RdRP</td>
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<td>RNA helicases</td>
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<td>RNA unwinding</td>
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<td>QDE-3</td>
<td>DNA helicase</td>
<td>DNA unwinding</td>
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<td>RDE-4</td>
<td>dsRNA binding, RNase D</td>
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<td>MUT-7</td>
<td>KH, RGG</td>
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<td>[24]</td>
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<td>Fragile X related protein (dFXR)</td>
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<td>Vasa intronic gene (VIG)</td>
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<td>Protein–protein interaction, Histone lysine methyltransferase</td>
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<td>SGS3</td>
<td>WD-40 (MES-6)</td>
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VIRAL SUPPRESSORS OF GENE SILENCING:

• Viral synergism- mixed infections between PVX and PVY result in much more severe symptoms than those elicited by each virus individually (Vance et al., 1995 Virology 206:583).

• In coinfected tissues, the levels of PVX, but not PVY were dramatically enhanced.

• A factor produced by PVY was responsible for the increased PVX levels.

• Among the PVY proteins tested only HCPro was able to increase PVX levels.

• The mechanism was proposed to be suppression of a host defense that normally restricts accumulation of PVX in infected cells.
The host defense mechanism suppressed by HCPro was shown to be PTGS by demonstrating that crossing co-suppressed plants with HCPro transgenic plants resulted in release of PTGS in the offspring.
Assays to identify viral suppressors of gene silencing
Reversal of gene silencing assay:

(A) possible outcomes

- candidate virus or PVX-vector expressing candidate suppressor
- virus replication

(B) possible outcomes

- GFP-silenced plant
- GFP silencing remains
- GFP silencing reversed
Silencing suppression with stably transformed lines:

Genetic crosses:

Grafting assay:
Two major classes of suppressors have been identified:

• Suppressors that affect small RNA metabolism
  P19 binds siRNAs, sequestering them and blocking their function.
  HCPPro blocks the accumulation of siRNAs.

• Suppressors that affect systemic silencing
  CMV2b primarily targets systemic silencing, it blocks movement of the systemic signal.
Viral suppressors of silencing:

<table>
<thead>
<tr>
<th>Virus genus</th>
<th>Virus</th>
<th>Suppressor</th>
<th>RNA binding</th>
<th>Other viral function</th>
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<td>Cannaovirus</td>
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<td>2b</td>
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<td>Closterovirus</td>
<td>Beet yellows virus</td>
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<td>ND</td>
<td>Replicational enhancer</td>
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<td>Hordeiviruses</td>
<td>Barley yellow mosaic virus</td>
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<td>Long-distance movement</td>
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<td>C2</td>
<td>ND</td>
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Structure of the complex between P19 and siRNA:
Applications RNAi:

- RNAi can be used as a powerful tool for creating loss-of-function mutations for rapid analysis of gene function
- May be used to silence genes that cause disease
- May be used to silence pathogen genes

Limitations:

- A sequence shared between related genes might interfere with several members of the gene family
- Low level expression may resist RNA mediated interference
- In mammals, introduction of dsRNA longer than 30 nt induces a sequence specific interferon response
FUNCTION of PTGS:

• PTGS might be a mechanism that has evolved as a defense response to parasitic DNA sequences such as transposons or RNA sequences, such as viruses.

• In plants, RNA viruses induce and can be strongly inhibited by RNA silencing, resulting in a race of viral replication and spread against the induction of spread of antiviral RNA silencing.

• At the DNA level, RNA silencing is linked to transcriptional silencing of chromosomal genes.

• Pathways related to RNA silencing are required to process numerous endogenous precursor RNAs into 22nt microRNAs (miRNAs) that regulate developmental events.
Discovery of microRNAs (miRNAs):

Definition:

- Endogenous small RNAs that regulate gene expression.

- miRNAs are single stranded RNAs of 18-24 nt in length which are generated by Dicer from an endogenous transcript that contains a local hairpin structure.

- They direct the cleavage or translational inhibition of cellular mRNAs.
Differences between microRNAs (miRNAs) and siRNAs

**miRNAs:**
Encoded by their own genes or present in introns of other genes.

Pol II transcribes the miRNA genes into precursor RNAs.

Precursors form hairpin Structures.

They regulate other genes.

**siRNAs:**
Generated from exogenous dsRNA made from endogenous transcripts by RdRp.

Regulate the loci that gives rise to the siRNAs.
Similarities between microRNAs (miRNAs) and siRNAs:

- Similar in structure.
- The biogenesis of both types of small RNAs requires Dicer.
- Reside in similar protein complexes to function.
- Functions of miRNAs and siRNAs are similar:
  - Target the cleavage of complementary mRNA.
  - Inhibit the translation of mRNA that are not completely complementary to the small RNAs.
  - Target chromatin modifications such as histone methylation and DNA methylation.
Biogenesis of microRNAs (miRNAs) and siRNAs: