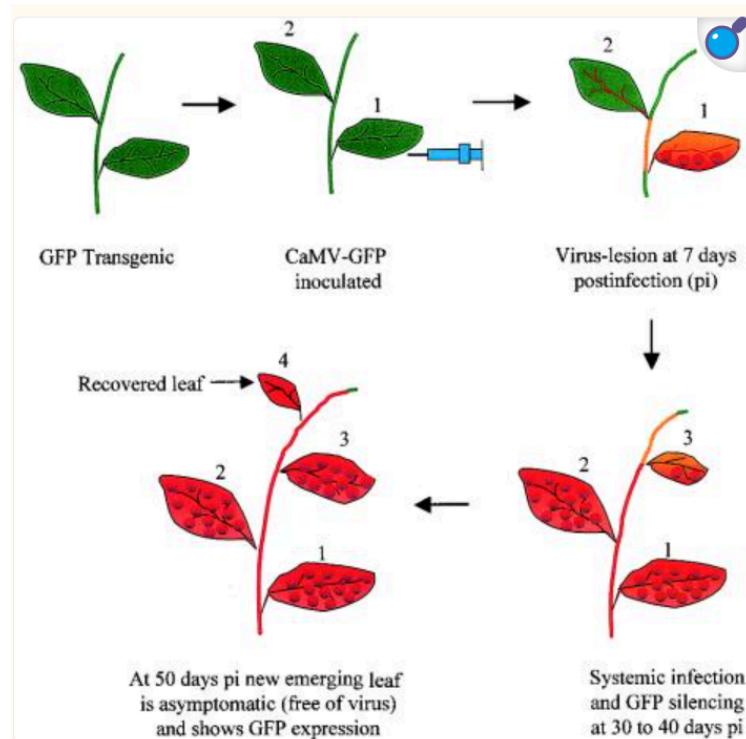


Gene Silencing, RNA Interference, and miRNA Functions



CaMV (Cauliflower Mosaic Virus) and GFP (Green Fluorescent Protein) are often used together in plant biology research, with the CaMV 35S promoter commonly driving GFP expression for visualizing gene activity and protein localization.

Gene silencing involves various proteins and processes. Genetic and biochemical studies in organisms like *Neurospora crassa*, *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* have identified key components involved in gene silencing, including initiators, effectors, amplifiers, and transmitters.

History of Discovery

- In the 1990s, scientists observed **gene silencing** when **inserting foreign genes** into plants, notably in chalcone synthase regulation (Napoli & Jorgensen).
- Around the same time, Lee et al. discovered a **small RNA (lin-4)** in *C. elegans*, marking the **first microRNA (miRNA)**, which controlled developmental timing.
- In 1998, Mello & Fire demonstrated **RNA interference (RNAi)** using **double-stranded RNA (dsRNA)** to silence genes in *C. elegans*, winning the **2006 Nobel Prize**.
- In 2001, the term **miRNA was introduced**, distinguishing **siRNA (small interfering RNA)** for viral defense and **miRNA** for gene regulation.
- Further studies identified key proteins:
 - Dicer (cuts RNA into smaller pieces)
 - Argonaute (helps silence target genes)

Gene Count and Non-Coding RNAs

In the 1990s, scientists estimated that the human genome contained 35,000–100,000 genes. However, full sequencing later revealed only about 20,000–25,000 protein-coding genes—less than 2% of the genome. Surprisingly, over **90% of the genome is still transcribed into non-coding RNAs (ncRNAs)**, which were once thought to be useless byproducts of transcription. However, growing evidence shows that these ncRNAs play essential roles in cell functions and disease processes, challenging the traditional view of RNA as merely a messenger between DNA and proteins.

Types of ncRNAs

With advances in sequencing technology, many ncRNAs have been discovered. They are broadly classified into:

Small ncRNAs (<200 nucleotides)

Long ncRNAs (lncRNAs) (≥ 200 nucleotides)

Small ncRNAs

tRNA (transfer RNA): Helps build proteins.

snoRNA (small nucleolar RNA): Modifies and processes rRNA.

snRNA (small nuclear RNA): Plays a role in RNA splicing.

gRNA (guide RNA): Involved in RNA editing in certain organisms.

miRNA (microRNA): Regulates gene expression, controlling more than 60% of protein-coding genes.

Long ncRNAs (lncRNAs)

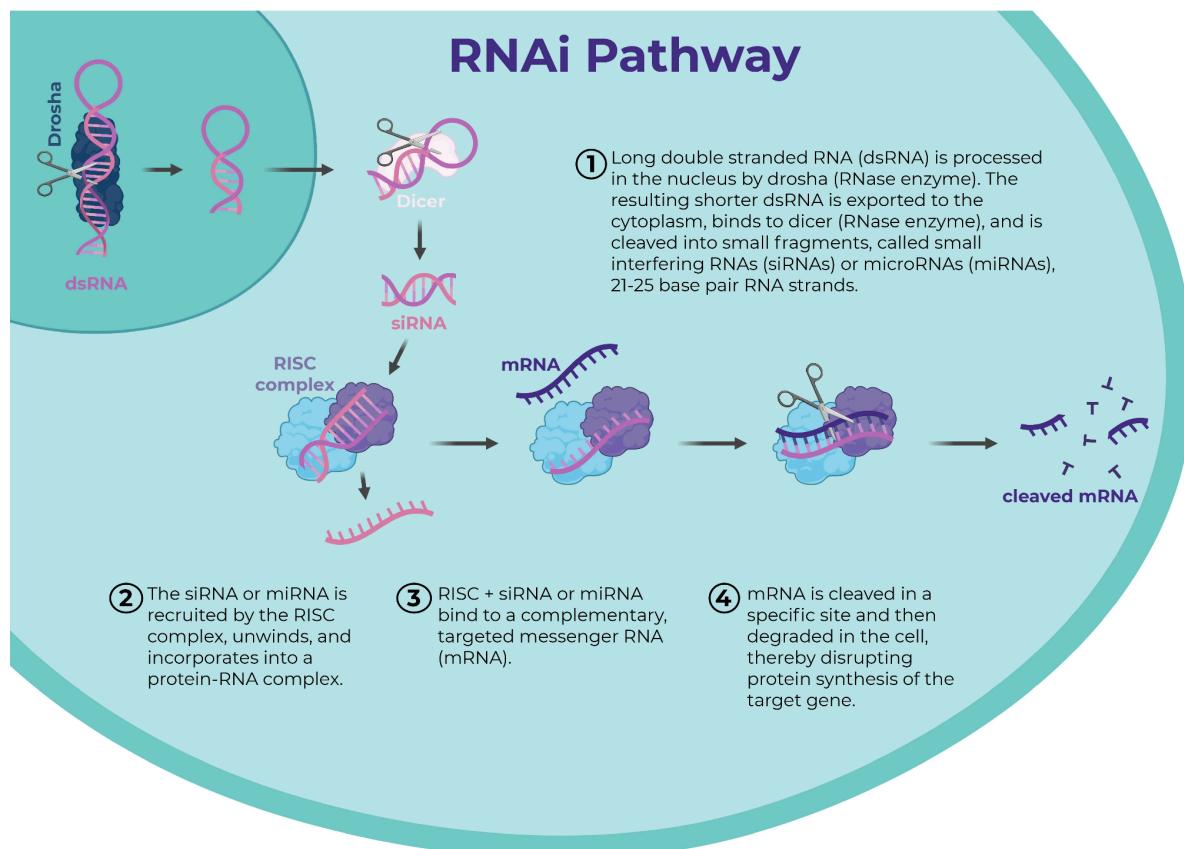
lncRNAs are the largest class of ncRNAs, but their study is relatively recent. Some well-known lncRNAs include:

- XIST & H19: Discovered in the 1980s and linked to gene regulation.
- HOTAIR: Associated with metastatic breast cancer, suggesting a role in chromatin modification.
- HULC & PTENP1: May act as decoys to bind miRNAs, affecting gene regulation.

While much remains unknown, lncRNAs are now believed to play key roles in cellular functions rather than being accidental byproducts.

Table 13.1: ncRNA classifications (based on [6, 8, 13, 20, 24])

Name	Abbreviation	Function
Ribosomal RNA	<i>Housekeeping RNAs</i>	
Transfer RNA	rRNA	translation
Small nucleolar RNA	tRNA	translation
Small Cajal body-specific RNA	snoRNA (~60-220 nt)	rRNA modification
Small nuclear RNA	scaRNA	spliceosome modification
Guide RNA	snRNA (~60-300 nt)	RNA splicing
	gRNA	RNA editing
MicroRNA	<i>Small ncRNAs (<200 nt)</i>	
Small interfering RNA	miRNA (~19-24 nt)	RNA silencing
Piwi interacting RNA	siRNA (~21-22 nt)	RNA silencing
Tiny transcription initiation RNA	piRNA (~26-31 nt)	Transposon silencing, epigenetic regulation
Promoter-associated short RNA	tRNA (~17-18 nt)	Transcriptional regulation? unknown
Transcription start site antisense RNA	PASR (~22-200 nt)	Transcriptional maintainence? not clear
Termini-associated short RNA	TSSa-RNA (~20-90 nt)	not clear
Antisense termini associated short RNA	TASR	not clear
Retrotransposon-derived RNA	aTASR	not clear
3'UTR-derived RNA	RE-RNA	not clear
x-ncRNA	uaRNA	not clear
Small NF90-associated RNA	x-ncRNA	not clear
Unusually small RNA	snaR usRNA	not clear
Vault RNA	vtRNA	not clear
Human Y RNA	hY RNA	not clear
Large intergenic ncRNA	<i>Long ncRNAs (>200 nt) lncRNA</i>	Epigenetics regulation
Transcribed ultraconserved regions	T-UCR	miRNA regulation?
Pseudogenes	none	
Promoter upstream transcripts	PROMPT	miRNA regulation? Transcriptional activation? telomeric heterochromatin main- tenance
Telomeric repeat-containing RNA	TERRA	not clear
GAA-repeat containing RNA	GRC-RNA	not clear
Enhancer RNA	eRNA	not clear
Long intronic ncRNA	none	not clear
Antisense RNA	aRNA	not clear
Promoter-associated long RNA	PALR	not clear
Stable excised intron RNA	none	not clear
Long stress-induced non-coding transcripts	LSINCT	not clear



Dicer and Gene-Silencing Techniques

After the discovery of RNA interference (RNAi), scientists quickly realized it could be used as a tool to silence genes in the lab. RNAi technology developed faster than the understanding of its biology. Early methods used **long double-stranded RNA (dsRNA), which could be introduced into organisms like invertebrates to suppress specific genes.** In some cases, organisms like nematodes could absorb dsRNA from their environment, triggering gene silencing. The process requires **Dicer, an enzyme that cuts the long dsRNA into small interfering RNAs (siRNAs)**, which are the key molecules that silence genes.

The use of RNAi allows scientists to "knock down" the expression of almost any gene. The technology has advanced significantly, especially for use in mammalian cells, which is the focus here.

RNA Silencing Mechanisms:

1. RNAi Discovery: Initially discovered in plants as "post-transcriptional gene silencing," RNAi has since been found in a wide range of organisms, from plants to animals. dsRNA precursors are processed into small RNA duplexes (21-28 nucleotides), which guide the silencing of complementary RNA sequences, like mRNA or viral RNA.
2. Types of Small RNAs:
 - siRNAs (Short Interfering RNAs): Derived from dsRNA (e.g., from viruses), they guide mRNA degradation or chromatin modification.
 - rasiRNAs (Repeat-Associated siRNAs): Produced from repetitive sequences (e.g., transposons), they also guide mRNA degradation and chromatin modification.
 - miRNAs (MicroRNAs): Formed from hairpin-shaped dsRNAs in the cell, these mainly inhibit translation but can also degrade mRNA.
3. Sources of dsRNA:
 - Viral dsRNA: Produced by RNA viruses, leading to the generation of siRNAs.
 - Transgene or Transposon dsRNA: Caused by overlapping transcripts of repetitive sequences, generating siRNAs or rasiRNAs.
 - Endogenous dsRNA: Formed by inverted repeats in genes that fold into dsRNA hairpins, which are processed into miRNAs.

Role in Gene Expression Regulation:

RNA silencing mechanisms were first recognized as antiviral defenses against RNA viruses and to control the integration of transposable elements. However, its role in gene regulation became apparent when it was discovered that specific genes encode small dsRNA molecules, such as miRNAs, which regulate gene expression.

- In Plants: miRNAs mainly function as siRNAs, guiding cleavage of complementary mRNAs.
- In Animals (e.g., *Caenorhabditis elegans*): miRNAs mainly repress translation by targeting partially complementary sequences in the 3' untranslated region (UTR) of mRNAs.

Mechanisms of RNA Processing:

1. Processing of dsRNA Precursors:

- The maturation of small RNAs is a multi-step process, primarily catalyzed by RNase-III enzymes (Drosha and Dicer).
- Drosha processes miRNA precursors in the nucleus, cutting them to create a 5' phosphate and a 2-nucleotide 3' overhang.
- The miRNA precursor is exported to the cytoplasm where it is further processed by Dicer.

2. Dicer Processing:

- Dicer is responsible for cutting dsRNA into small RNA duplexes (~21 nucleotides), which can be incorporated into RNA silencing complexes.
- Dicer has multiple forms across species:
 - In *Drosophila*, Dicer-1 processes miRNA precursors, while Dicer-2 processes long dsRNAs for siRNA production.
 - In *Arabidopsis thaliana*, four Dicer-like proteins (DCL1–DCL4) are involved in processing dsRNA from different sources (miRNA, viral RNA, and transposons).

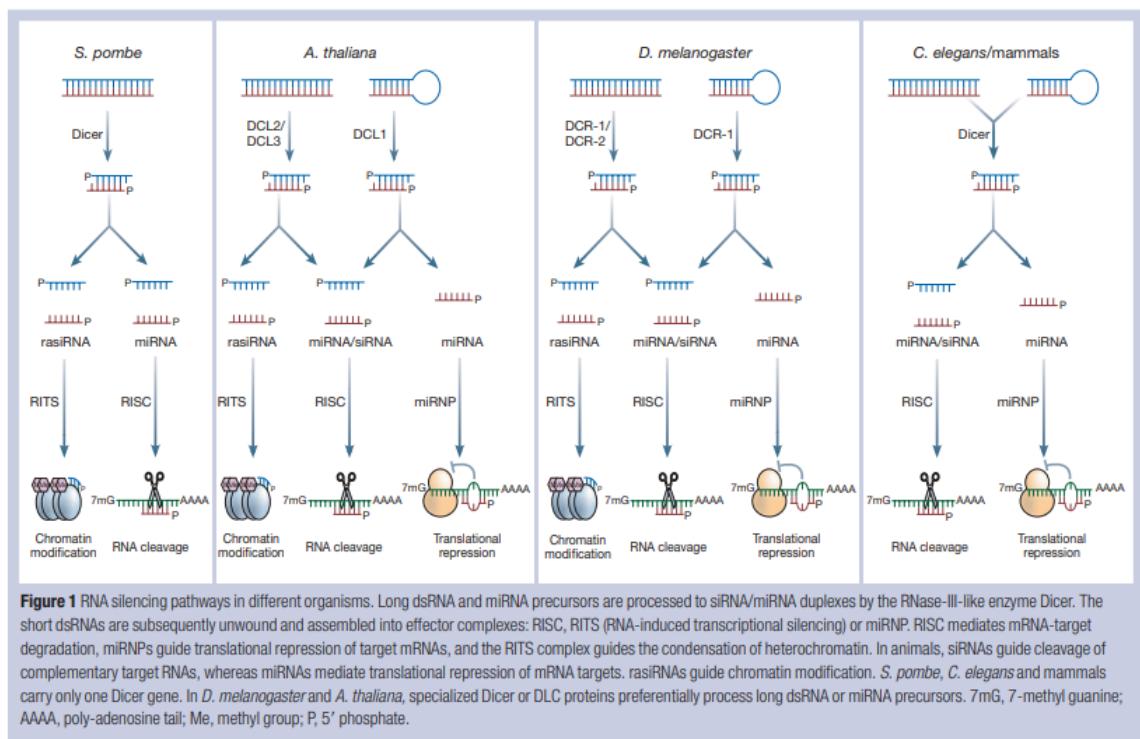
3. Dicer in Different Organisms:

- In *C. elegans*, a single Dicer (DCR-1) is involved in RNA interference (RNAi), working with proteins like RDE-4 to recognize dsRNA.
- Mammals and *C. elegans* may have additional Dicer-interacting proteins, allowing for flexibility in recognizing various dsRNA sources.

RNA silencing is an evolutionarily conserved mechanism that regulates gene expression through the processing of dsRNA into small RNAs. While the basic mechanisms are conserved, variations exist among species in the origin of dsRNA and the proteins involved in RNA silencing. Understanding these processes is key to developing tools for gene regulation and therapeutic interventions.

Assembly of RNA Silencing Effector Complexes

Once small RNA duplexes (siRNAs and miRNAs) are processed, they are integrated into ribonucleoprotein particles (RNPs) that form the RNA-induced silencing complex (RISC) or miRNA RNPs (miRNPs). These complexes contain single-stranded siRNAs or miRNAs and are crucial for RNA silencing functions, including gene silencing through mRNA cleavage or translational repression.



Other Proteins in RISC and miRNPs:

In addition to Ago proteins, other proteins have been identified in larger RISC and miRNP complexes:

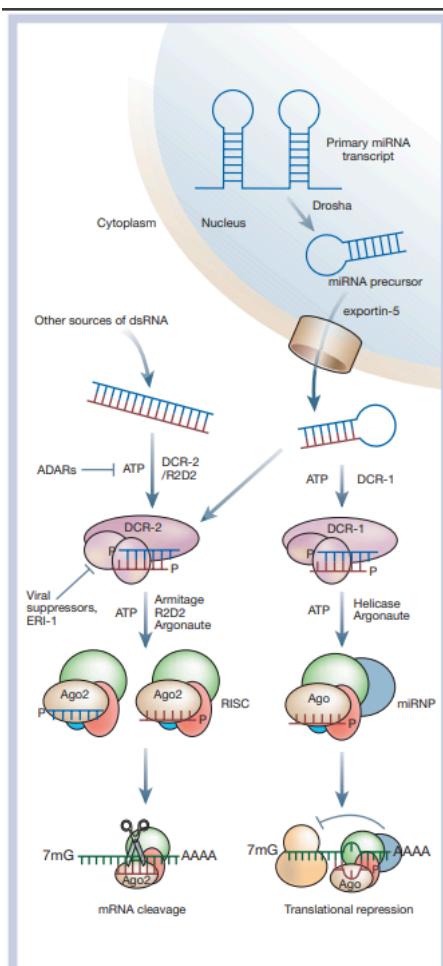
- **Drosophila RISC:** Includes Vasa intronic gene product (VIG), fragile-X-related protein (dFXR), and Tudor-SN protein.
- **Drosophila miRNPs:** Includes AGO2, dFXR, and the RNA helicase RM62, indicating a role in translational repression.

- **Human miRNAs:** Contain AGO2, and RNA helicases like Gemin3 and Gemin4. While these additional proteins are important, their precise functions in RNA silencing are still unclear.

Translational Repression by miRNAs:

- Early Evidence: The first evidence of translational repression by miRNAs came from studies in *C. elegans*, where miRNAs targeted specific genes and reduced protein synthesis without affecting mRNA levels. These miRNAs bound to sites in the 3' untranslated region (UTR) of the target mRNA and were found on polyribosomes, suggesting a block in translation elongation or termination.
- Mechanism in Mammals: In mammalian cells, miRNAs are also associated with polysomes, further supporting the idea that miRNAs may interfere with translation elongation or termination rather than initiation.

siRNA and miRNA Overlap: miRNAs can sometimes act like siRNAs and vice versa, depending on the context and complementarity between the small RNA and its target. This blurs the lines between the two types of RNA, suggesting that the complexes they form might have distinct functions based on the protein factors they recruit.



● **Simultaneous Mechanisms:** It's likely that miRNA-guided translational regulation and mRNA degradation are coordinated mechanisms. Both processes help control gene expression at different levels, depending on the context and the specific small RNA involved.

1. **miRNA Production:** Drosha processes primary miRNAs in the nucleus, and exportin-5 transports them to the cytoplasm.
2. **miRNA Processing:** Dicer processes miRNA precursors into small RNA duplexes in the cytoplasm.
3. **RISC Assembly:** Small RNA duplexes unwind and form RISC complexes with Ago proteins for gene silencing.
4. **Other dsRNA Sources:** Long dsRNA comes from viral RNAs, artificial dsRNA, or RdRP-generated dsRNAs.
5. **dsRNA Processing:** Dicer processes long dsRNAs into 21-23 nt intermediates for RISC loading.
6. **Regulation:** ADARs and ERI-1 regulate dsRNA stability and Dicer recognition.

Regulators of RNAi

Amplification of Silencing by RNA-Dependent RNA Polymerases (RdRPs):

- In plants and nematodes, RNA silencing is amplified by RNA-dependent RNA polymerases (RdRPs), which synthesize dsRNA from target mRNAs or their cleavage products. This amplification enhances the silencing effect.
- Absence in Animals: In species like *D. melanogaster* and vertebrates, no equivalent RdRP proteins are present, and there is no amplification mechanism for RNA silencing triggers.

Immune Response Against RNA Viruses:

- RNA silencing is part of an innate immune response against RNA viruses and transposable elements. Viruses, however, often develop countermeasures to suppress RNA silencing:
 - Viral Inhibitors: Some viruses produce proteins, such as dsRNA-binding proteins, that interfere with the host's RNA silencing machinery. For example, the viral suppressor protein p19 from plant tombusviruses binds to siRNA duplexes, preventing their recognition and action by the host cell.
- Spreading Silencing Signal: In plants, RNA silencing can spread from infected cells to neighboring cells. This systemic silencing requires proteins that facilitate the transfer of the silencing signal, though the exact nature of the signal (dsRNA, siRNA, RISC, or other factors) remains debated.
- Regulation of RNA Silencing in Animals: Negative Regulators:
 - ADARs (Adenosine Deaminases): In animals, dsRNA-specific ADARs can edit the target mRNA, converting adenosine to inosine. This editing reduces the complementarity between the dsRNA and mRNA, making the mRNA a poor substrate for Dicer.
ERI-1: In *C. elegans*, the ERI-1 protein inhibits RNAi, particularly in neurons. Loss-of-function mutants show enhanced RNAi activity in neurons.
RRF-3: Another negative regulator of RNAi in *C. elegans*, RRF-3 competes with RNA amplification machinery, inhibiting the production of secondary small RNAs.

RNA silencing is a complex and finely regulated process involving multiple proteins and mechanisms. While the cleavage of target mRNAs by RISC is a well-studied aspect, the broader mechanisms of translational repression and the regulation of RNA silencing pathways (both positive and negative) highlight the complexity of gene regulation. Understanding these processes is crucial for harnessing RNA silencing in therapeutic applications and defending against viral infections.

RNAi silencing in Plants

Key RNA Silencing Pathways:

Cytoplasmic siRNA Silencing: Important in virus-infected plants, where dsRNA forms during viral replication or as a secondary structure of RNA. This silences genes in response to viral infections and transgene expression.

miRNA-Mediated Silencing: miRNAs regulate gene expression by binding to specific mRNAs, leading to RNA cleavage or stopping protein production. These 21-24 nucleotide RNAs are processed by Dicer from precursor RNAs.

Chromatin-Level Silencing: Involves DNA methylation and histone modification, often guided by siRNAs. This pathway is important in defending against transposons and maintaining genome stability.

Protein Families Involved: Argonaute (Ago) Proteins: Key in all RNA silencing pathways, binding to siRNAs and miRNAs for gene silencing.

Dicer Proteins: Involved in processing RNA into siRNAs or miRNAs. Different Dicers handle different pathways, like miRNA biogenesis or chromatin silencing.

Plant miRNA Differences:

- miRNA processing occurs in the nucleus in plants, unlike in animals (which process miRNAs in both the nucleus and cytoplasm).

Plant miRNAs are more perfectly matched to their targets and typically cleave the mRNA, while animal miRNAs mainly suppress translation.

Plant miRNAs often target coding sequences or 5' UTR, while animal miRNAs target the 3' UTR.

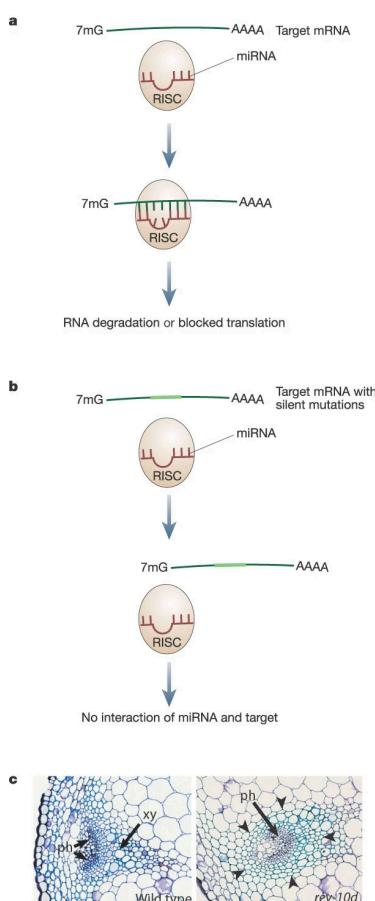
miRNA Target Validation in Plants:

- Several plant miRNAs have been linked to transcription factors involved in key developmental processes like leaf morphology, hormone responses, and floral development.

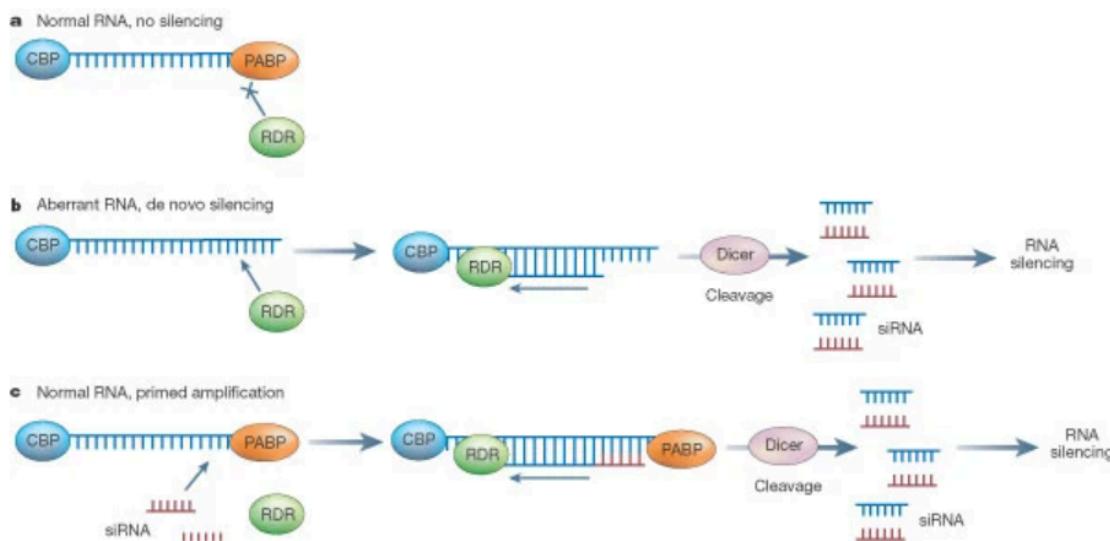
In a **normal plant (a)**, an **miRNA** in the **RISC complex** binds to its target mRNA, causing either degradation of the RNA or blocking translation. However, if a **transgene** is introduced with **mutations** in the miRNA target sequence

(b), the miRNA can't bind, and the protein from that mRNA is **overexpressed**.

• In **transgenic plants (c)**, the **rev-10d transgene** makes the **revoluta transcription factor** resistant to miR165 and miR166 targeting. In wild-type plants, the **xylem (xy)** is inside the **phloem (ph)**, while in the rev-10d plants, the xylem forms a **radial pattern** around the phloem, indicating that miR165 and miR166 are essential for correct positioning of xylem and phloem tissues.



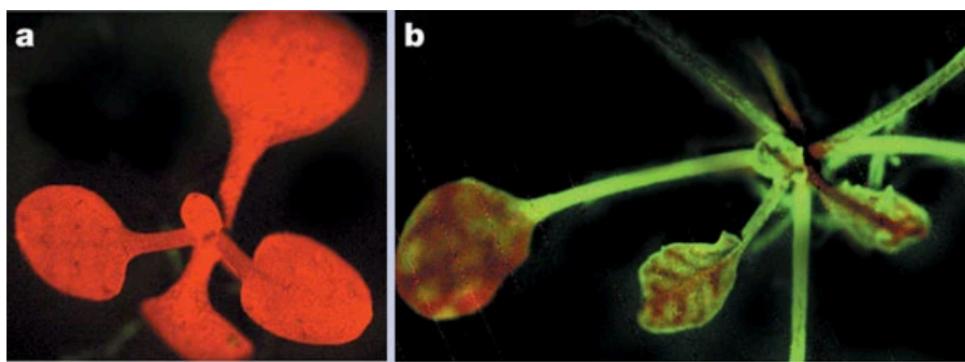
In **a**, RNAs are usually not silenced because the **RDR proteins** cannot access the RNA due to the presence of a **5' cap** and **3' poly-A tail**. These structures restrict access.



In **b**, if the RNA lacks these structures (cap and poly-A tail), the **RDR protein** can access it, leading to the production of **dsRNA**. This dsRNA then enters the **siRNA pathway**, amplifying the silencing process.

In **c**, if there's a small amount of **primary siRNA** from a virus, transposon, or cellular RNA, the **antisense strand** of this siRNA can bind to a target RNA. This forms a primer for **RDR**, which produces more **dsRNA**. This dsRNA is cleaved by **Dicer**, amplifying the process by generating many **secondary siRNAs** from each primary siRNA.

There are two panels showing *A. thaliana* plants with two transgenes:

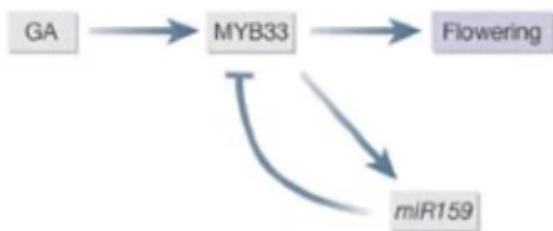


- **Panel a:** In the wild-type plant, a **GFP transgene** is always transcribed, but its fluorescence is suppressed because a second transgene producing **GFP dsRNA** is expressed in the **phloem cells**. The silencing signal spreads from the phloem and silences the GFP transgene throughout the leaf. Under UV light, the plant appears red due to **chlorophyll fluorescence**.
- **Panel b:** In a plant with a **RDR6 mutation**, the silencing signal only works in cells near the phloem. The GFP transgene is not silenced in cells farther than about 20 cells from the

phloem, causing those cells to appear green under UV light.

miR159 and flowering regulation:

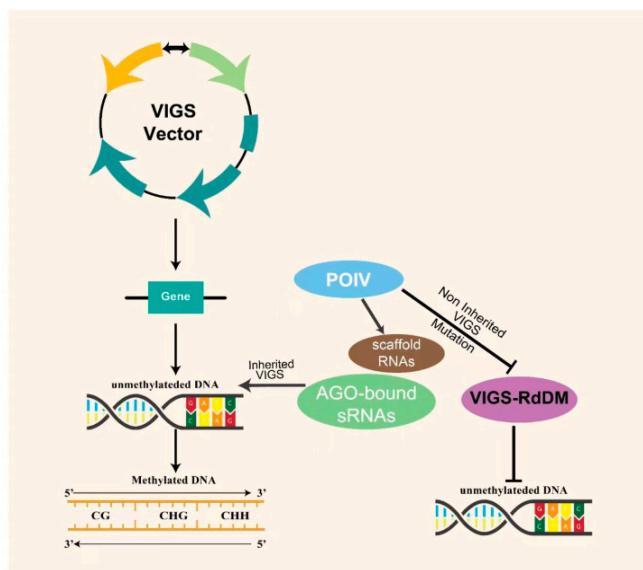
- miR159 and its target, MYB33 (a transcription factor), are **both regulated by GA (gibberellic acid)**.
- GA stimulates MYB33, promoting **flowering**. This triggers an increase in **miR159**, which then suppresses MYB33, acting as a negative feedback loop to **dampen the GA response**.



miR159 regulation is influenced by the plant hormone **GA**. **MYB33** transcription factor is regulated by both **miR159** and **GA**. **GA** increases **MYB33 mRNA** and **miR159** levels. High **miR159** then suppresses the **GA-induced MYB33 increase**, creating a feedback loop that amplifies the silencing effect. Investigation of *miR159* and *miR319* may tell us about the potential importance of secondary targets because the two miRNAs differ at only three nucleotide positions and have *MYB* or *TCP* mRNAs, respectively, as distinct primary targets.

Popular RNAi Methods for Gene Silencing in Plants and Their Limitations

- **VIGS (Virus-Induced Gene Silencing)** is a widely used method to silence genes in plants, helping researchers study genes involved in stress responses (e.g., drought, salinity, oxidative stress).



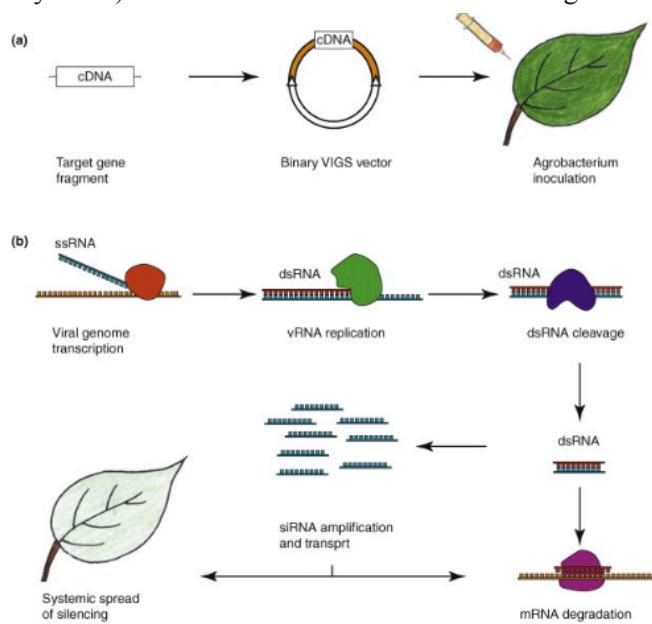
Epigenetic Silencing in VIGS:

1. **Vector Insertion:** The VIGS vector is inserted into the cell targeting the gene for silencing.

2. **Scaffold RNA Production:** POIV synthesizes **scaffold RNAs** that provide binding sites for AGO-associated siRNAs.

3. **Gene Silencing:** The AGO-siRNAs direct **DNA methylation**, which leads to **epigenetic gene silencing**.

4. **Effect of Mutation:** A mutation in **PolV** causes a loss of **VIGS-RdDM** (RNA-directed DNA methylation) and results in **non-heritable** silencing.



- Gene Insertion (a):** A fragment of the target gene (like PDS) is inserted into the viral genome (e.g., Tobacco Rattle Virus or TRV), which is carried by a binary vector with T-DNA. Agrobacterium tumefaciens is used to transfer this modified plasmid into the plant.
- Viral Infection (b):** Once the plant is infected, the viral genome (carried by T-DNA) integrates into the plant's DNA. The plant's **RNA Polymerase** (red) transcribes the viral genome.
- dsRNA Production:** **RNA-dependent RNA Polymerase** (green) generates **dsRNA** from the viral RNA.
- siRNA Formation:** The **Dicer-like enzymes** (blue) chop the dsRNA into small interfering RNAs (**siRNAs**).
- Gene Silencing:** These siRNAs are incorporated into the **RISC complex** (purple), which then uses the siRNAs to target and degrade the corresponding plant mRNA, silencing the gene.
- Spread of Silencing:** The siRNAs spread through the plant, triggering gene silencing in distant parts of the plant, resulting in visible phenotypes like **photobleaching** on leaves.

This method enables gene silencing in plants, even in tissues far from the original infection site.

Viral-Vector	Targeted-Gene	Next-Generation Efficiency	Delivery Method	Reference
Cotton leaf crumple virus (CLCrV)	<i>BRI1, GL2, PDS</i>	4.35–8.79%	<i>Agrobacterium</i> -mediated transient transformation	[64]
Tobacco rattle virus (TRV)	<i>AtPDS3</i>	30–60%	<i>Agrobacterium</i> -based flooding method	[65]
Potato virus X PVX	<i>NbXT2B, NbPDS3, NbFT</i>	100% for <i>NbXT2B</i> and 20% and 30% for <i>NbPDS3</i> and <i>NbFT</i>	Agroinfiltration	[68]
Pea early-browning virus PEBV	<i>PDS</i>	57 to 63%	<i>Agrobacterium</i> transformation	[69]
Beet necrotic yellow vein virus (BNYVV)	<i>NbPDS</i>	85%	<i>Agrobacterium</i> -mediated transformation method	[70]
Barley stripe mosaic virus (BSMV)	<i>TaPDS, TaGASR7, and TaGW2</i>	12.9% to 100%	<i>Agrobacterium</i> -mediated gene delivery	[67]

	RNAi	CRISPR
Benefits	<ul style="list-style-type: none"> Pre-designed reagents readily available Useful for studying the effect of essential genes on phenotypes Studies where temporary loss-of-function is desired (e.g., to mimic the effect of a drug) 	<ul style="list-style-type: none"> Precise gene targeting with fewer off-target effects Permanent gene disruption results in robust signal Lower risk of immune response (some formats) Flexible time frame for assay
Drawbacks	<ul style="list-style-type: none"> Temporary gene disruption may require a narrow assay window Incomplete silencing (knockdown) may not produce a strong signal Associated with more off-target effects Silencing of multiple transcripts possible (introducing noise) Introduced RNA may stimulate immune response Laborious analysis and verification of true hits 	<ul style="list-style-type: none"> Cannot be used to study essential genes

RNAi: The Knockdown Method

RNAi (RNA interference) was discovered in the 1990s and involves **double-stranded RNA (dsRNA)** or **small RNAs (siRNAs/miRNAs)** silencing genes. The process:

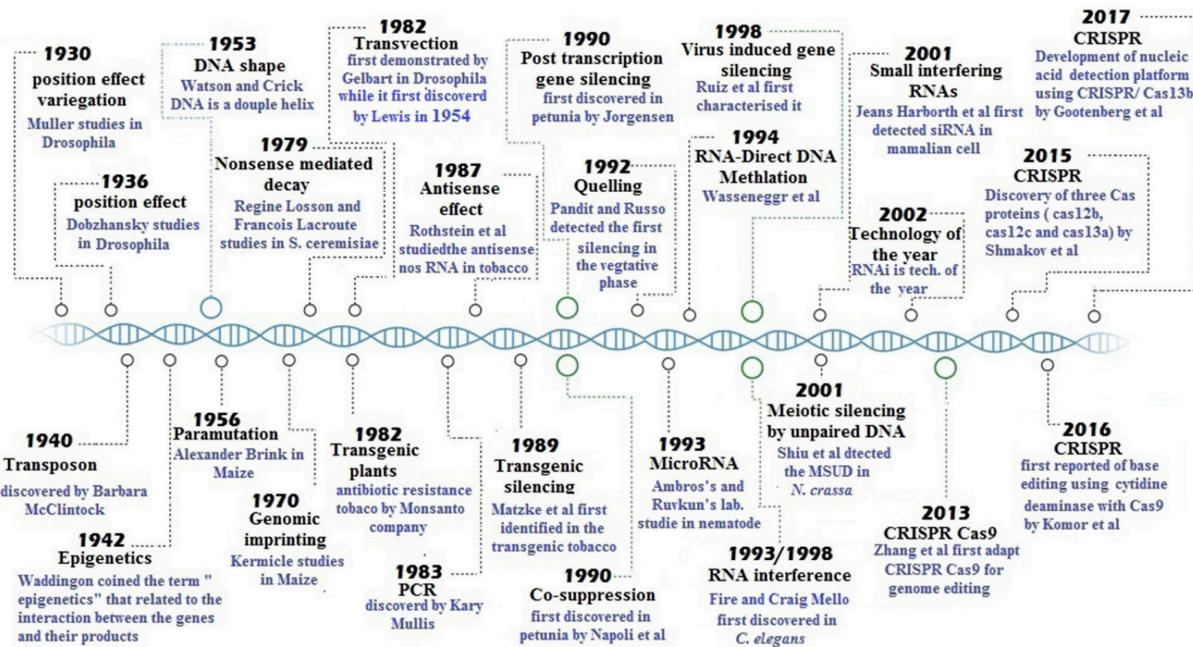
1. dsRNA or pre-miRNA is processed into short fragments by Dicer.
2. These fragments guide the RISC complex, which cleaves target mRNA to block protein production.
3. If there's an imperfect match, translation is blocked without mRNA cleavage.

CRISPR: The Knockout Method

CRISPR-Cas9 was developed in 2012 and involves editing DNA directly. The process:

1. A guide RNA directs Cas9 protein to a specific DNA sequence.
2. Cas9 cuts the DNA, leading to insertions or deletions (indels) during the DNA repair process (usually via NHEJ).
3. This results in a genetic knockout, stopping protein production.

<https://www.youtube.com/watch?v=U3Z4u0DKbx0>



	RNAi	TALE Repression	TALEN	Cas9 Nuclease	CRISPRi	CRISPRa
Loss-of-function mechanism	Post-transcriptional RNA degradation	Repression of transcription	Frame shift DNA mutation	Frame shift DNA mutation	Repression of transcription	Activation of transcription
Result	Reversible knockdown	Reversible knockdown	Permanent knockout	Permanent knockout	Reversible knockdown	Reversible activation
Transgenes	si/shRNA	TALE-KRAB	TALEN	Cas9 nuclease sgRNA	dCas9-KRAB sgRNA	dCas9-VP64 sgRNA
Guiding sequence	si/shRNA	DBD	DBD	sgRNA	sgRNA	sgRNA
Required sequence information	Transcriptome	Annotated TSS	Transcriptome	Transcriptome	Annotated TSS	Annotated TSS
Off-target space	Transcriptome	Window around TSS	Genome; requires FokI dimerization	Genome; cuts as monomer	Window around TSS	Window around TSS
Transcript variants	All variants via conserved region	Only variants from the same TSS	All variants via conserved region	All variants via conserved region	Only variants from the same TSS	Only variants from the same TSS