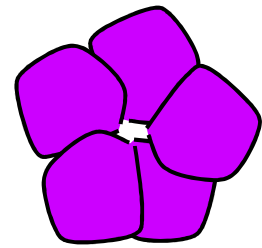
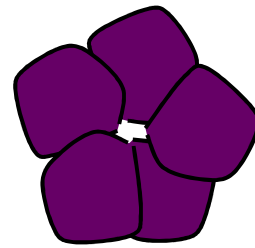
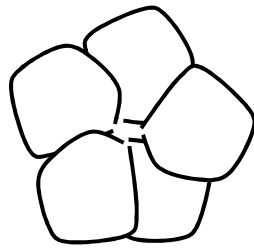
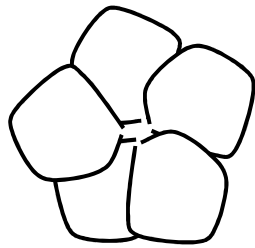
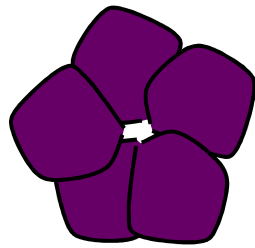
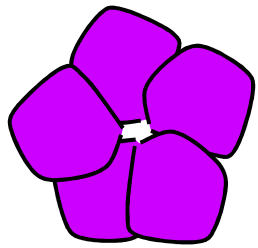


Small RNAs

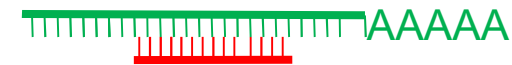


What are small RNAs?

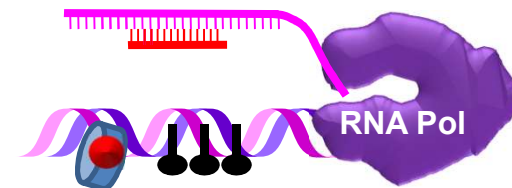
- Small RNAs are a pool of 21 to 24 nt RNAs that generally function in **gene silencing**



- Small RNAs contribute to **post-transcriptional gene silencing** by affecting mRNA translation or stability



- Small RNAs contribute to **transcriptional gene silencing** through epigenetic modifications to chromatin

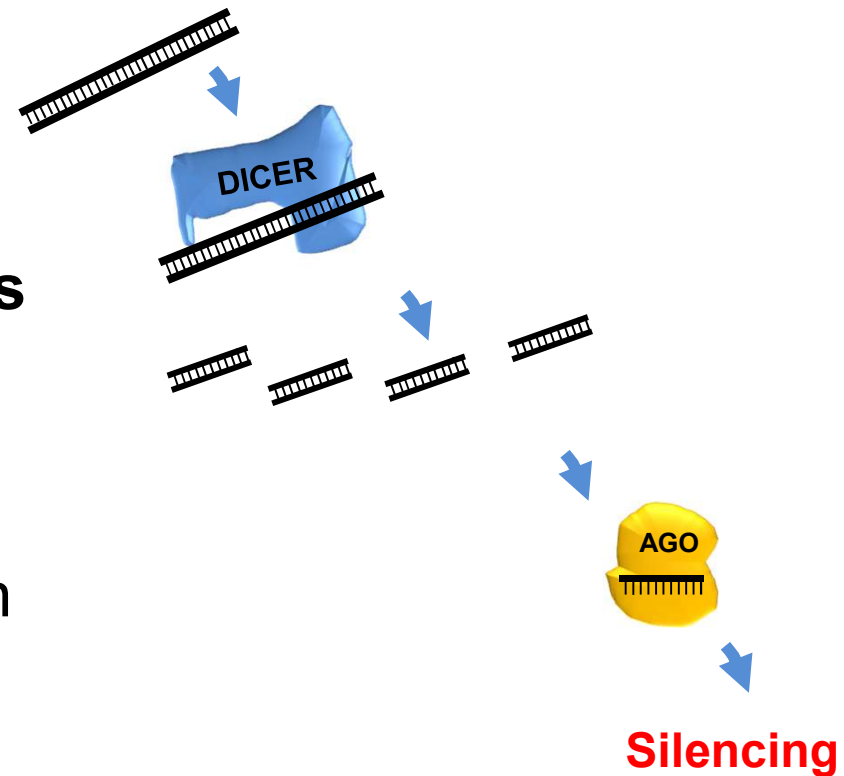


Histone modification, DNA methylation

The core of RNA silencing: Dicers and Argonautes

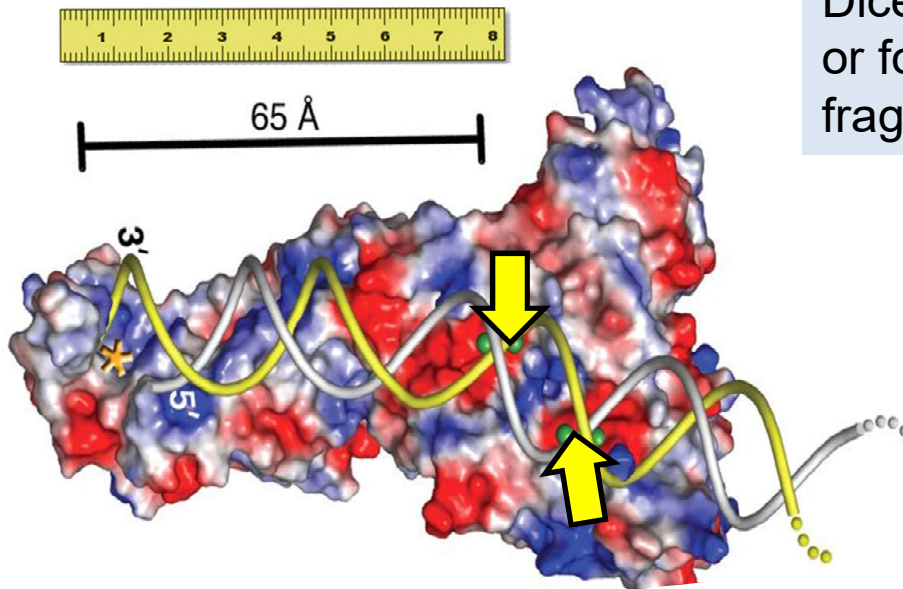
RNA silencing uses a set of core reactions in which **double-stranded RNA (dsRNA)** is processed by **Dicer** or **Dicer-like proteins** into **short RNA duplexes**.

These small RNAs subsequently associate with **ARGONAUTE** proteins to confer silencing.



Dicer and Dicer-like proteins

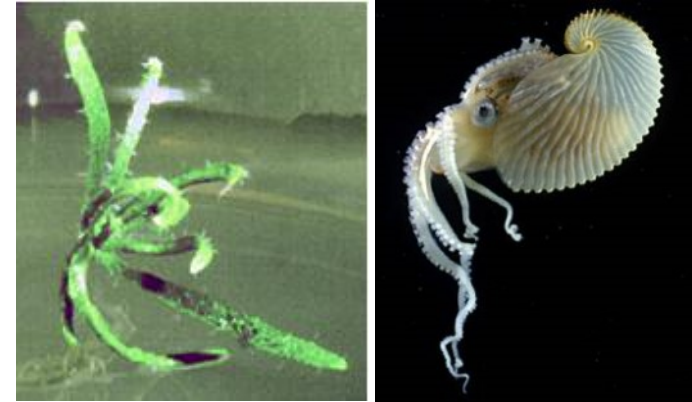
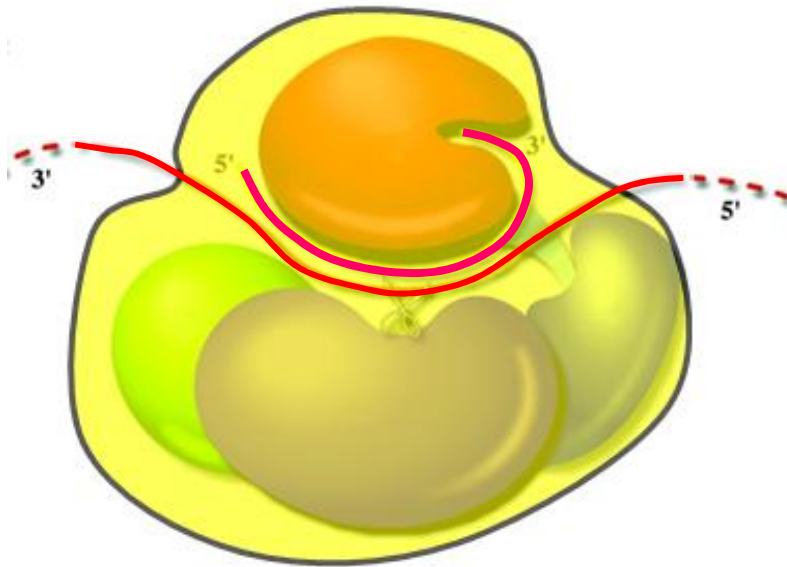
In siRNA and miRNA biogenesis, Dicer or Dicer-like (DCL) proteins cleave long dsRNA or foldback (hairpin) RNA into ~ 21 – 25 nt fragments.



Dicer's structure allows it to measure the RNA it is cleaving. Like a cook who “dices” a carrot, Dicer chops RNA into uniformly-sized pieces.

Argonaute proteins

ARGONAUTE proteins bind small RNAs and their targets.



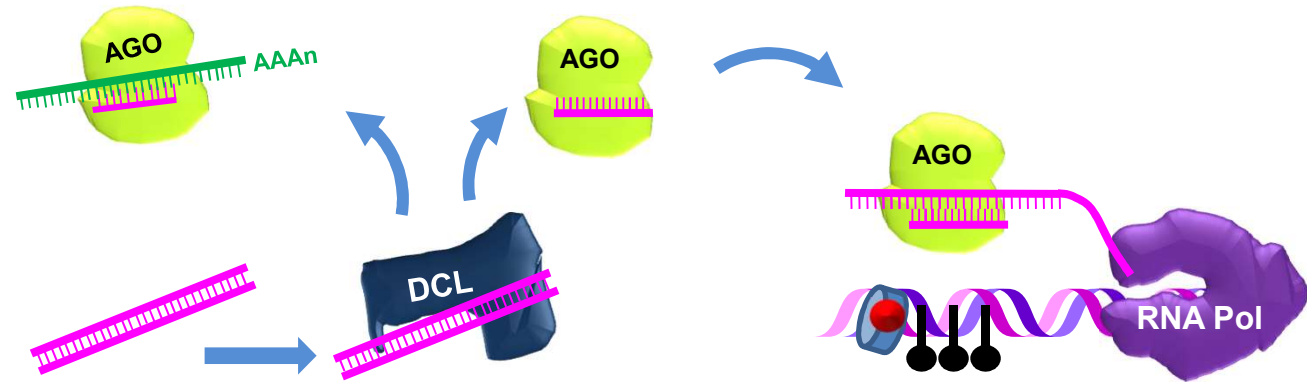
The *Arabidopsis ago1* mutant and the octopus *Argonauta argo*

ARGONAUTE proteins are named after the *argonaute1* mutant of *Arabidopsis*; *ago1* has thin radial leaves and was named for the octopus *Argonauta* which it resembles.

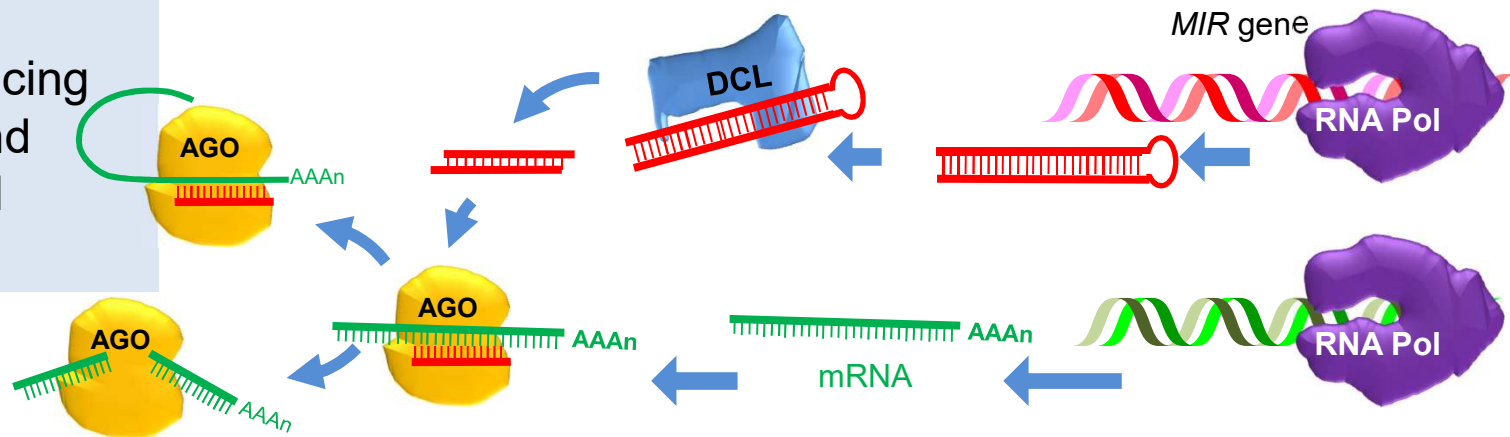
Reprinted by permission from Macmillan Publishers Ltd: EMBO J. Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J. 17: [170–180](#). Copyright 1998; Reprinted from Song, J.-J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. Science 305: [1434 – 1437](#). with permission of AAAS.

RNA silencing - overview

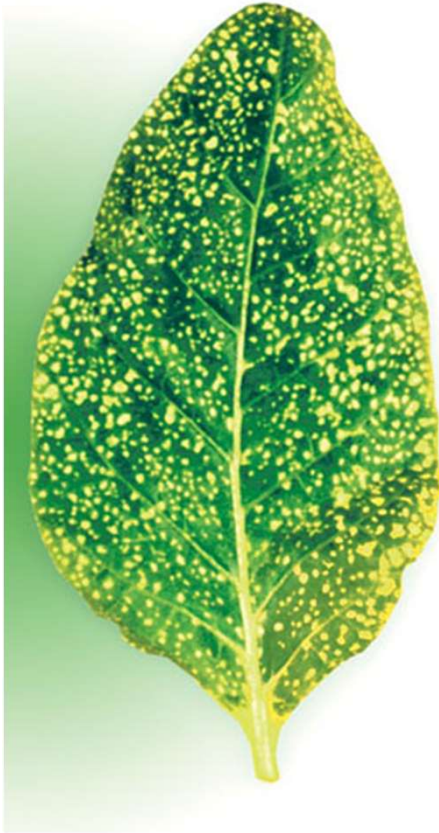
siRNA-mediated silencing via post-transcriptional and transcriptional gene silencing



miRNA - mediated slicing of mRNA and translational repression



siRNAs – Genomic Defenders

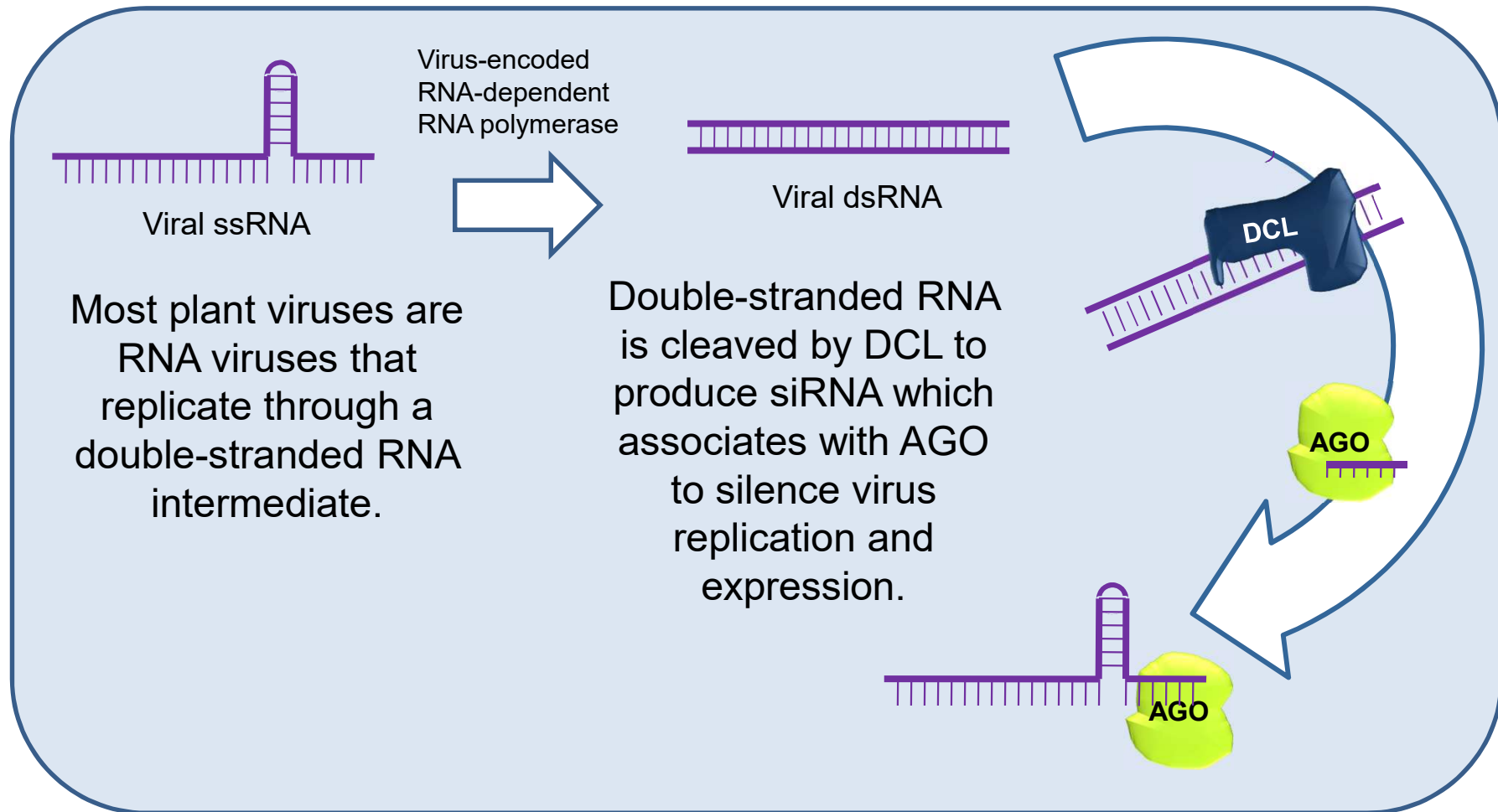


siRNAs protect the genome by

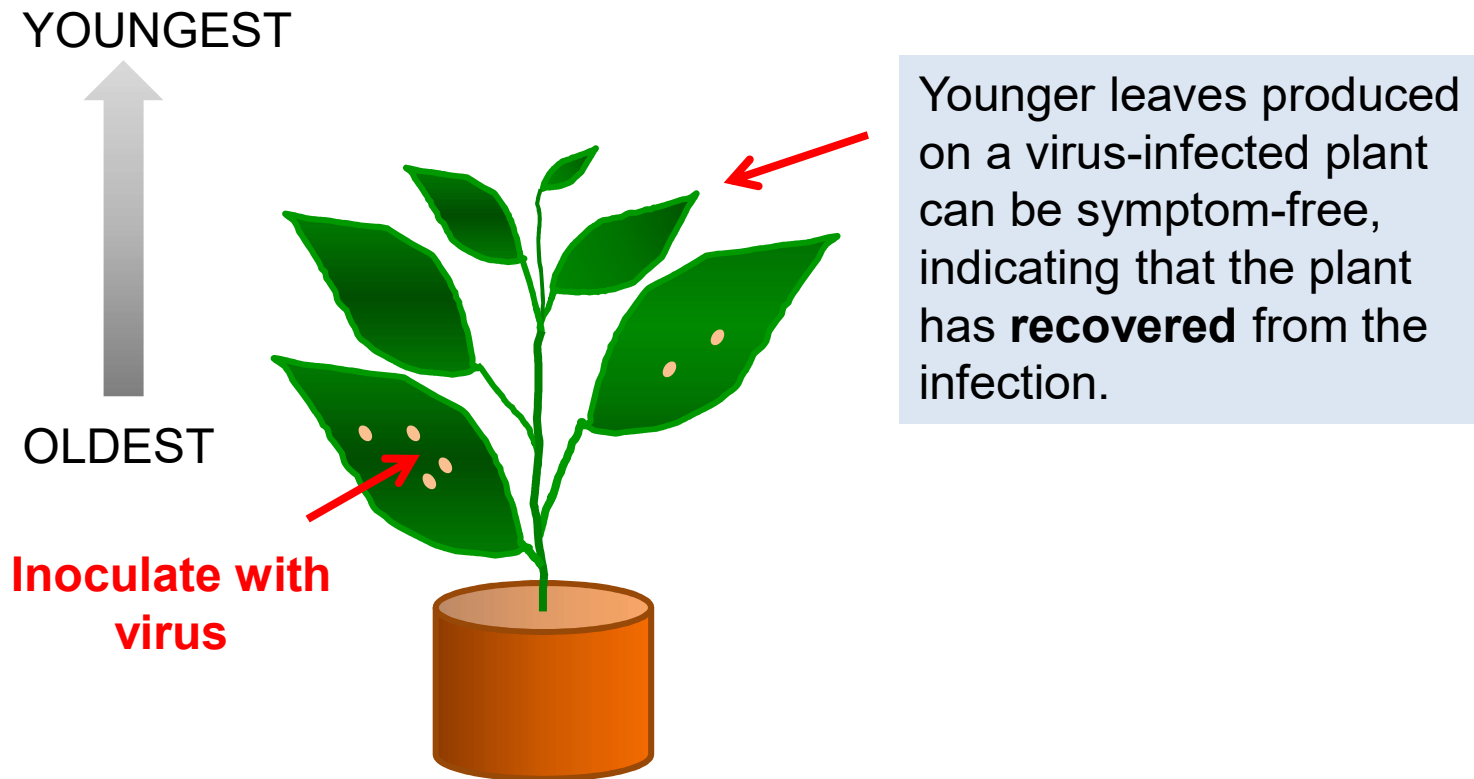
- Suppressing invading viruses
- Silencing sources of aberrant transcripts
- Silencing transposons and repetitive elements

siRNAs also maintain some genes in an epigenetically silent state

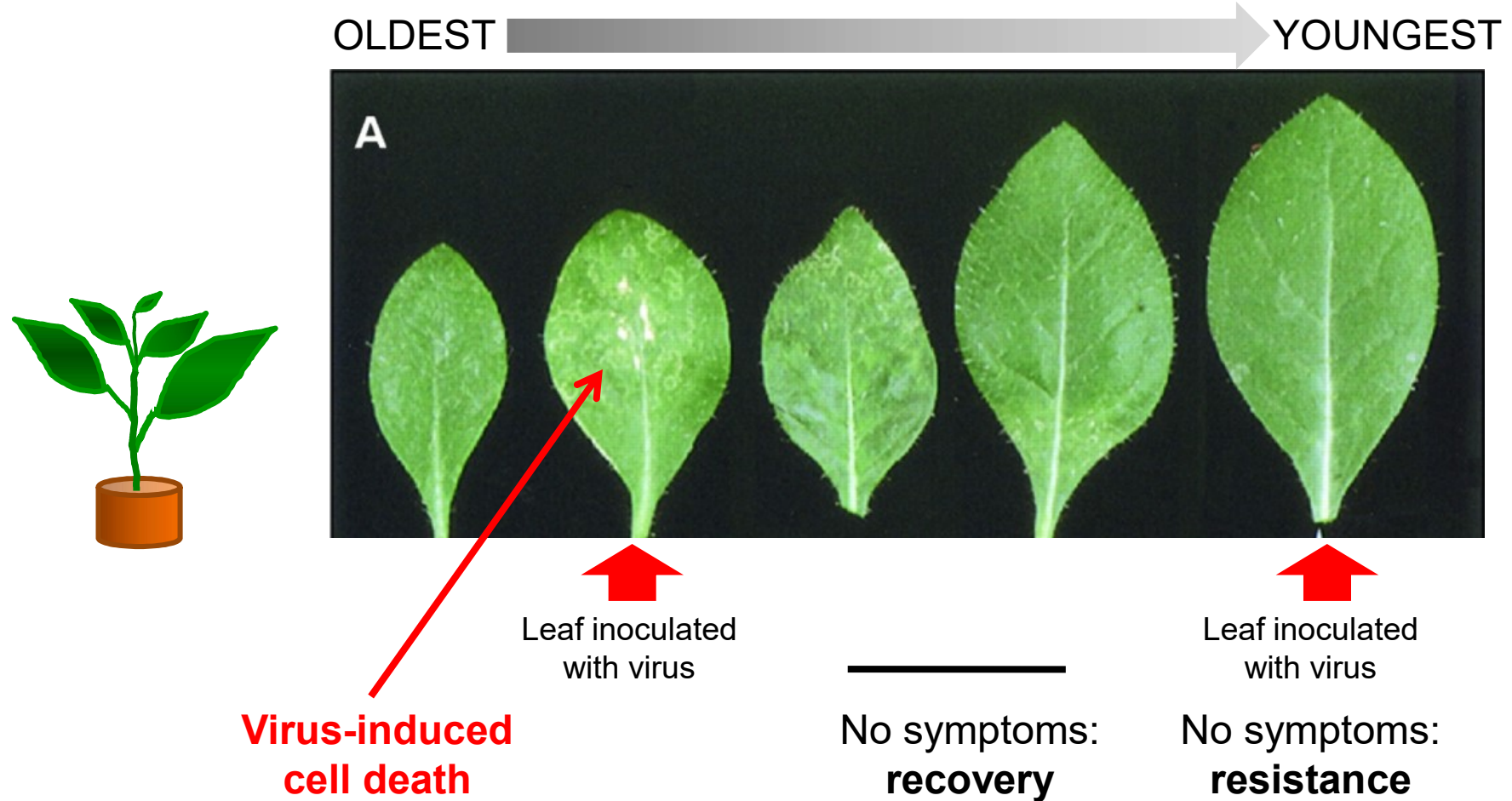
Viral induced gene silencing - overview



Plants can recover from viral infection and become resistant

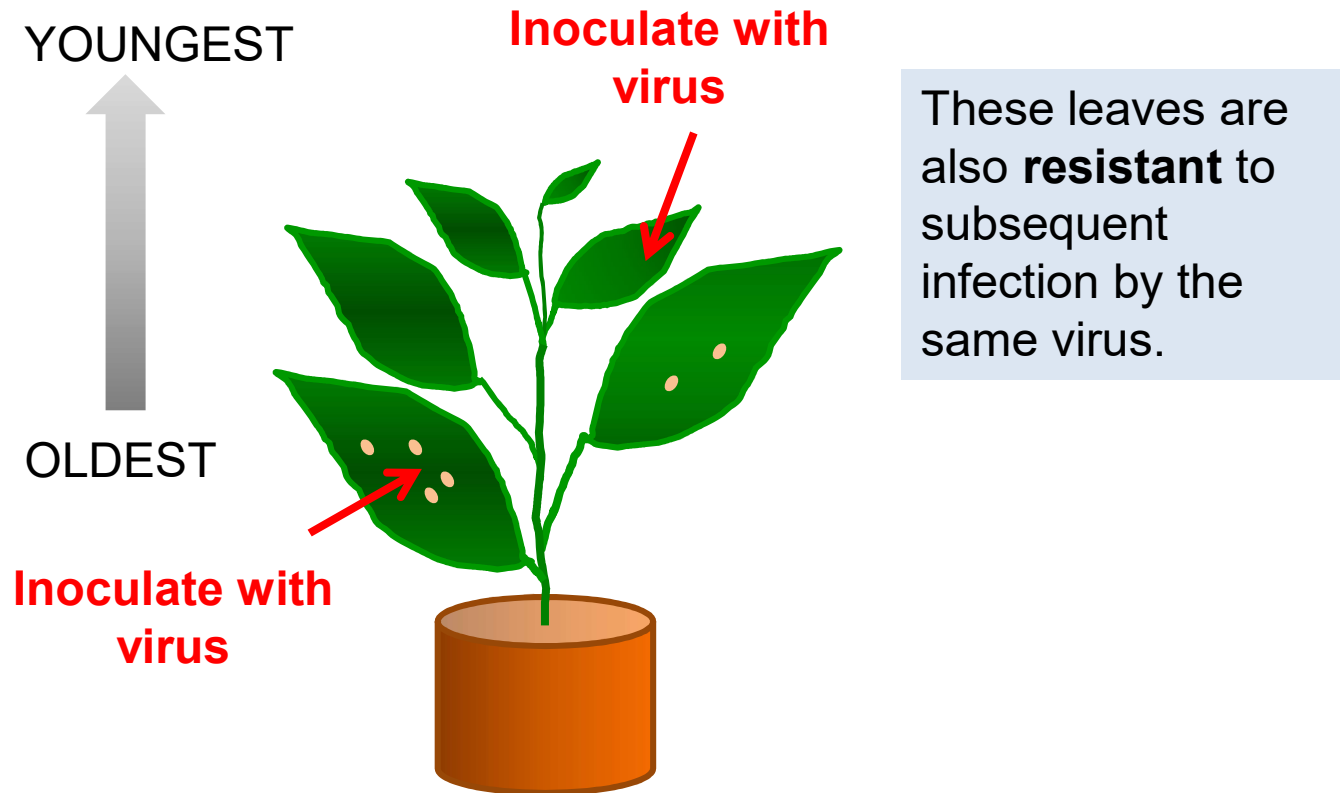


Plants can recover from viral infection and become resistant

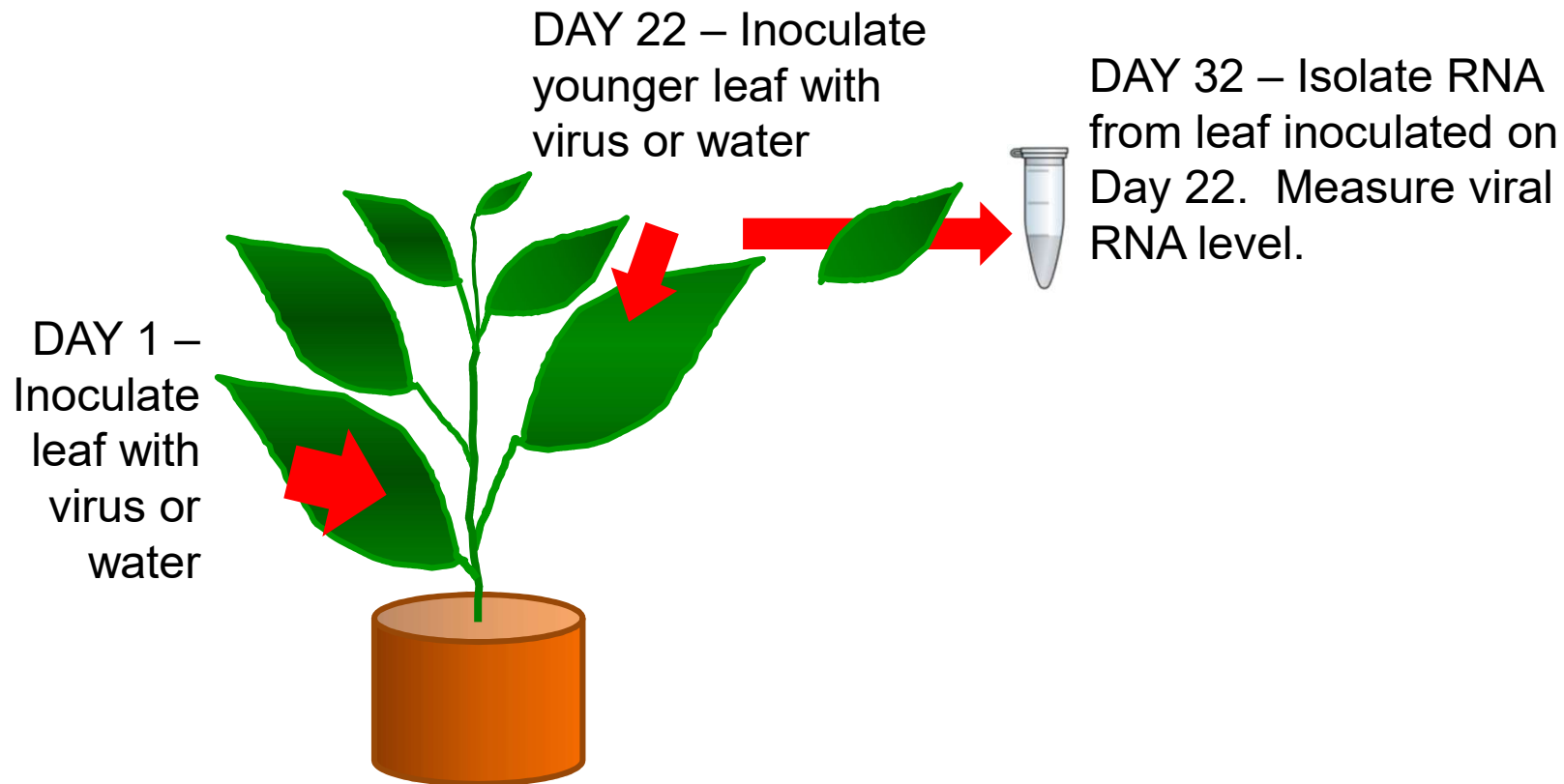


From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral and gene silencing in plants. *Science* 276: [1558-1560](#). Reprinted with permission from AAAS.

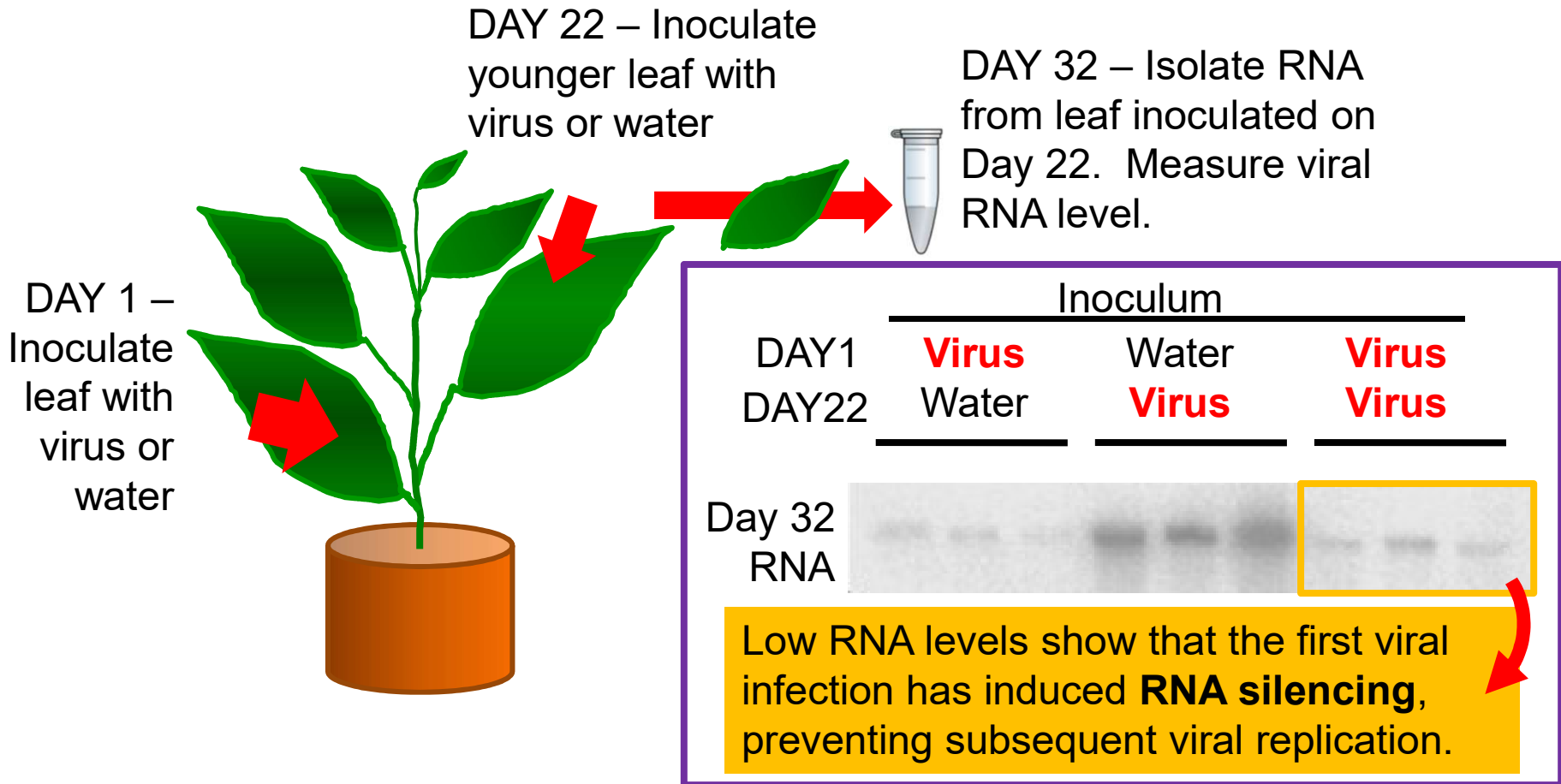
Plants can recover from viral infection and become resistant



Viral resistance involves siRNA-mediated silencing



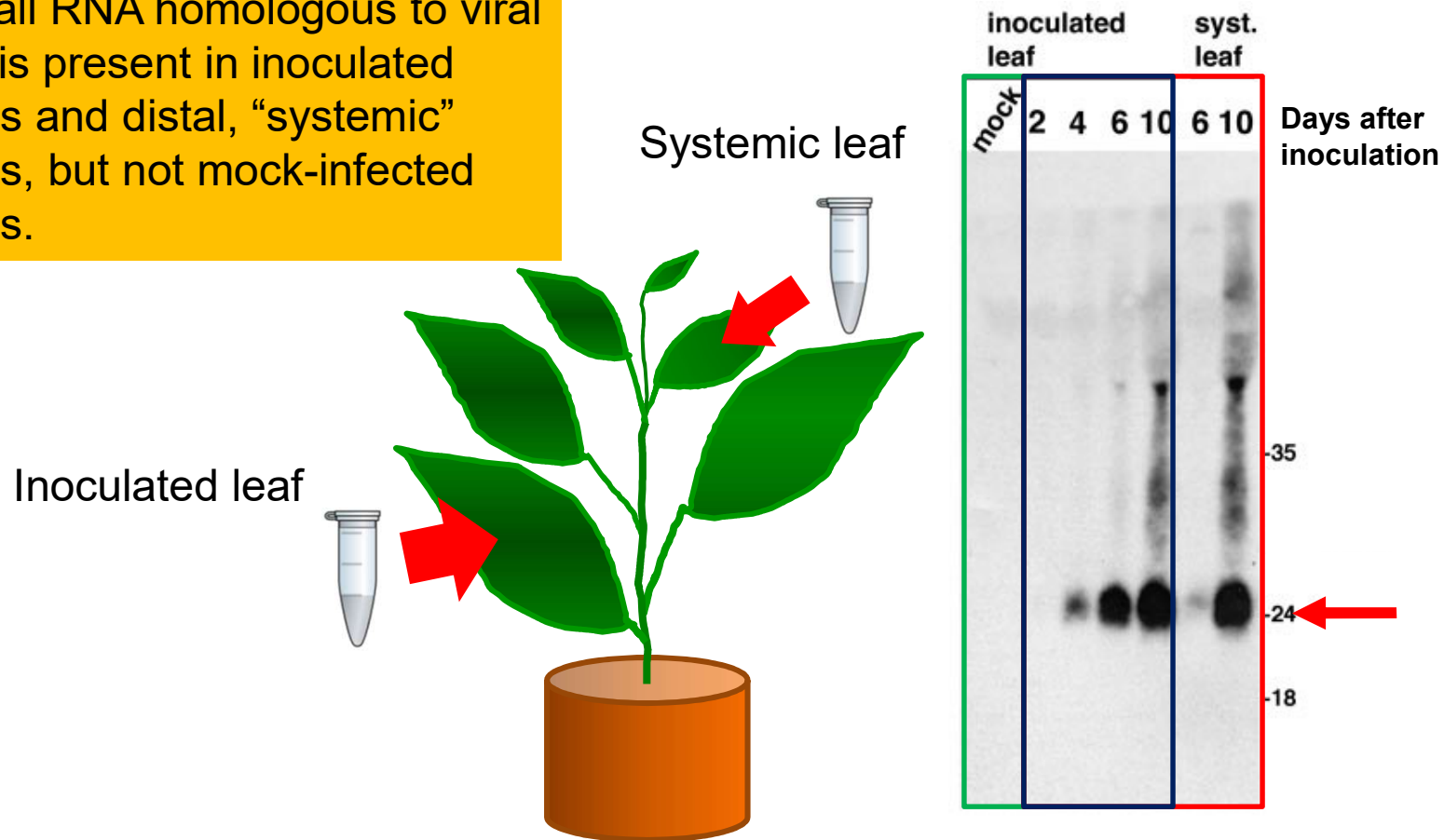
Viral resistance involves siRNA-mediated silencing



From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral defense and gene silencing in plants. *Science* 276: [1558–1560](#). Reprinted with permission from AAAS.

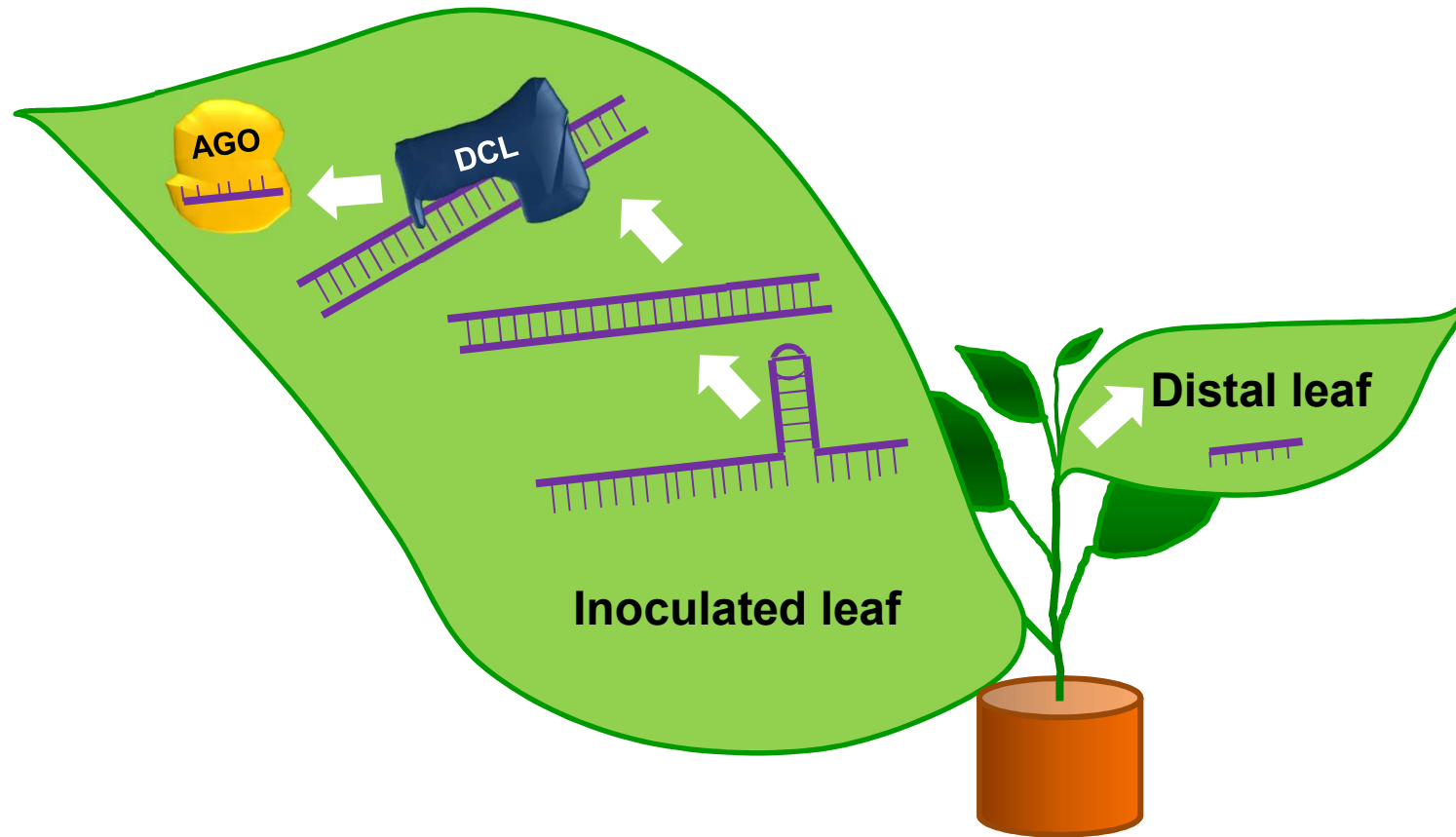
Small RNAs are correlated with viral-induced gene silencing

A small RNA homologous to viral RNA is present in inoculated leaves and distal, "systemic" leaves, but not mock-infected leaves.

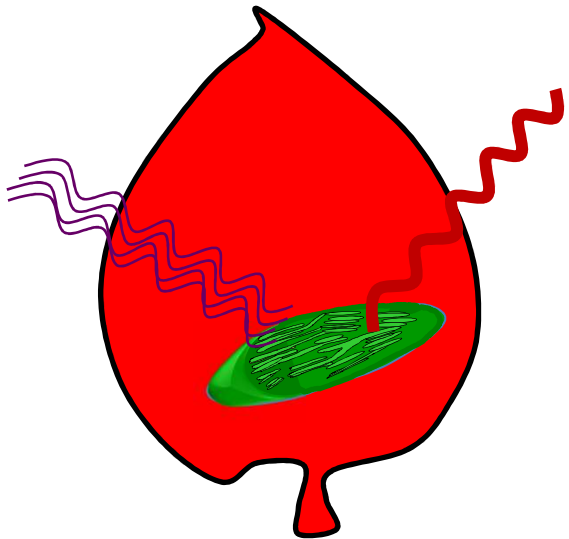


From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral defense and gene silencing in plants. *Science* 276: [1558–1560](#). Reprinted with permission from AAAS.

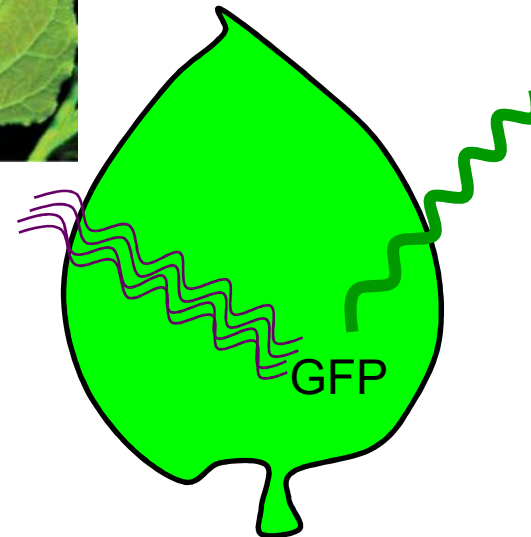
Virus infection causes systemic siRNA accumulation



How does RNA silencing spread systemically???

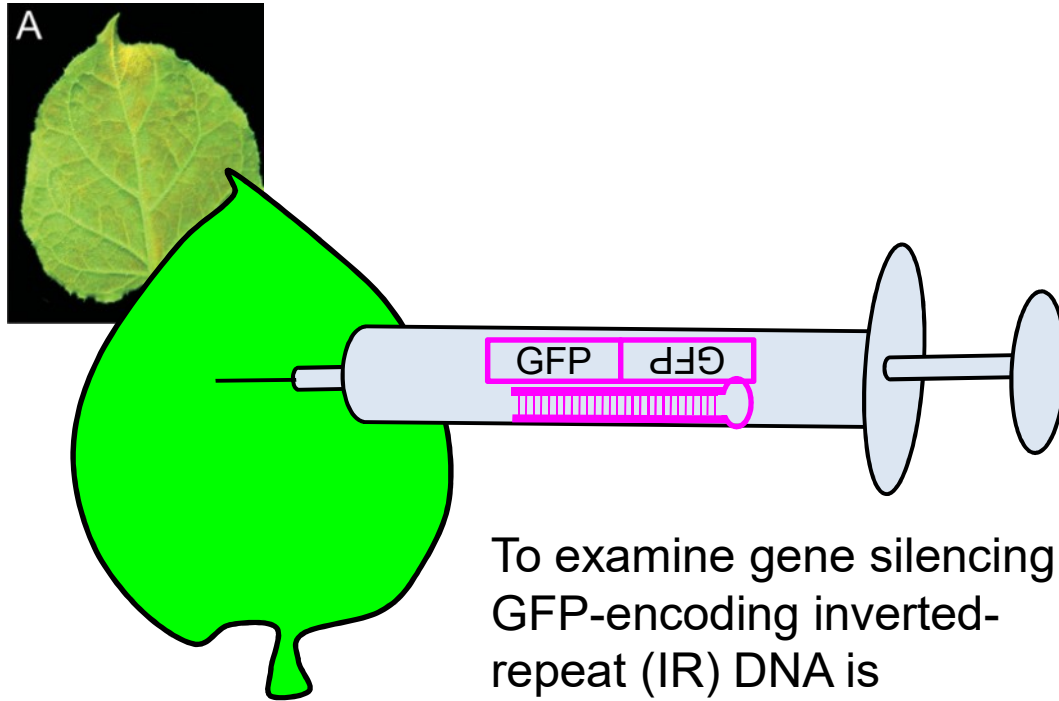


Under UV light, wild-type leaves fluoresce **red**, from chlorophyll in the chloroplasts.



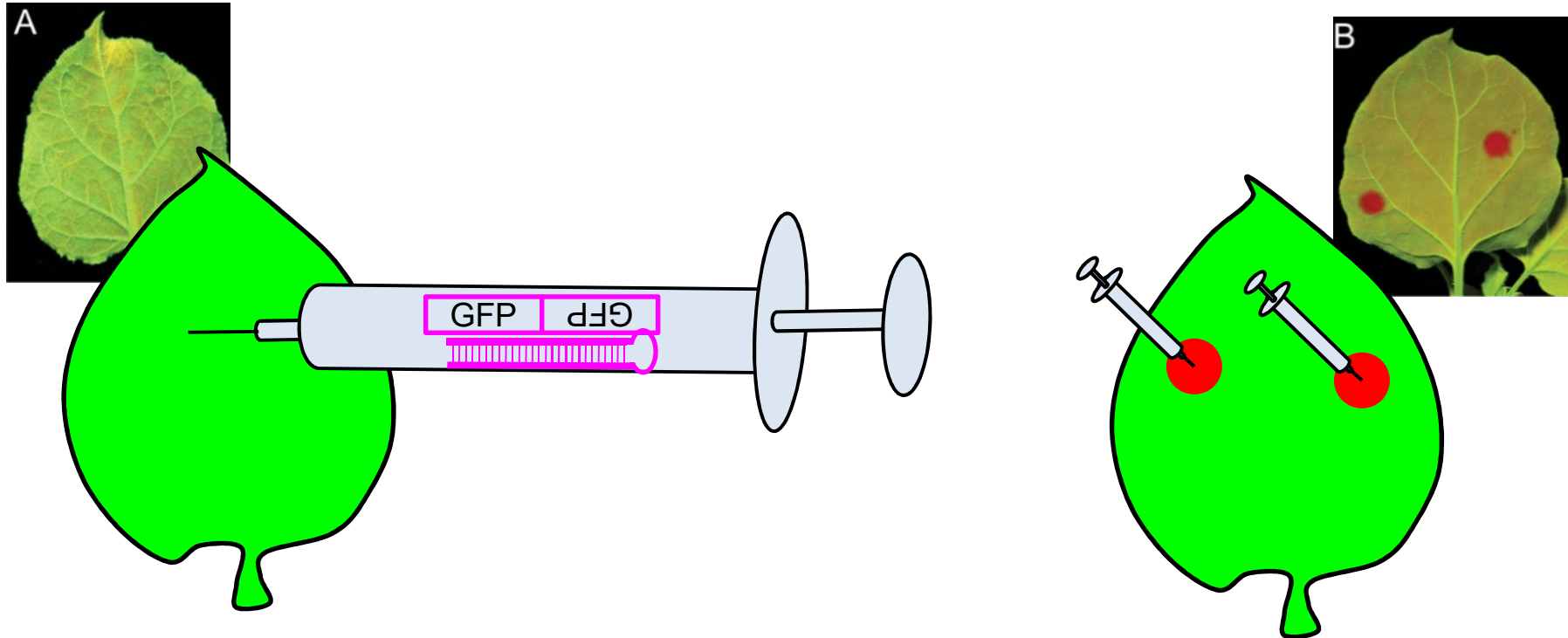
A plant expressing GFP fluoresces **green** under UV light.

Spreading of RNA silencing



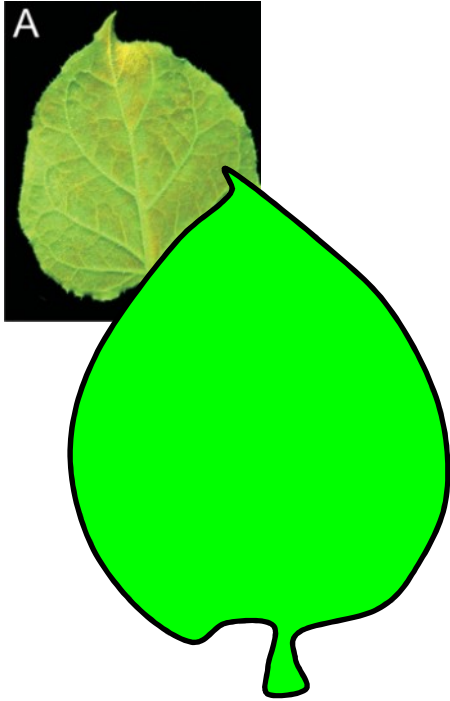
To examine gene silencing, GFP-encoding inverted-repeat (IR) DNA is introduced into the GFP-expressing cells.

Spreading of RNA silencing

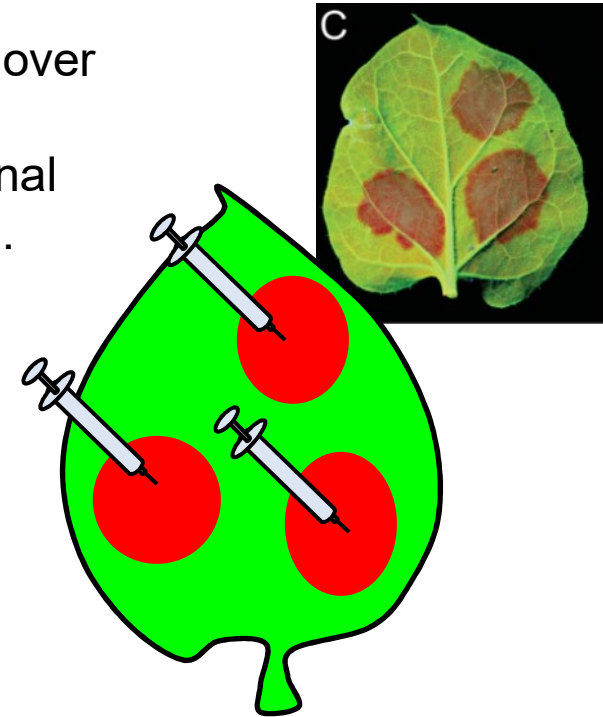


When GFP is silenced, the red chlorophyll fluorescence becomes visible.

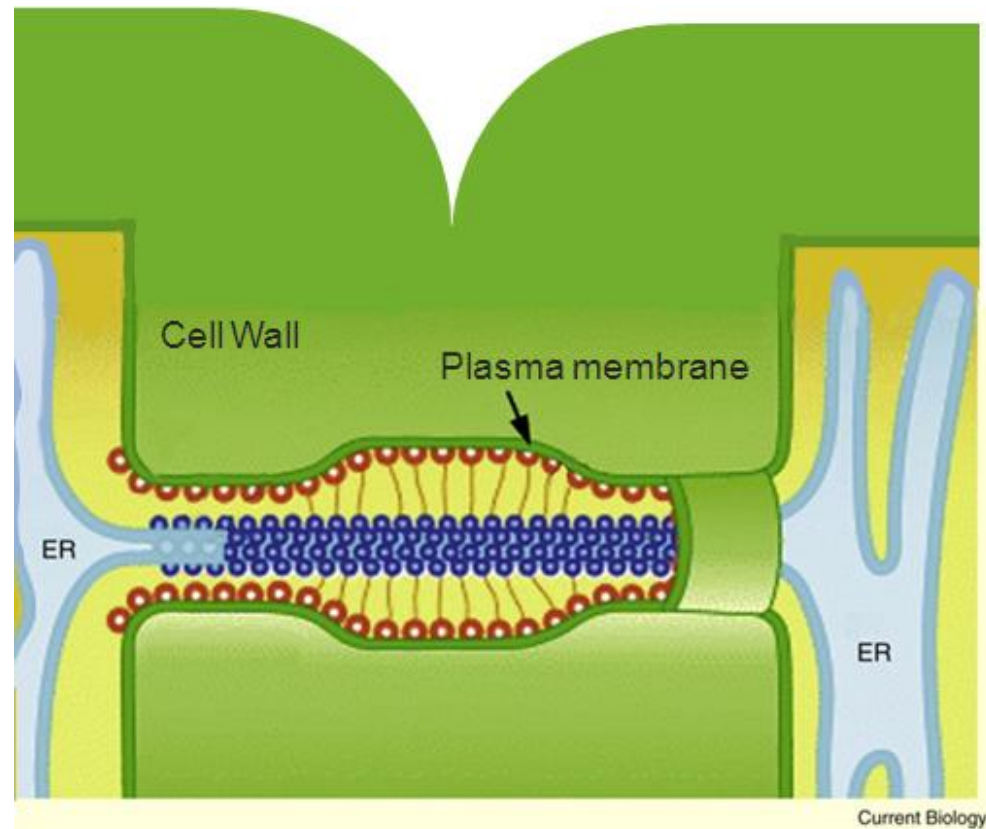
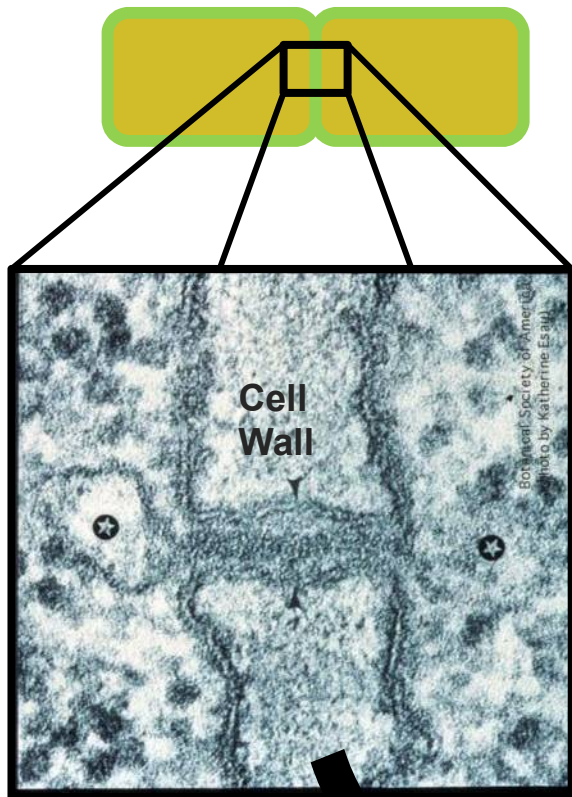
Silencing can spread locally



Often the silencing spreads over up to 15 cells, probably by diffusion of the silencing signal through the plasmodesmata.

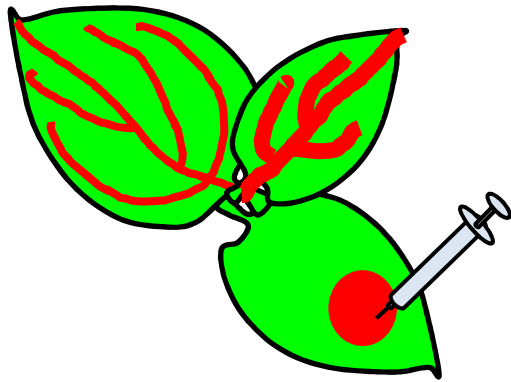


Plasmodesmata are regulated connections between plant cells

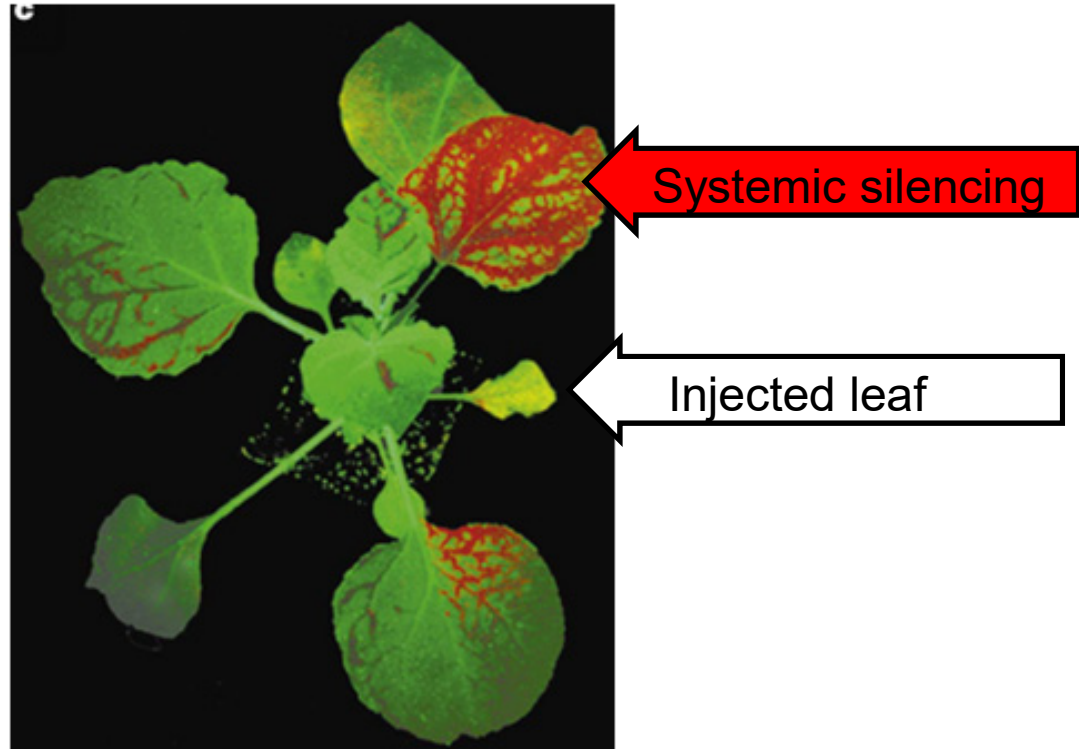


Reprinted from Zambryski, P. (2008) Plasmodesmata. *Curr. Biol.* 18: [R324-325](#) with permission from Elsevier. TEM image credit [BSA](#) Photo by Katherine Esau;

Silencing can spread systemically through the phloem

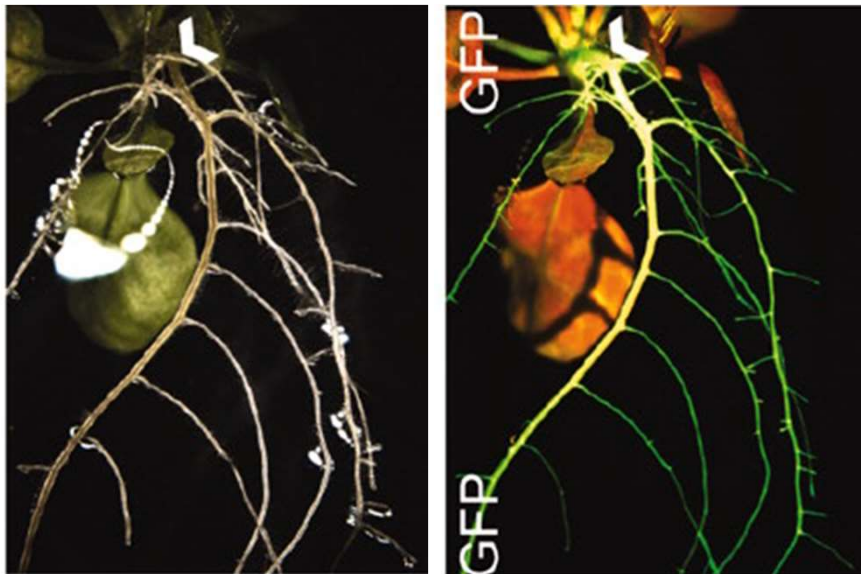


Recent experiments have shed light on the identity of the silencing signal...



Small RNAs can move from shoot to root in Arabidopsis

Control GFP expressing plant showing GFP in shoot and root



White light

Fluorescence

GFP-inverted repeat-expressing shoot grafted onto GFP root – newly formed roots do not express GFP (indicated by arrowheads)

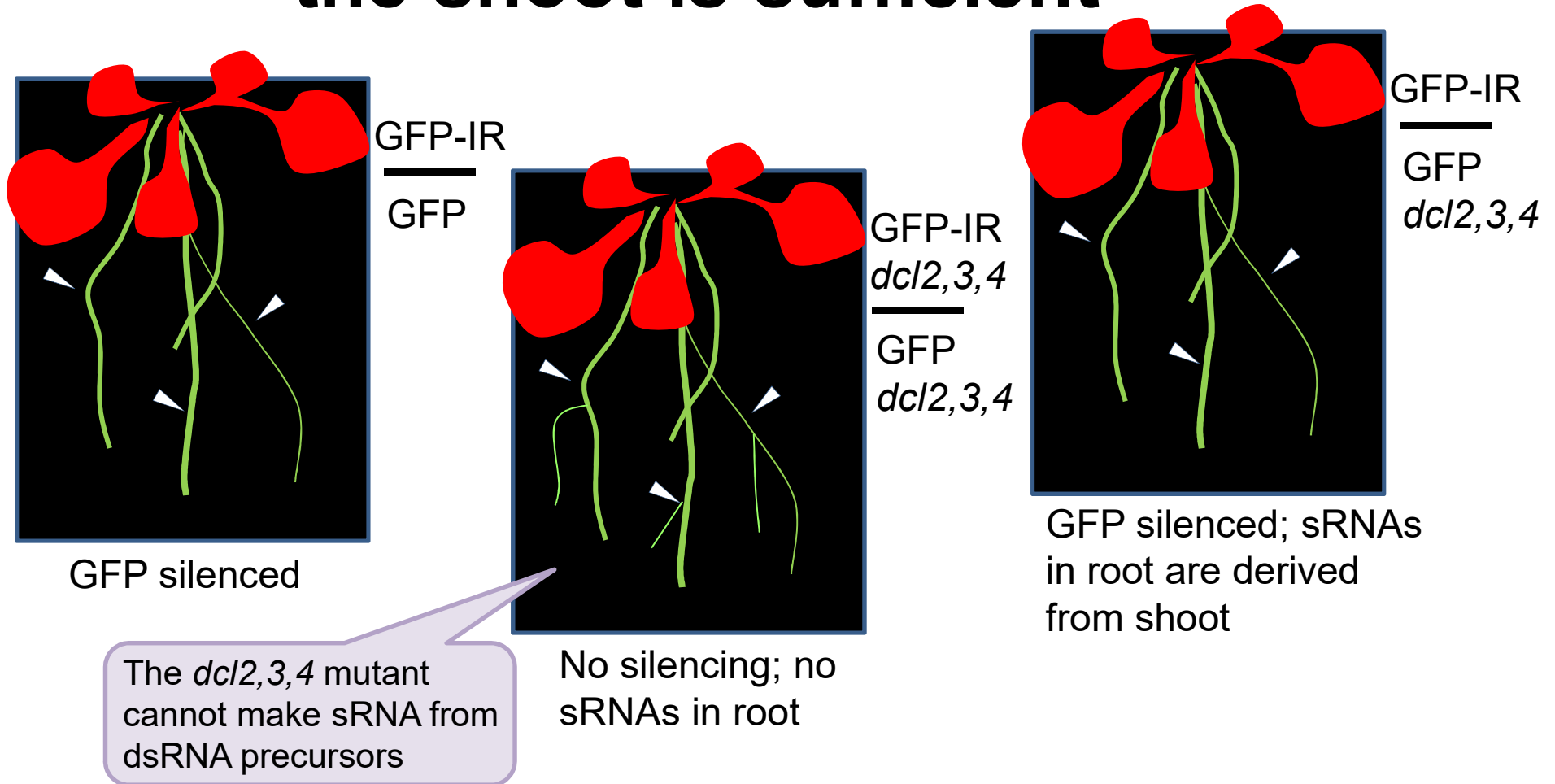


White light

Fluorescence

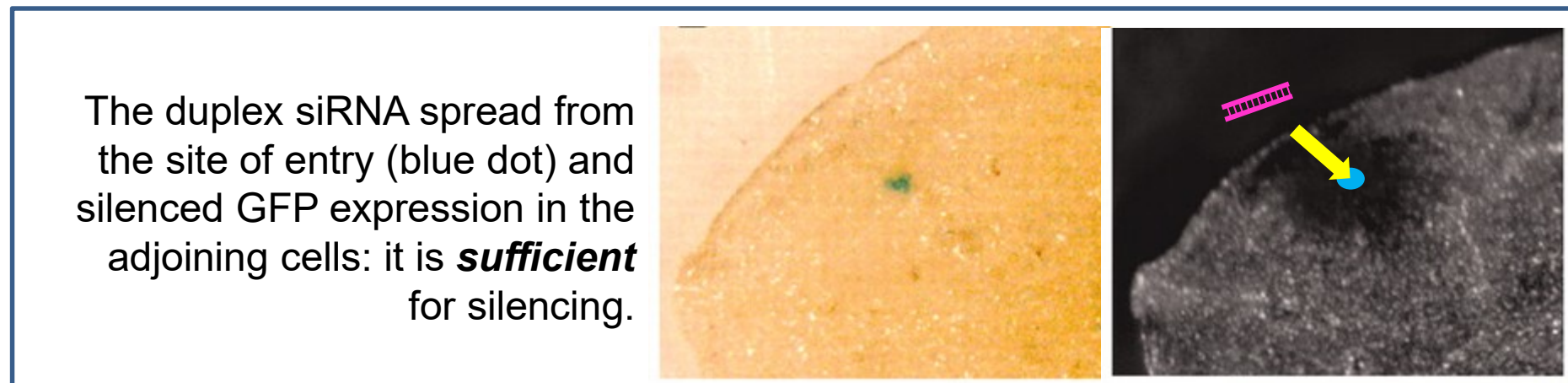
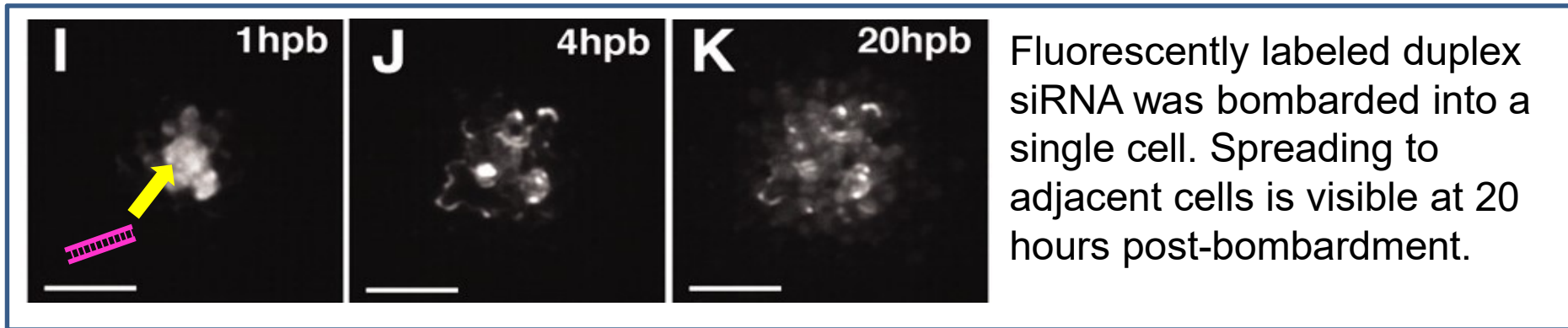
From Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**: [872-875](#); reprinted with permission from AAAS.

Dicer activity for sRNA production in the shoot is sufficient



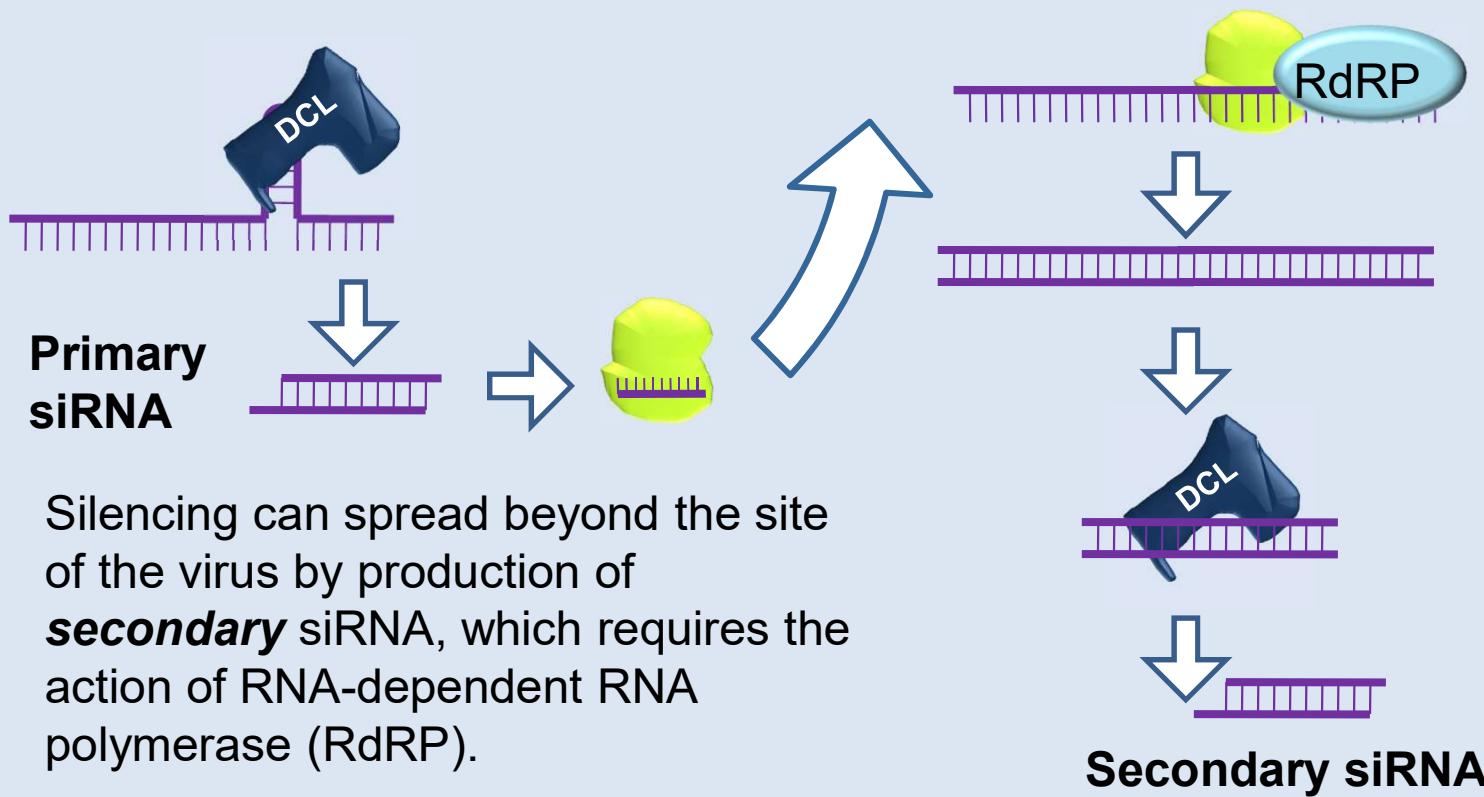
From Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**: [872-875](#); reprinted with permission from AAAS.

siRNA duplexes move between cells and are sufficient to confer silencing



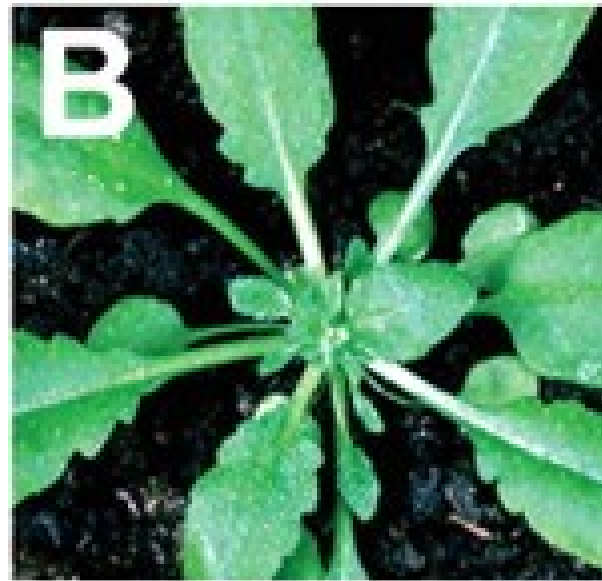
From Dunoyer, P., Schott, G., Himber, C., Meyer, D., Takeda, A., Carrington, J.C. and Voinnet, O. (2010). Small RNA duplexes function as mobile silencing signals between plant cells. *Science*. 328: [912-916](#). Reprinted with permission from AAAS.

Systemic silencing is enhanced by signal amplification



siRNA production mutants are more susceptible to viral disease

WT Arabidopsis
inoculated with TRV

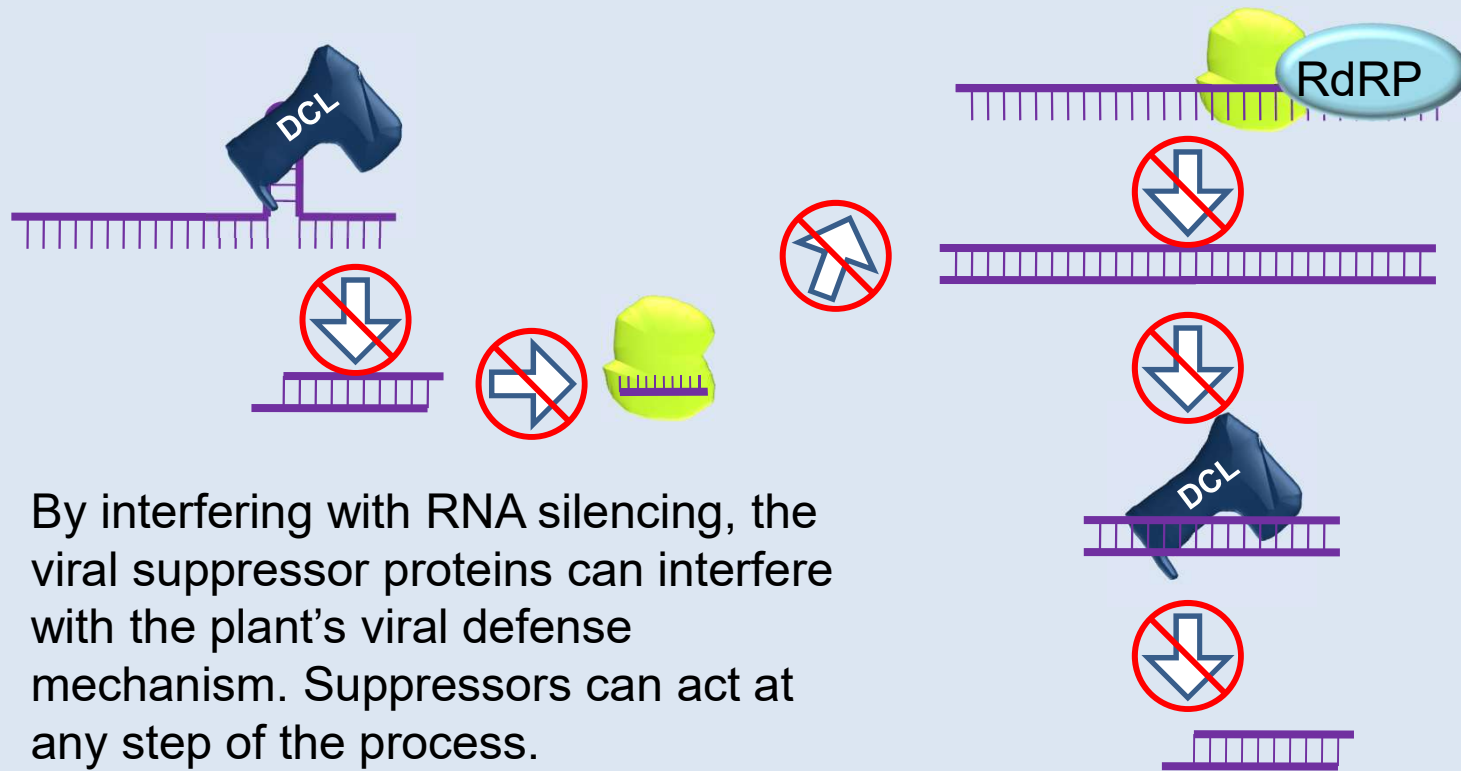


Double mutant of *dcl2-dcl4*
inoculated with
TRV



Tobacco Rattle Virus (TRV) silencing in wild-type Arabidopsis plants prevents disease symptoms. Mutants deficient in Dicer activity are unable to suppress viral infection.

Viruses have suppressor proteins that interfere with RNA silencing



A viral suppressor protein in action

Genes encoding functional, mutant, or no viral suppressor proteins were introduced into plants carrying a silenced GUS gene. The plants were inoculated with a virus expressing GUS. Blue spots indicate GUS expression.

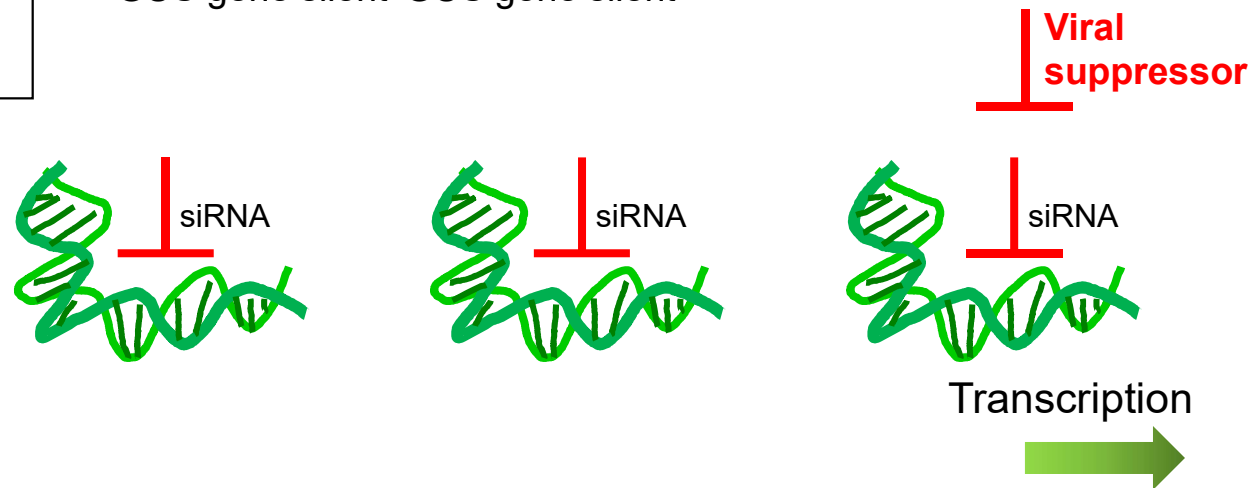


No viral suppressor:
GUS gene silent

Mutant viral suppressor:
GUS gene silent

Functional viral suppressor:
GUS gene expressed

The plant's RNA silencing efforts are suppressed by the viral protein.



Small RNAs also protect plants against bacterial pathogens

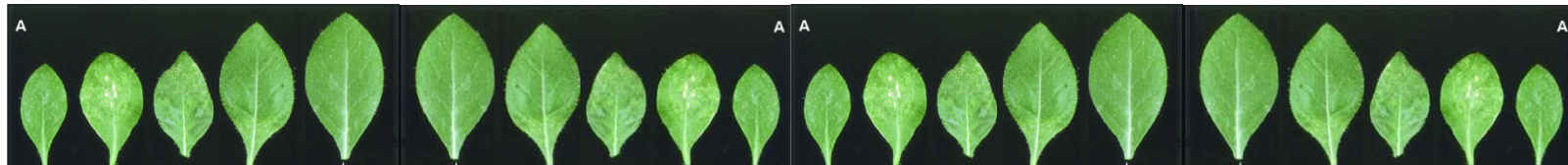


Wild-type (*La-er*) and small RNA processing mutants (*dcl1-9* and *hen1-1*) inoculated with *Pseudomonas* bacteria. The mutants show more visible disease symptoms and permit more bacterial replication.

Reprinted from Navarro, L., Jay, F., Nomura, K., He, S.Y., and Voinnet, O. (2008) Suppression of the microRNA pathway by bacterial effector proteins. (2008) *Science* 321: [964-967](#). Reprinted with permission from AAAS.

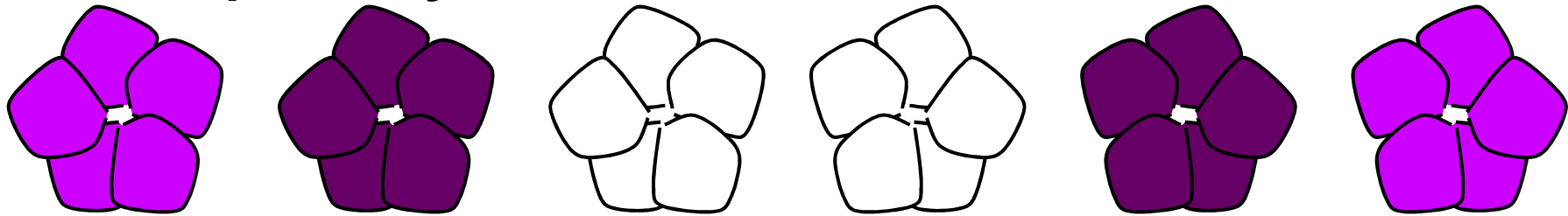
Viral-induced gene silencing summary

- RNA-mediated gene silencing is an important tool in plant defense against pathogens
- siRNAs interfere with viral replication
- siRNAs act systemically to aid in host plant recovery and resistance
- Most viruses produce suppressor proteins that target components of the plant's siRNA defense pathway; these proteins are important tools for dissecting RNA silencing pathways



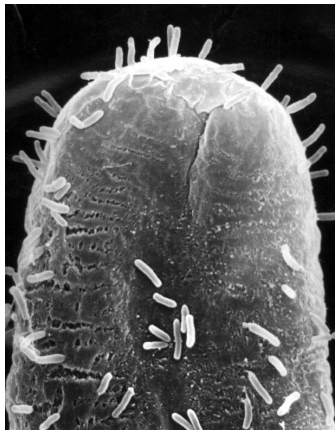
Silencing of transgenes

- Transgenes introduced into plants are frequently silenced by the siRNA pathway
- Silencing can be triggered by:
 - Very high expression levels
 - dsRNA derived from transgene
 - Aberrant RNAs encoded by transgenes
- Transgenes are silenced **post-transcriptionally** and **transcriptionally**

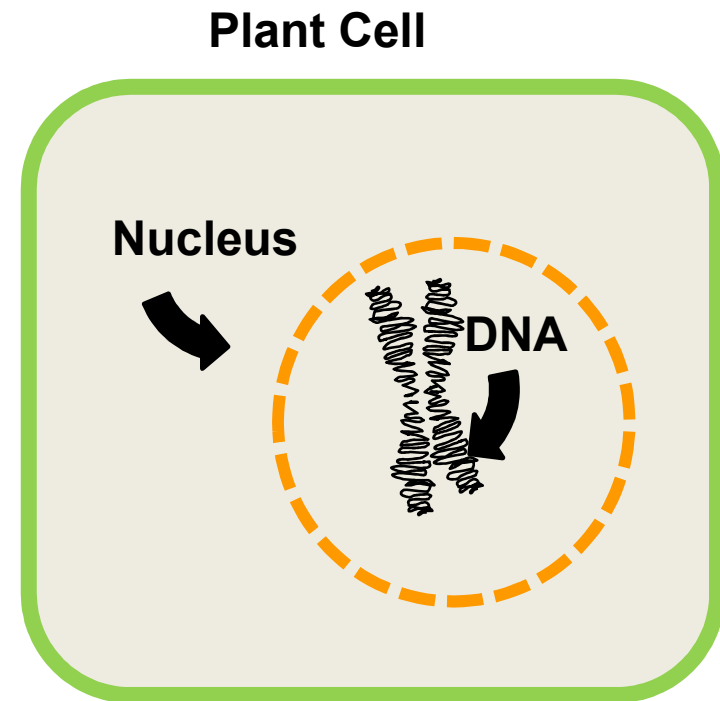


Transgene-induced gene silencing

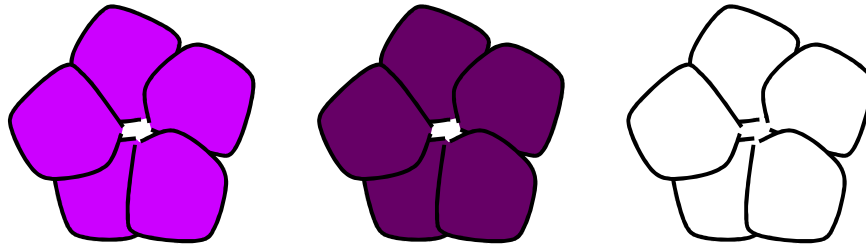
In the 1980s, scientists developed methods for introducing genes into plant genomes, using the bacterium *Agrobacterium tumefaciens*. The introduced genes are called transgenes.



Agrobacterium tumefaciens on the surface of a plant cell.



Transgene-induced post-transcriptional silencing

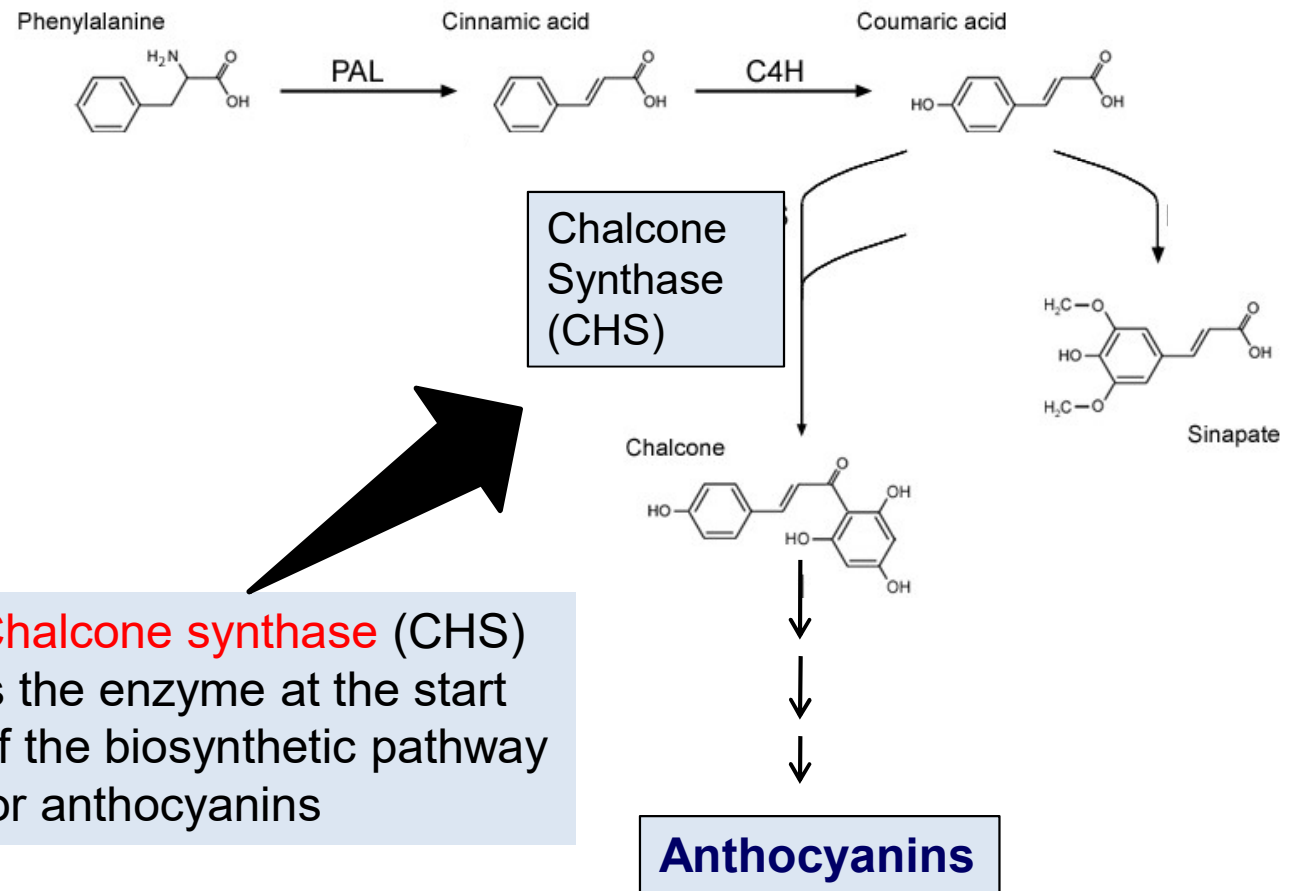


Experiments to modify flower color in petunia gave early evidence of RNA silencing.

Manipulation of chalcone synthase expression to modify pigmentation

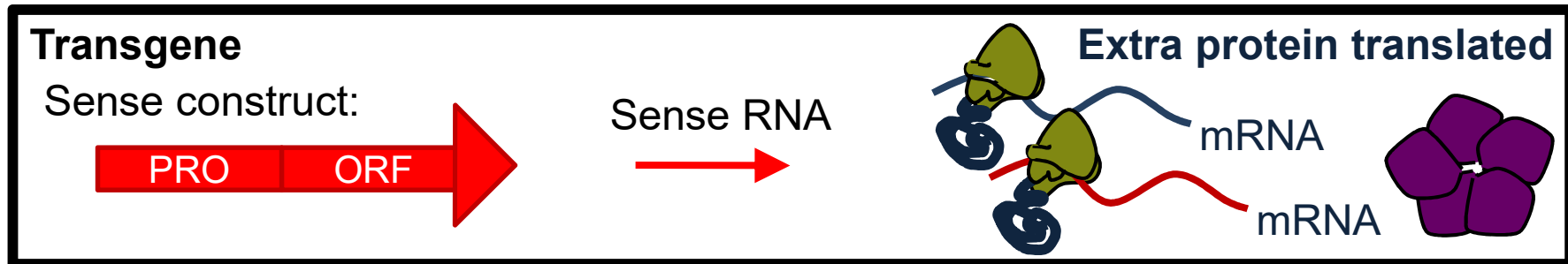
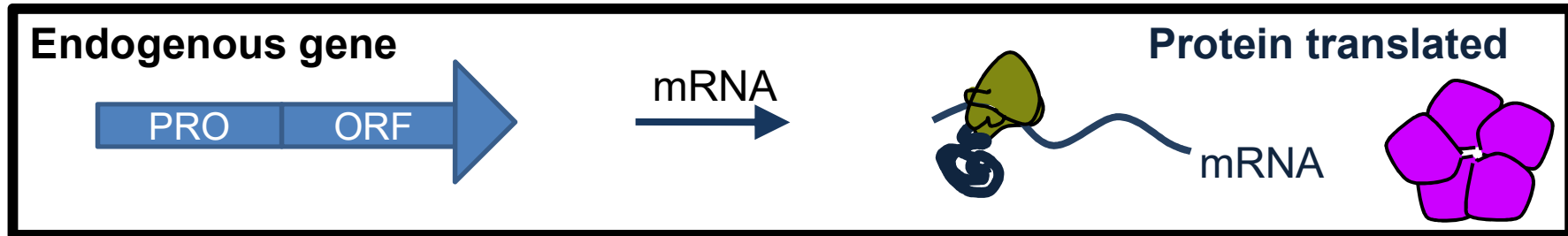


Wild-type petunia producing purple anthocyanin pigments

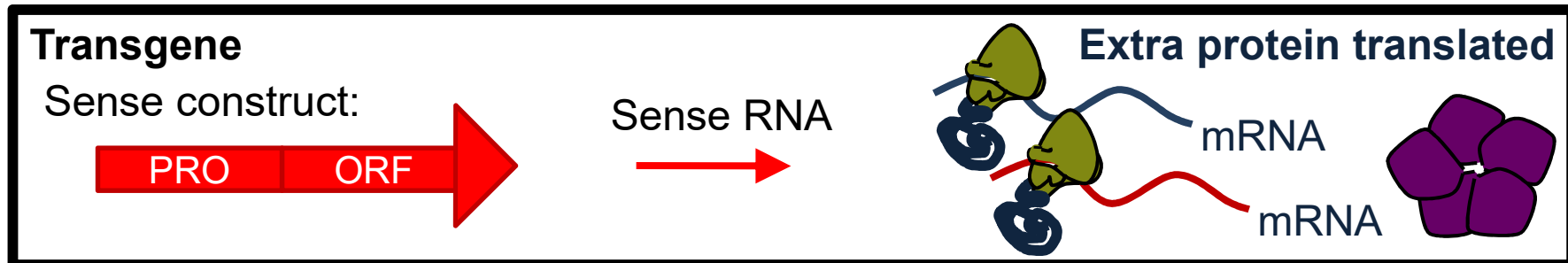
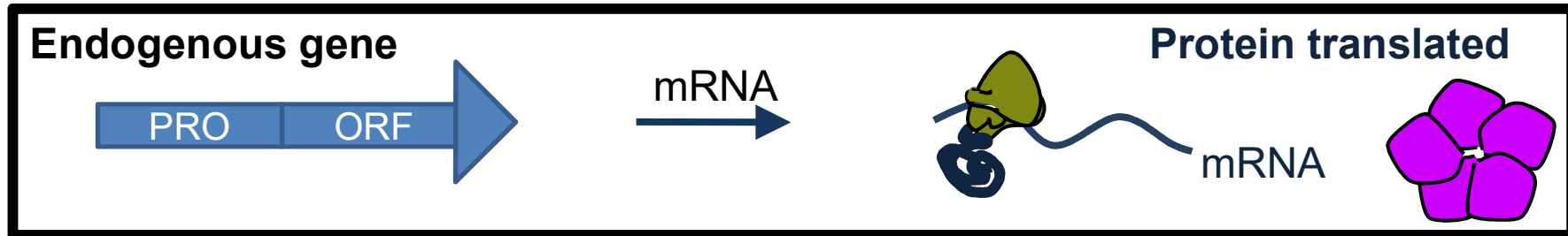


Chalcone synthase (CHS) is the enzyme at the start of the biosynthetic pathway for anthocyanins

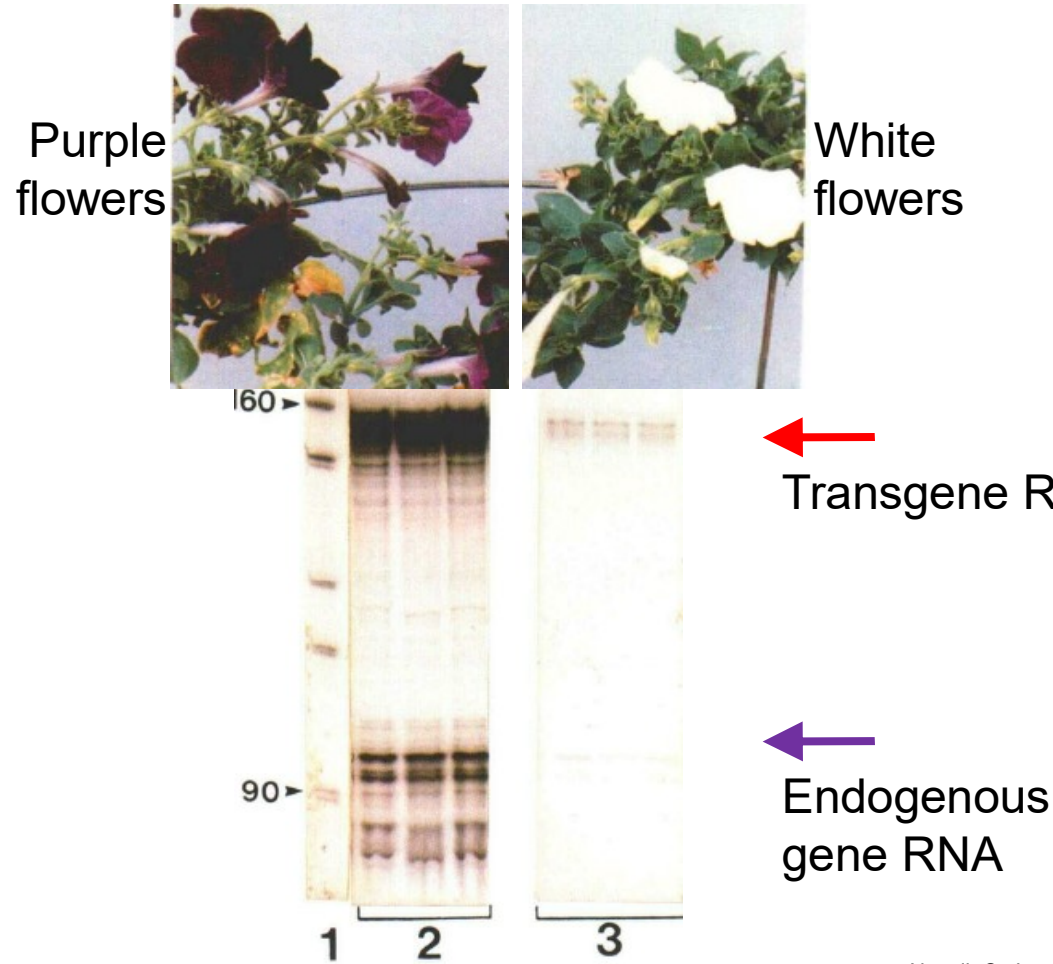
Expectation – sense RNA production would enhance pigmentation...



..and antisense RNA production would block pigmentation

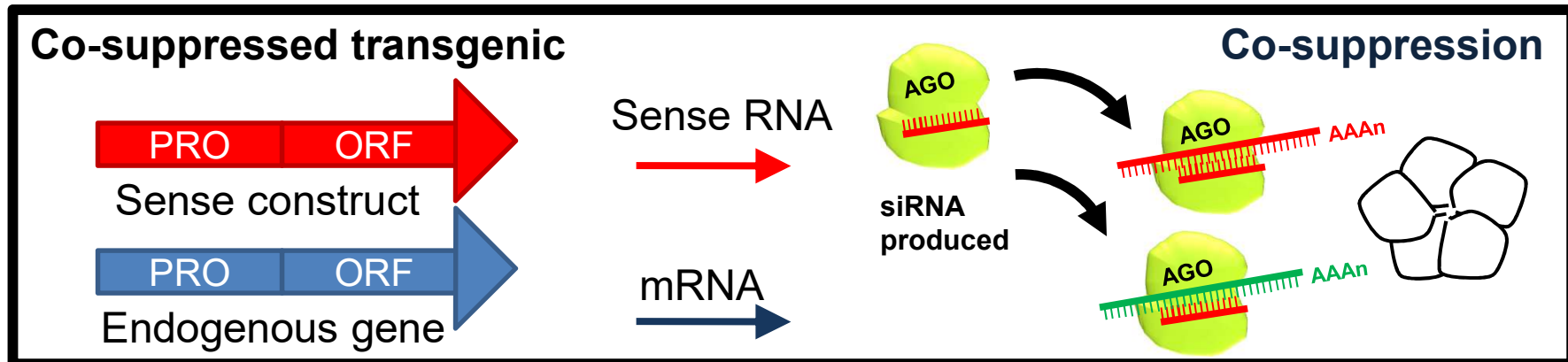
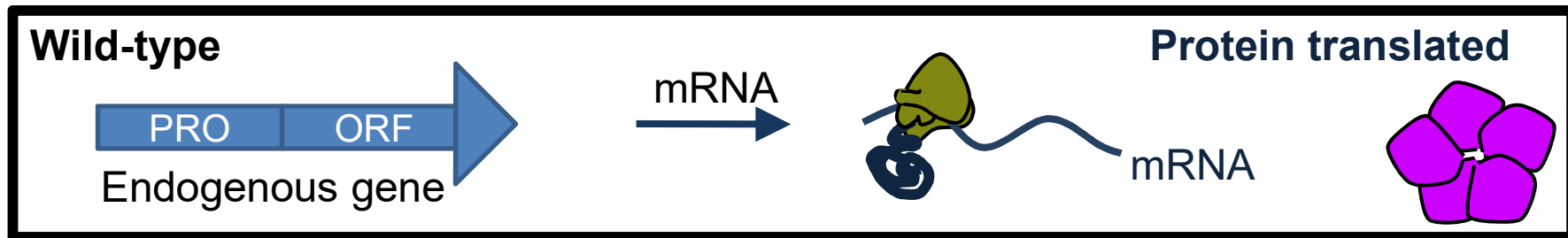


Silenced tissues do not express endogenous or introduced CHS



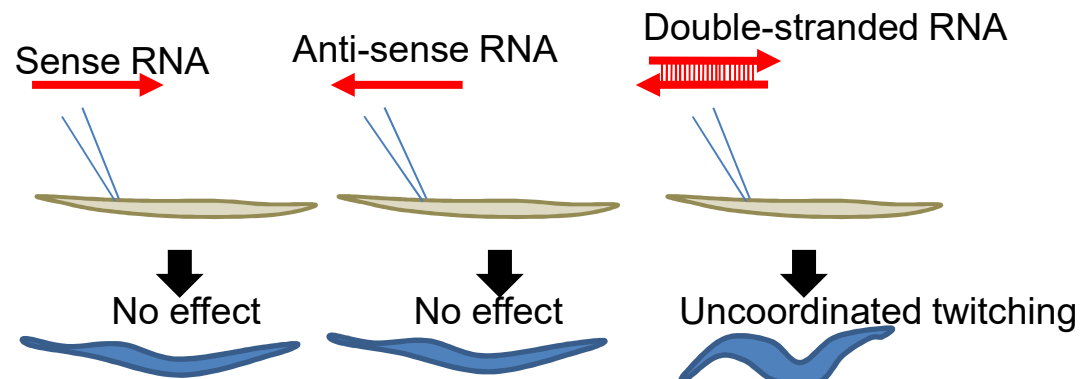
This phenomenon, in which both the introduced gene and the endogenous gene are silenced, has been called “co-suppression”.

Co-suppression is a consequence of siRNA production



Studies of *C. elegans* showed double-stranded RNA is the strongest trigger for gene silencing

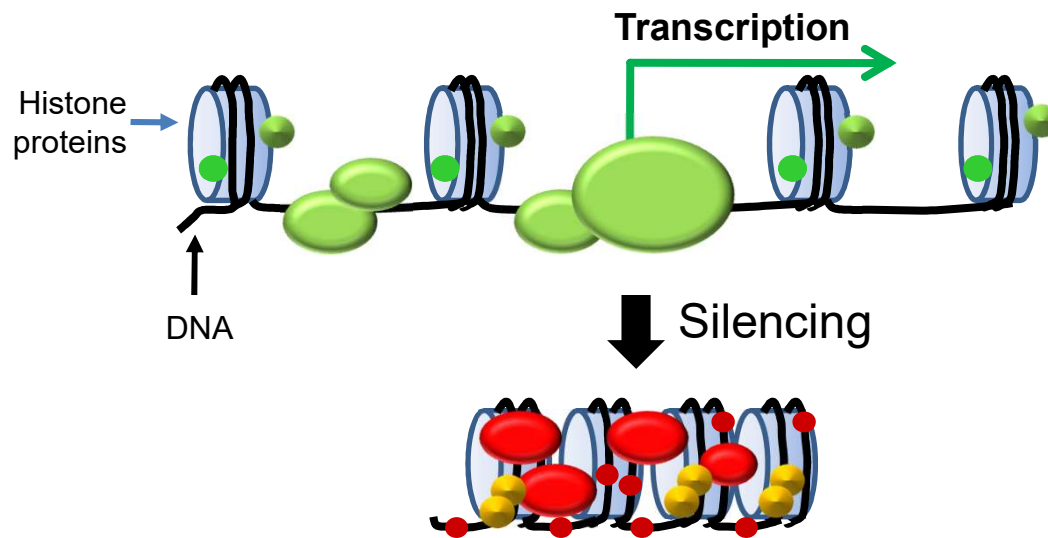
Sense, antisense or double-stranded RNAs homologous to the *unc-22* gene were introduced into worms. Silencing of *unc-22* causes loss of muscle control – hence its name, “*uncoordinated*”.



Derived [The Nobel Committee](#) based on Fire, A. et al., (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: [806-811](#).

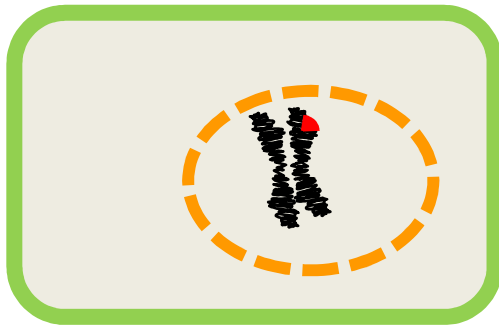
Transcriptional gene silencing

Small RNAs can initiate gene silencing through covalent modifications of the DNA or its associated histone proteins, interfering with transcription.



This form of silencing is frequently associated with stably silenced DNA including centromeres and transposons, but also occurs at genes.

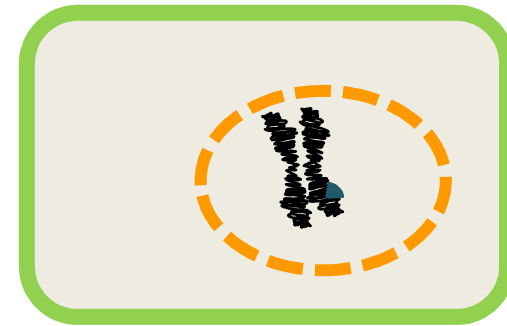
Transcriptional gene silencing



CaMV 35S pro : KAN

Expression of a gene that confers resistance to the antibiotic kanamycin

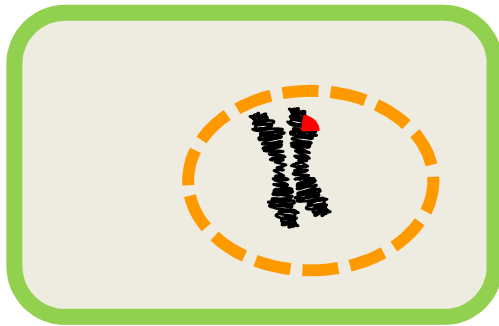
Transcriptional gene silencing was revealed through experiments to introduce more than one transgene into a plant by genetic crosses.



CaMV 35S pro : HYG

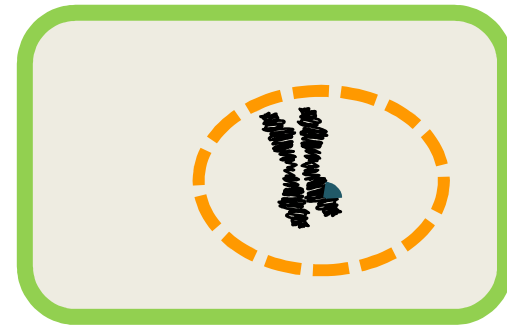
Expression of a gene that confers resistance to the antibiotic hygromycin

Transcriptional gene silencing



CaMV 35S pro : KAN

X



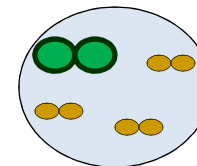
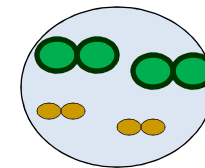
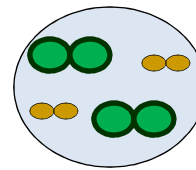
CaMV 35S pro : HYG

Expected Results

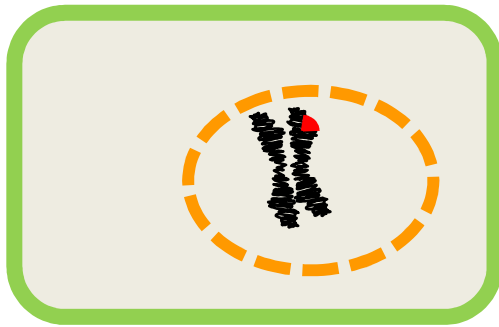
Selection on kanamycin only: 50% KanR

Selection on hygromycin only: 50% HygR

Selection on Kan + Hyg: 25% KanR and HygR

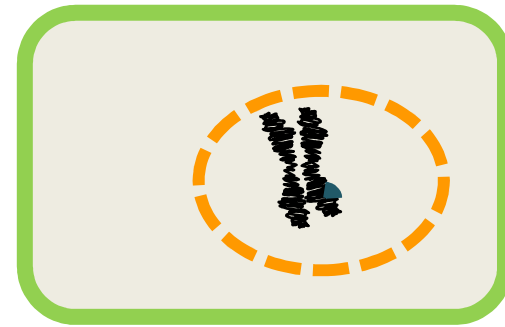


Transcriptional gene silencing



CaMV 35S pro : KAN

Sometimes one of the transgenes was silenced in the progeny carrying both genes.



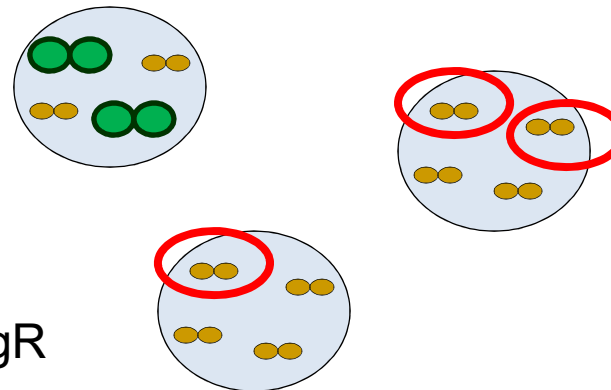
CaMV 35S pro : HYG

Observed Results

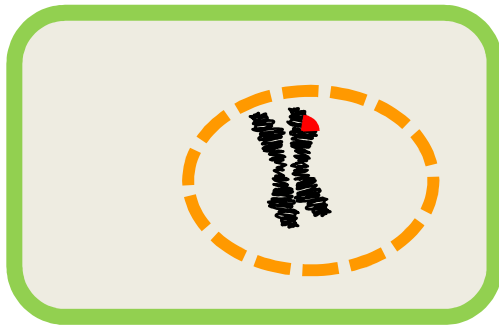
Selection on kanamycin only: 50% KanR

Selection on hygromycin only: 0% HygR

Selection on Kan + Hyg: 0% KanR and HygR

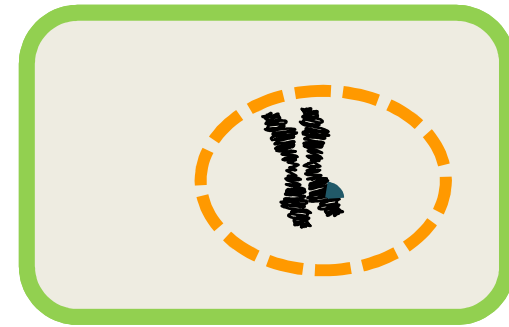


Transcriptional gene silencing

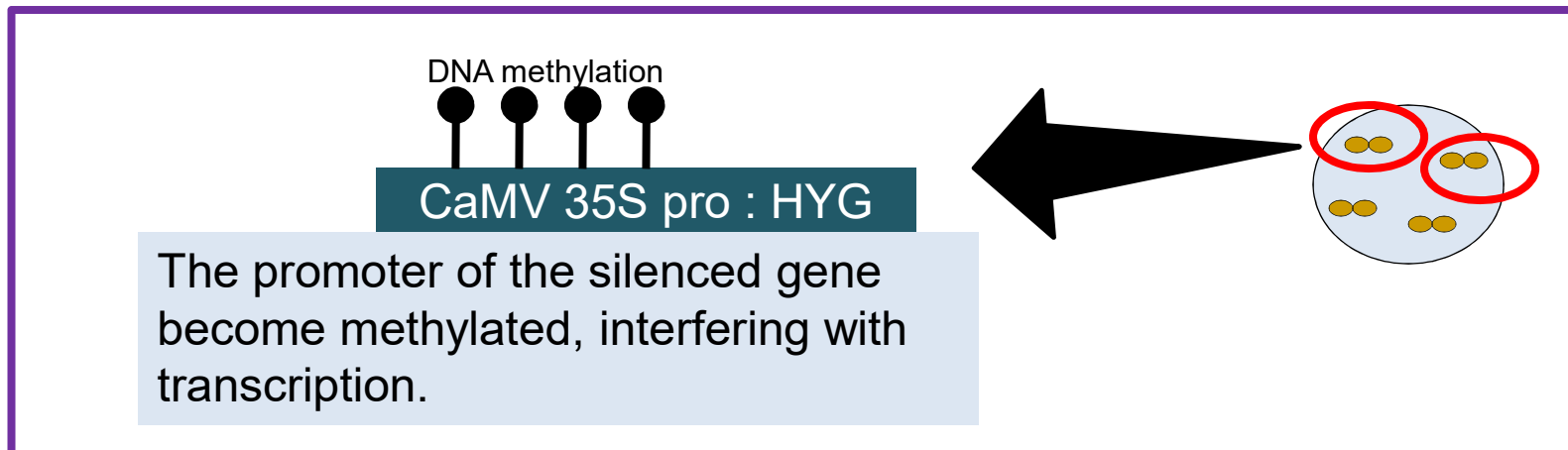


CaMV 35S pro : KAN

Sometimes one of the transgenes was silenced in the progeny carrying both genes.

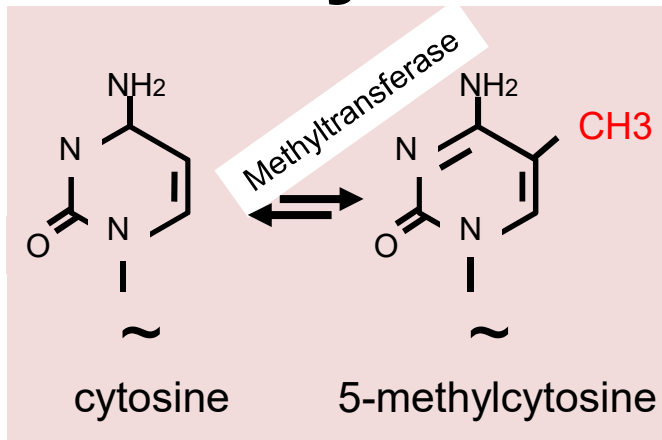


CaMV 35S pro : HYG



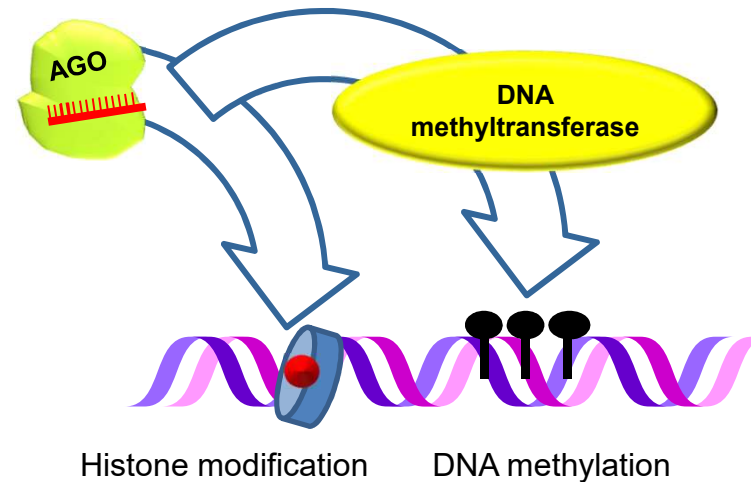
Based on Matzke, M., Primig, M., Trnovsky, J., Matzke, A. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed plants. EMBO J. 8: 643-649.

siRNAs can target DNA for silencing by cytosine methylation or by histone modification

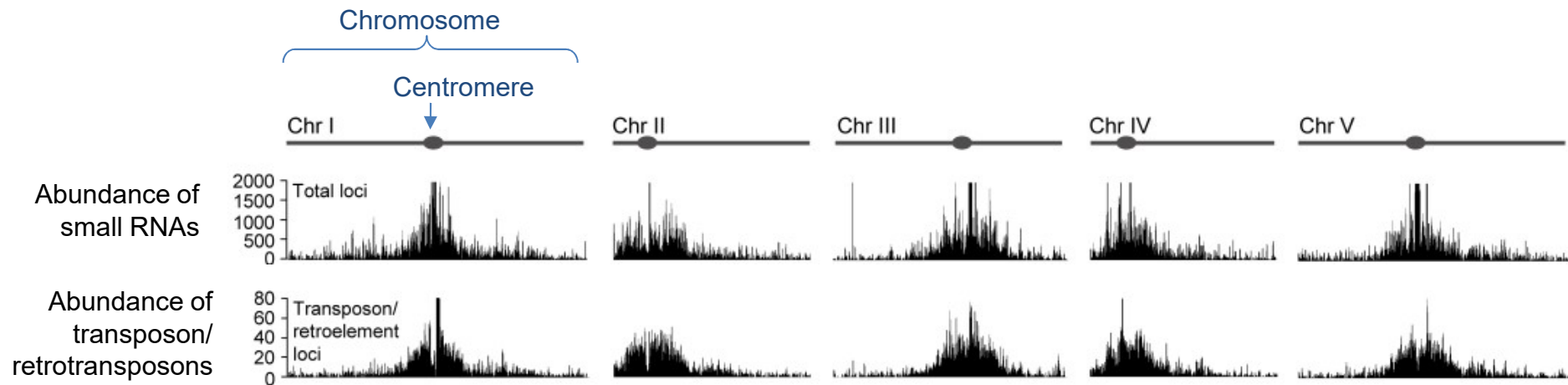


DNA can be covalently modified by cytosine methylation, carried out by DNA methyltransferases.

The precise mechanisms by which siRNAs target DNA for silencing are not known, but involve the action of two plant-specific RNA-polymerase complexes, RNA Polymerase IV (Pol IV) and RNA Polymerase V (Pol V).



Most siRNAs are produced from transposons and repetitive DNA



Most of the cellular siRNAs are derived from transposons and other repetitive sequences. In Arabidopsis, as shown above, there is a high density of these repeats in the pericentromeric regions of the chromosome.

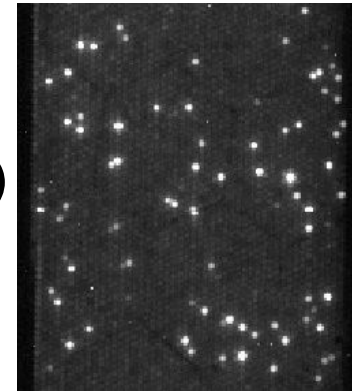
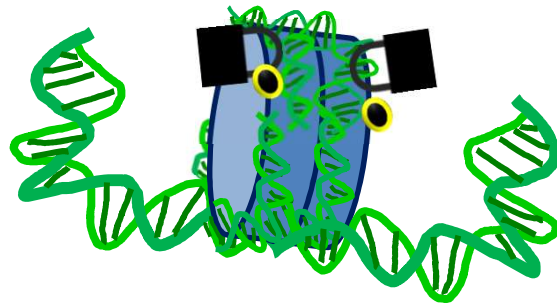
Methods for studying epigenetic modifications

DNA methylation– bisulfite sequencing



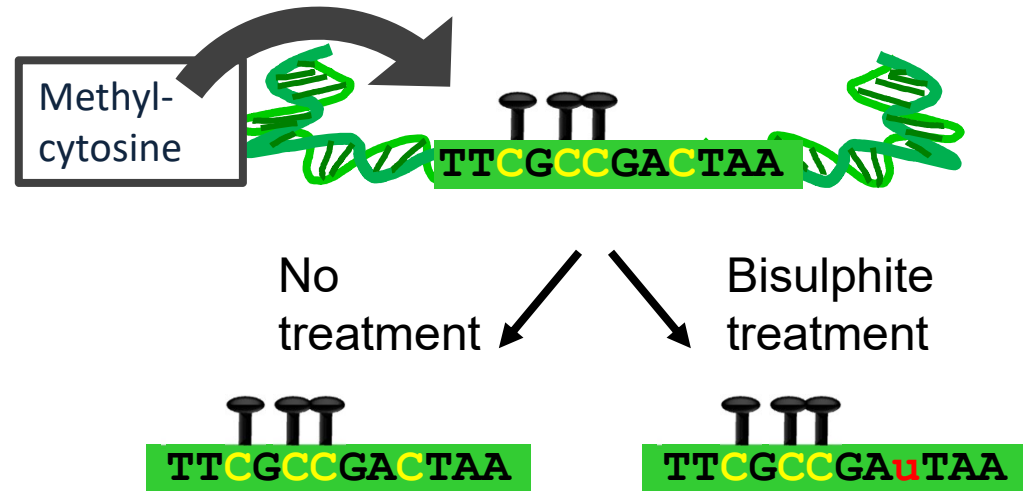
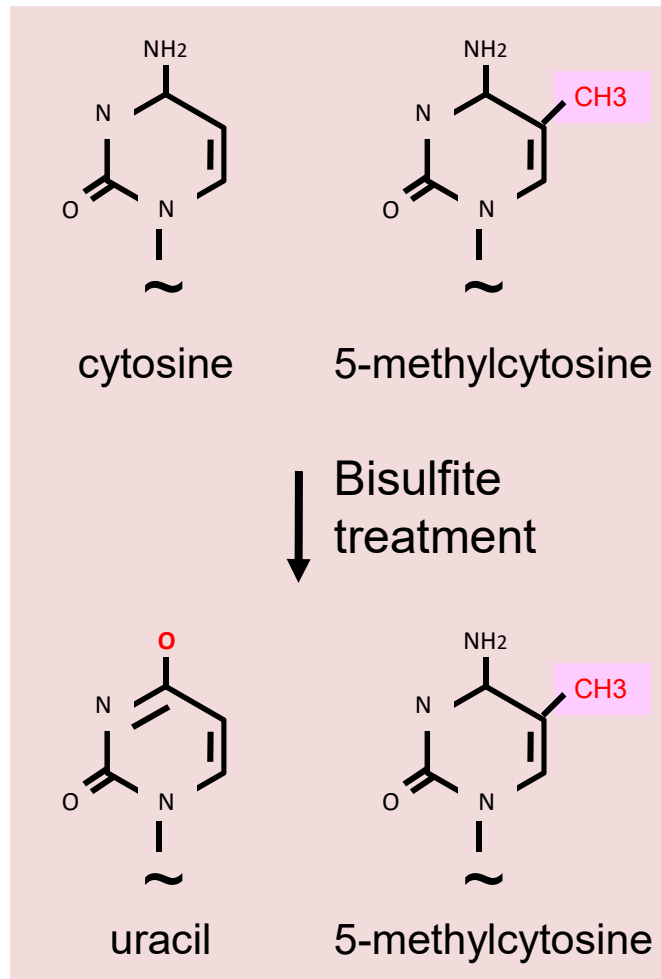
Histone modification

- chromatin immunoprecipitation (ChIP)
- DNA adenosine methylation identification (DamID)



siRNA production – deep sequencing

Bisulfite treatment differentiates cytosine and methylcytosine



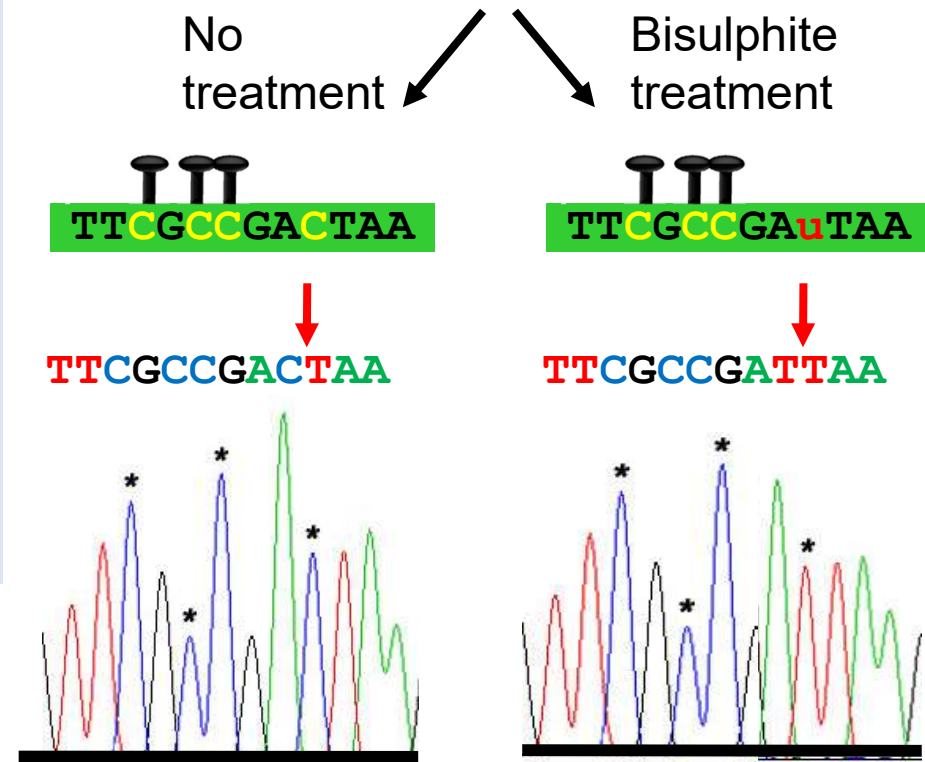
When DNA is bisulfite treated, unmethylated cytosine is converted to uracil. Methylcytosine is not affected.

Bisulfite treatment differentiates cytosine and methylcytosine

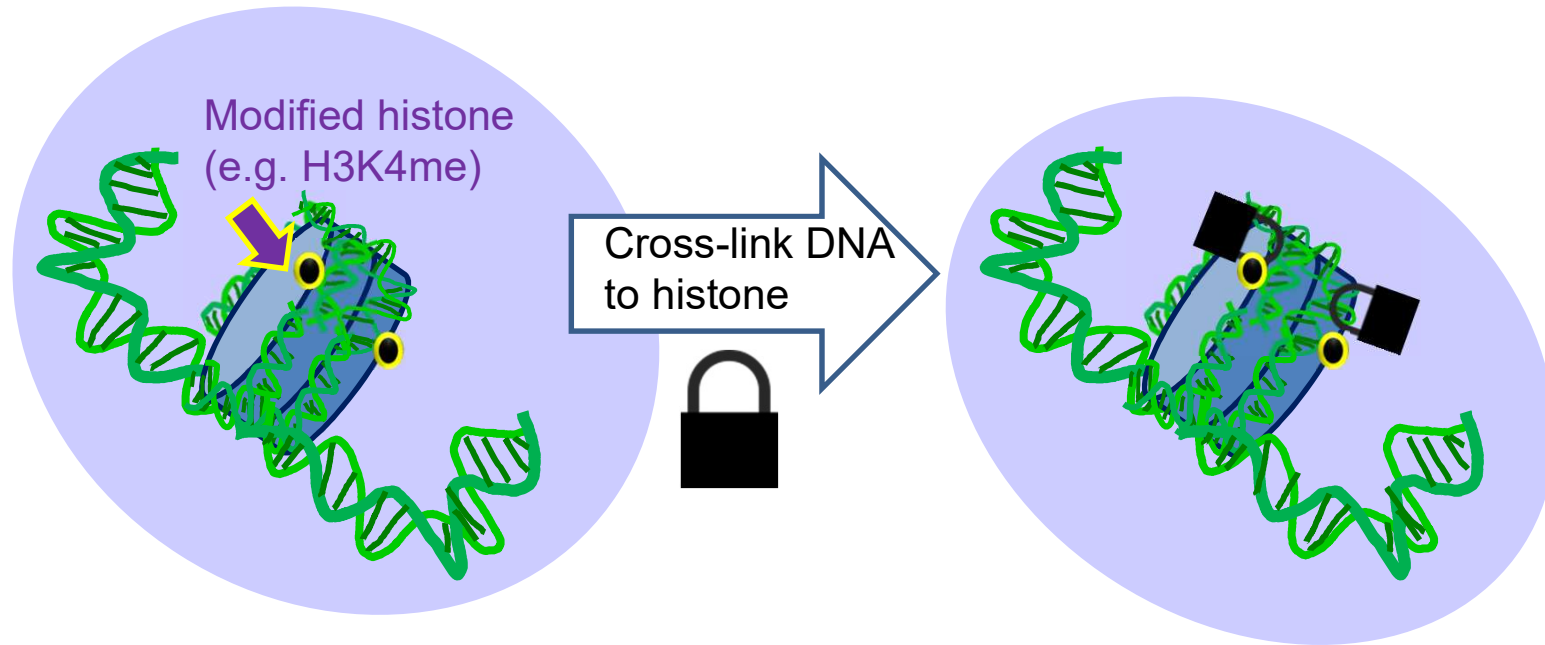


After bisulfite treatment, unmethylated Cs are read as T and so differ in the treated and untreated samples.

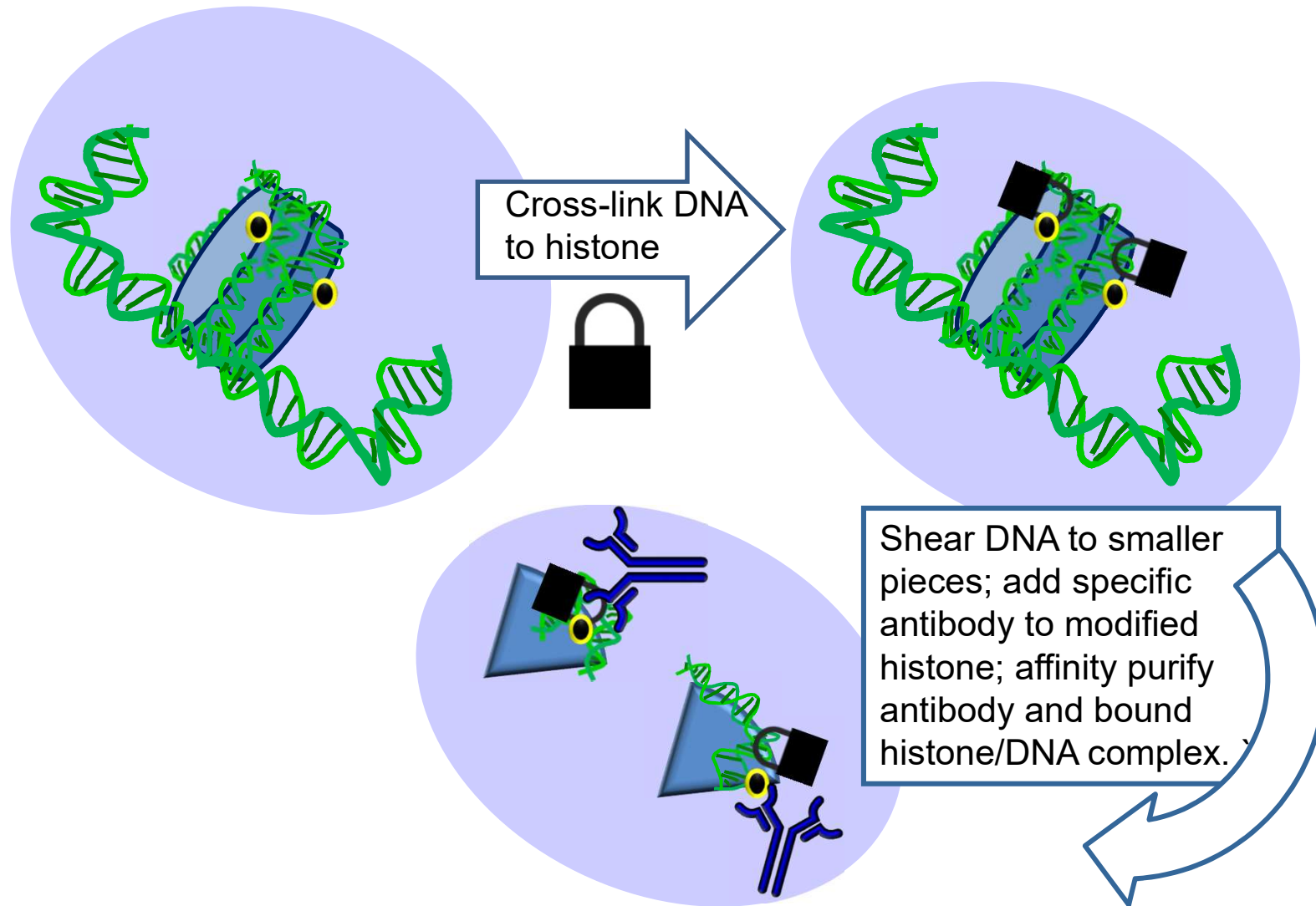
By contrast, **methyl-C** is read as C and is the same as the reference sequence.



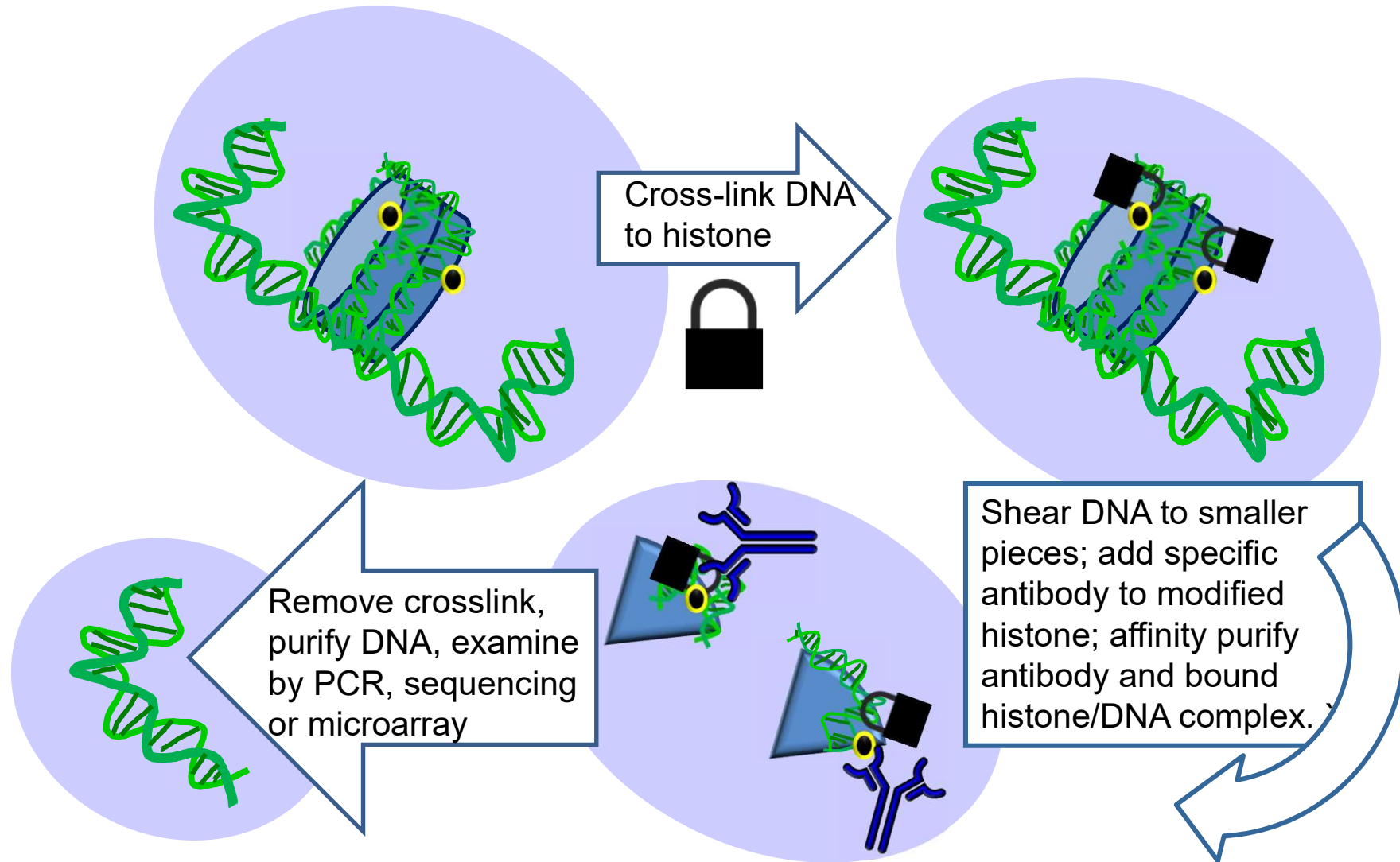
Chromatin Immunoprecipitation (ChIP)



Chromatin Immunoprecipitation (ChIP)

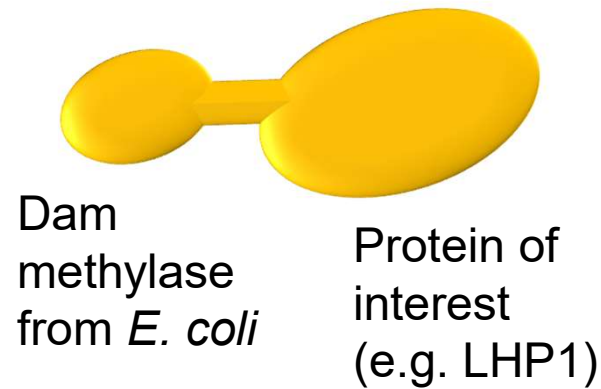


Chromatin Immunoprecipitation (ChIP)

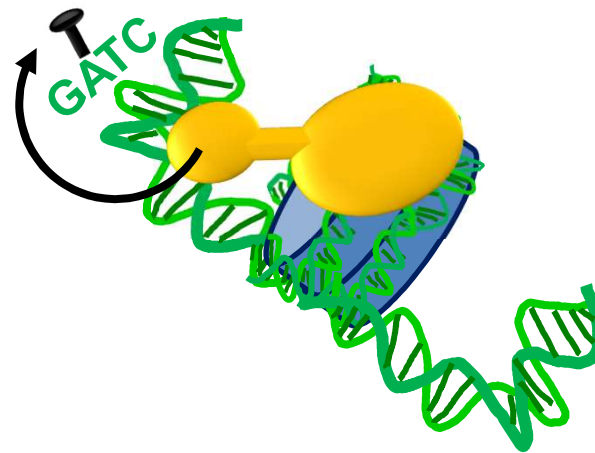


DNA adenine methylation ID (DamID)

A fusion protein is made of Dam and the protein of interest

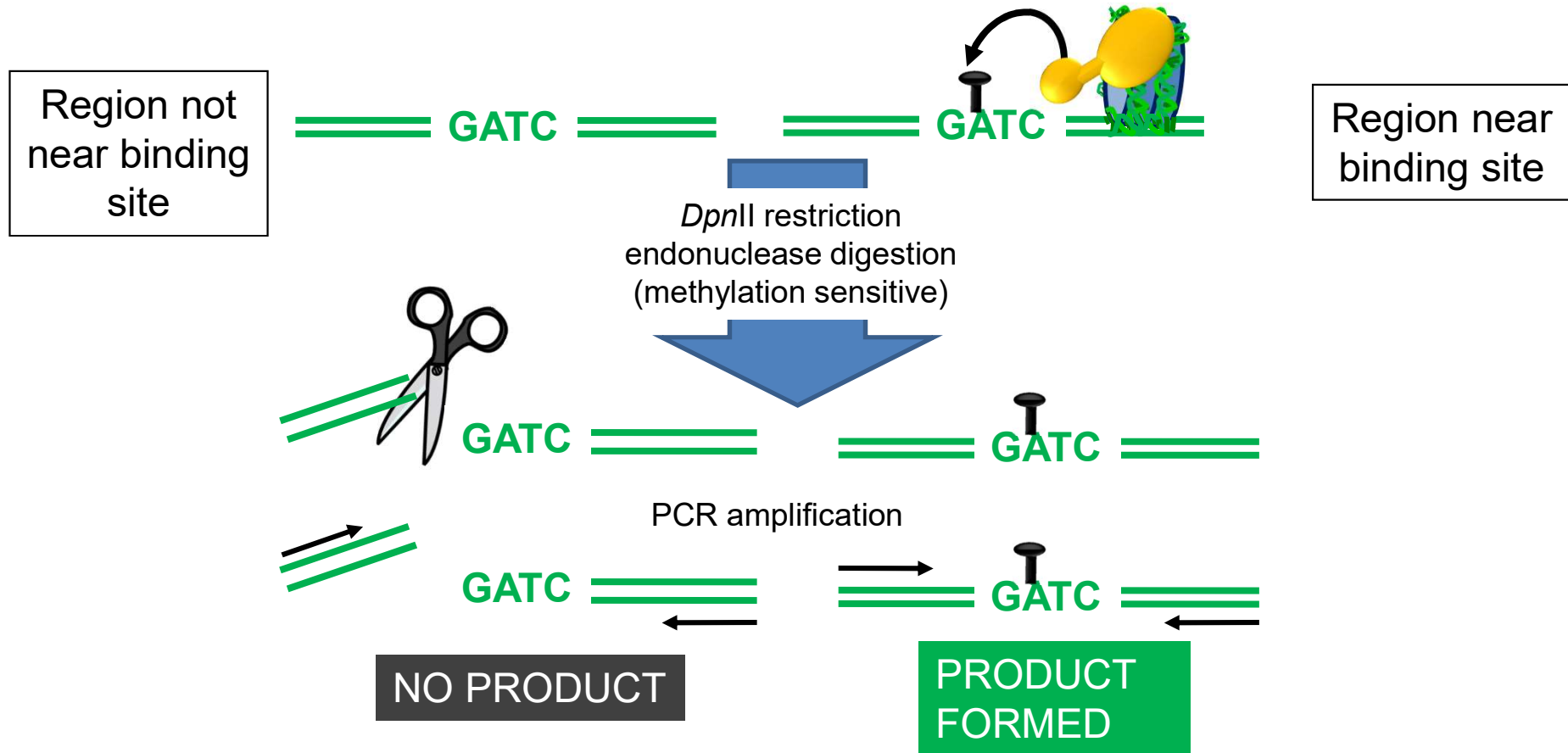


Dam is an adenine methyltransferase with specificity for the sequence **GATC**

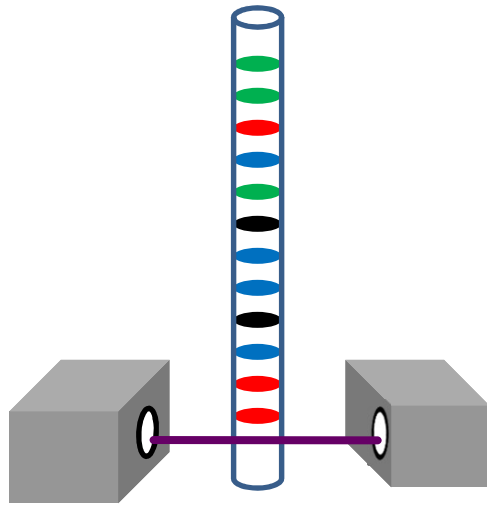


The fusion protein binds to selected regions of chromatin (e.g. H3K27me3) and methylates adenines at nearby GATC sites

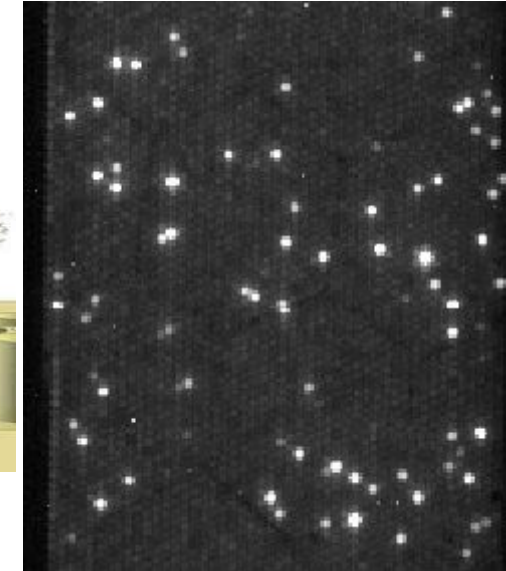
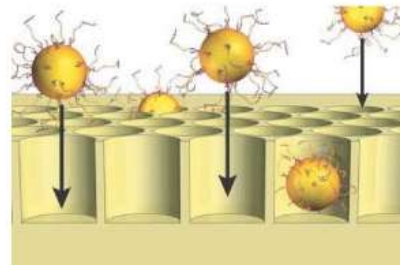
Methylation can be detected by methylation sensitive enzymes



Deep sequencing by “next generation” DNA sequencing methods

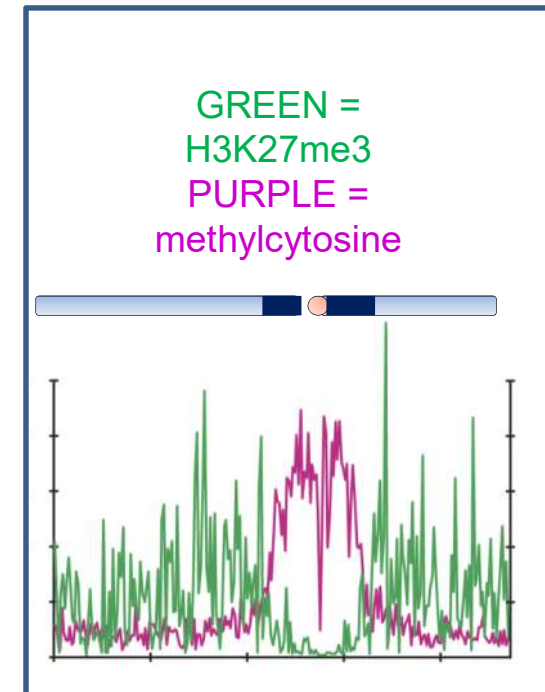
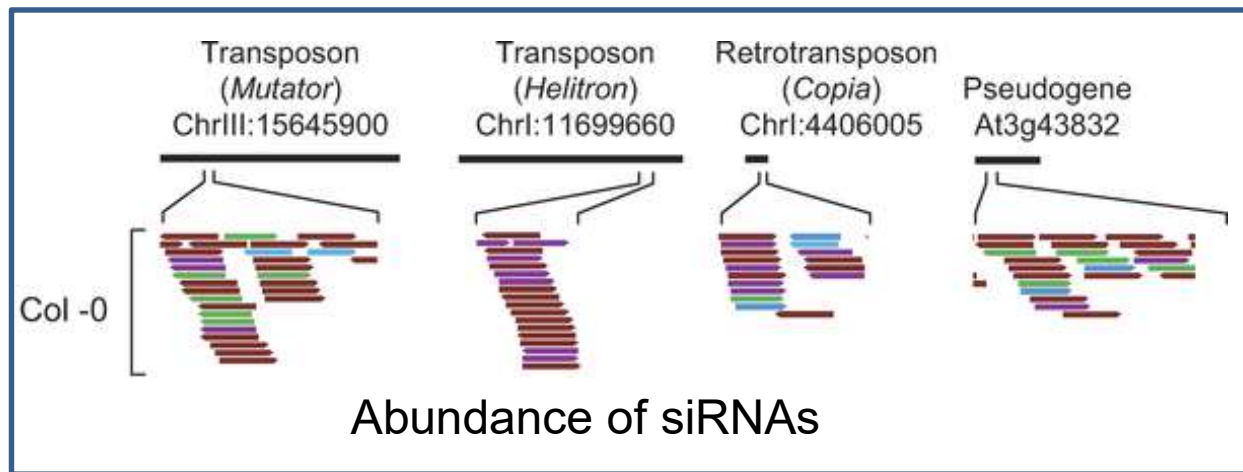


“Classical” DNA sequencing – one molecule examined at a time



“Next generation” DNA sequencing – one **million** molecules examined at a time

Using next-generation sequencing, epigenetic modifications can be identified genome-wide: EPIGENOMICS



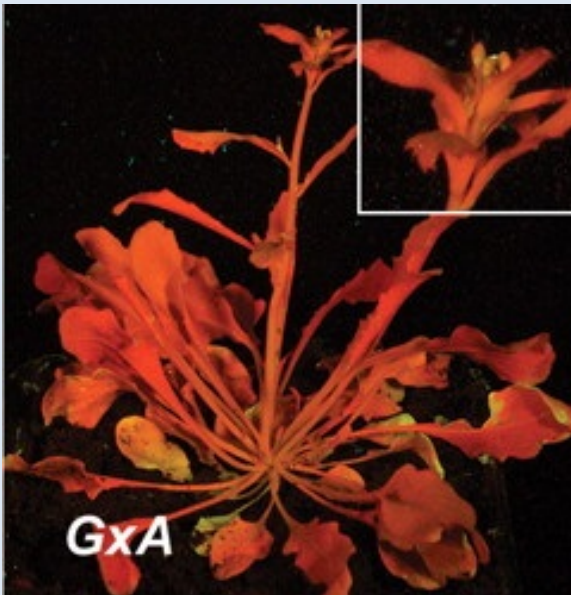
Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, et al. 2007 Genome-Wide Profiling and Analysis of *Arabidopsis* siRNAs. *PLoS Biol* 5(3): [e57](#).
Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y.V., Pellegrini, M., Goodrich, J., Jacobsen, S.E. (2007) Whole-genome analysis of histone H3 lysine 27 trimethylation in *Arabidopsis*. *PLoS Biol*. 5: [e129](#).

Plants have additional RNA Polymerase complexes that contribute to silencing

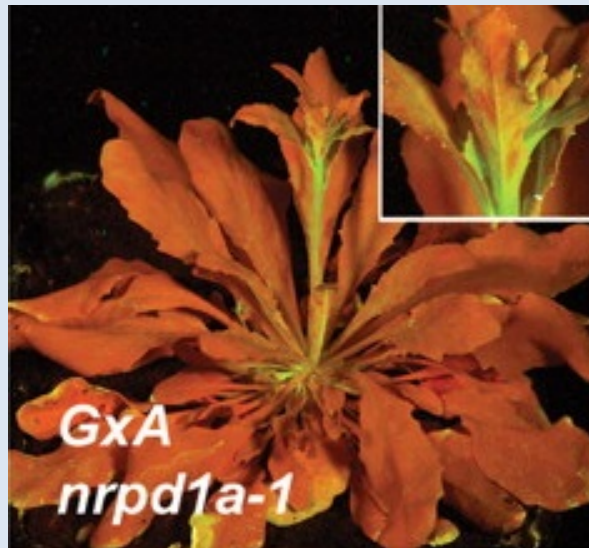
Complex	Distribution	Function
RNA Polymerase I	All eukaryotes	Production of rRNA
RNA Polymerase II	All eukaryotes	Production of mRNA, microRNA
RNA Polymerase III	All eukaryotes	Production of tRNA, 5S rRNA
RNA Polymerase IV	Land plants	Production of siRNA
RNA Polymerase V	Angiosperms	Recruitment of AGO to DNA

Loss of function of RNA Pol IV interferes with silencing

Arabidopsis plant with silenced GFP gene

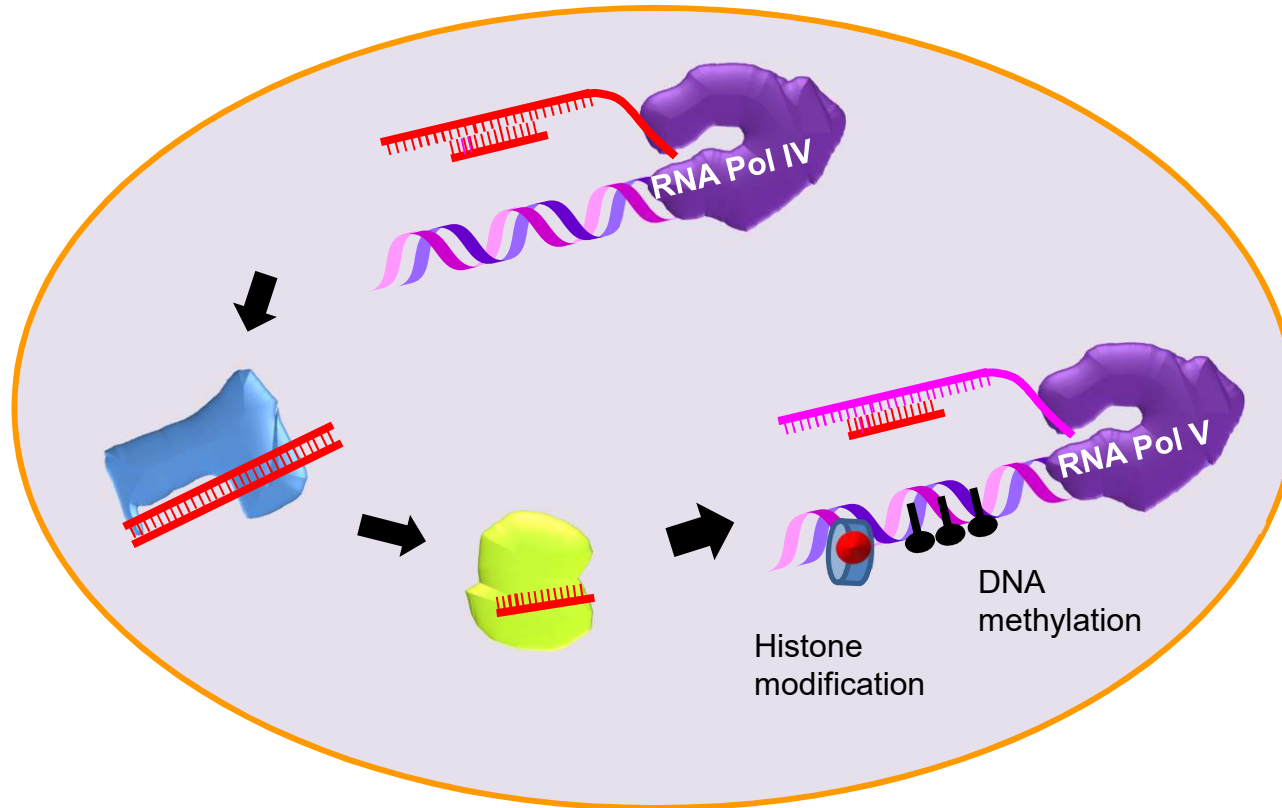


Loss-of-function mutant *nrpd1a-1*. *NRPD1A* encodes a subunit of RNA Polymerase IV.



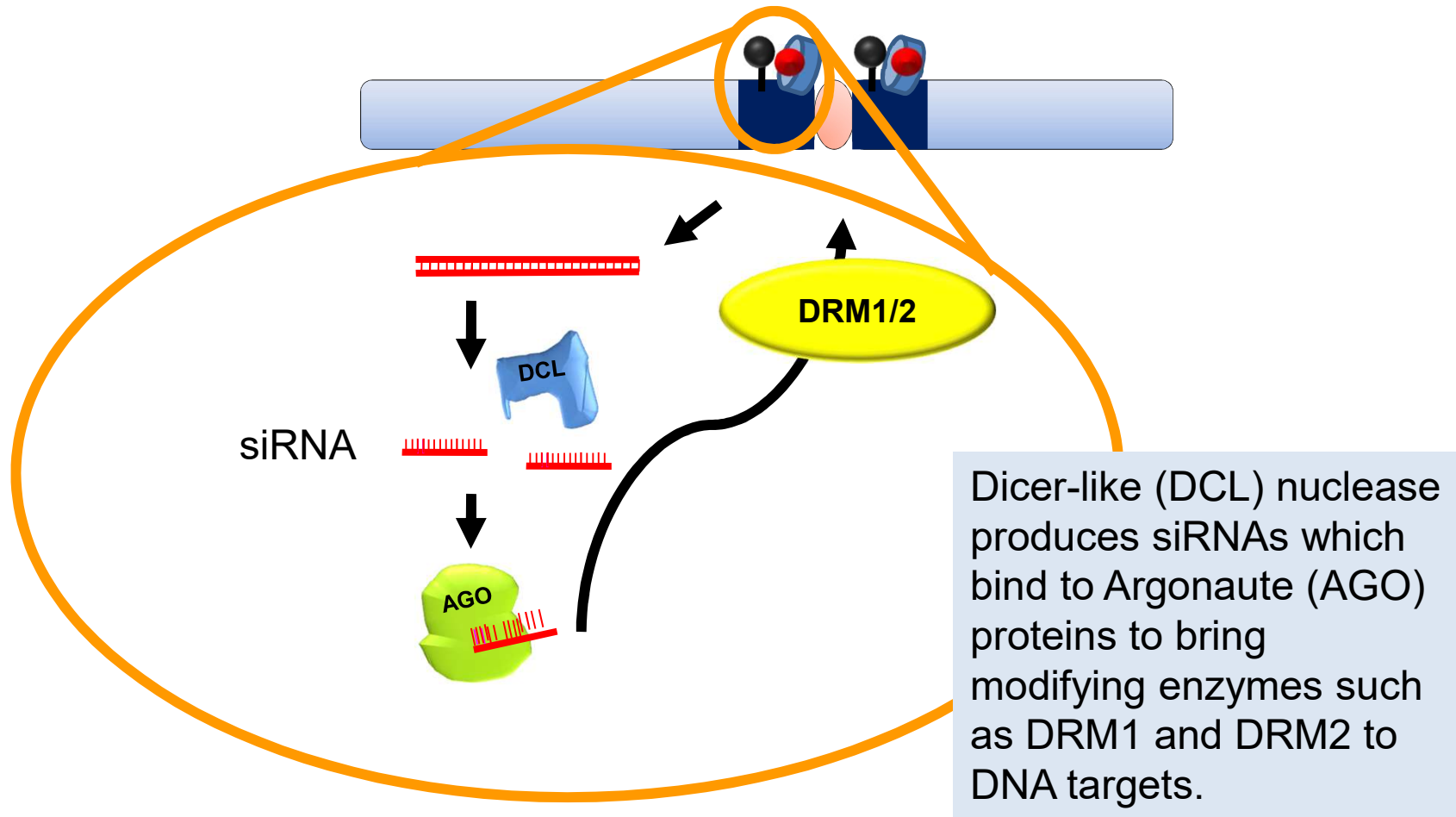
Green indicates GFP is expressed, showing that Pol IV is required for gene silencing.

Transcriptional silencing requires RNA Pol IV and V

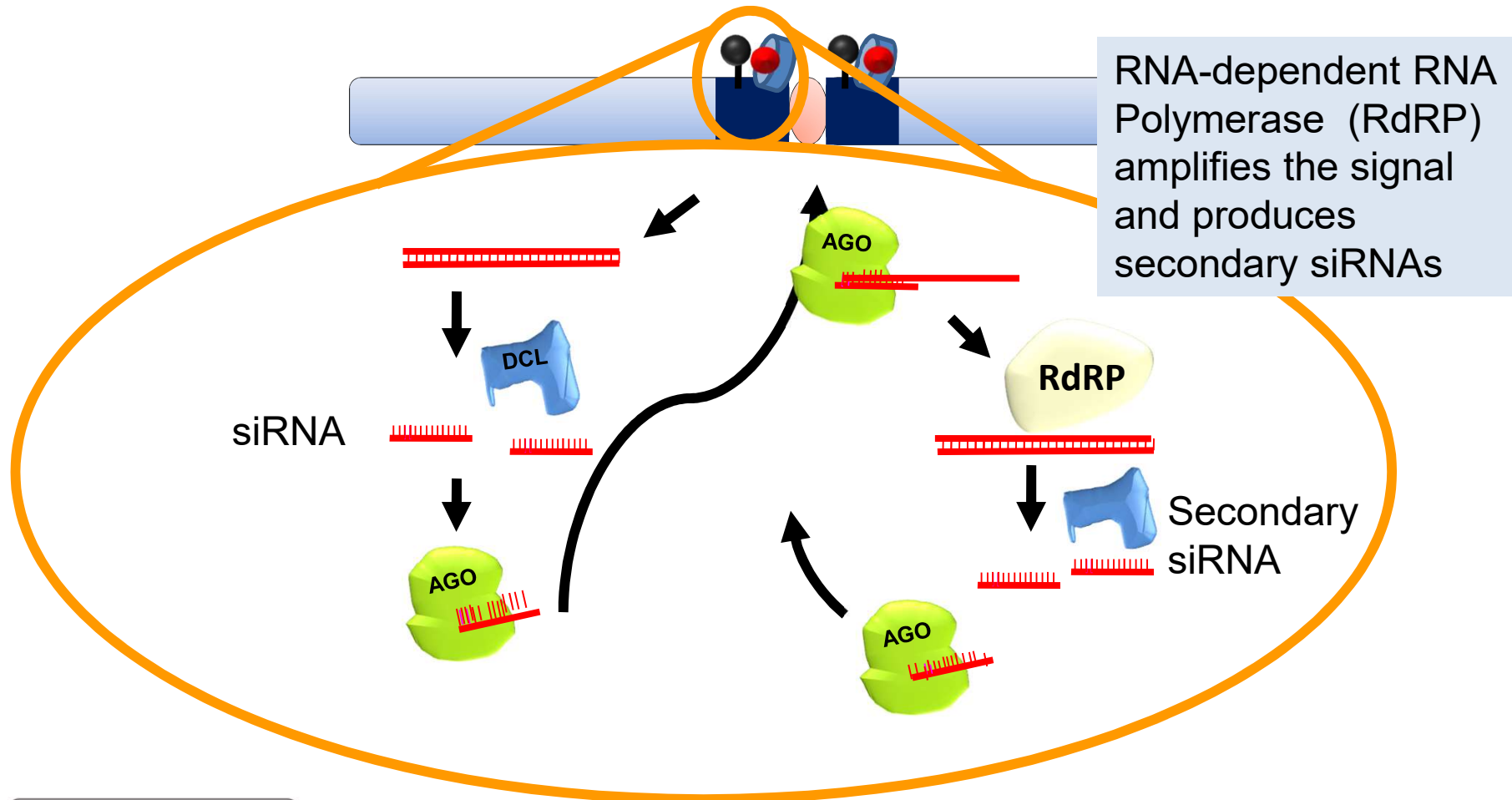


RNA Pol IV contributes to siRNA production. Non-coding RNAs produced by RNA Pol V direct silencing machinery to target sites.

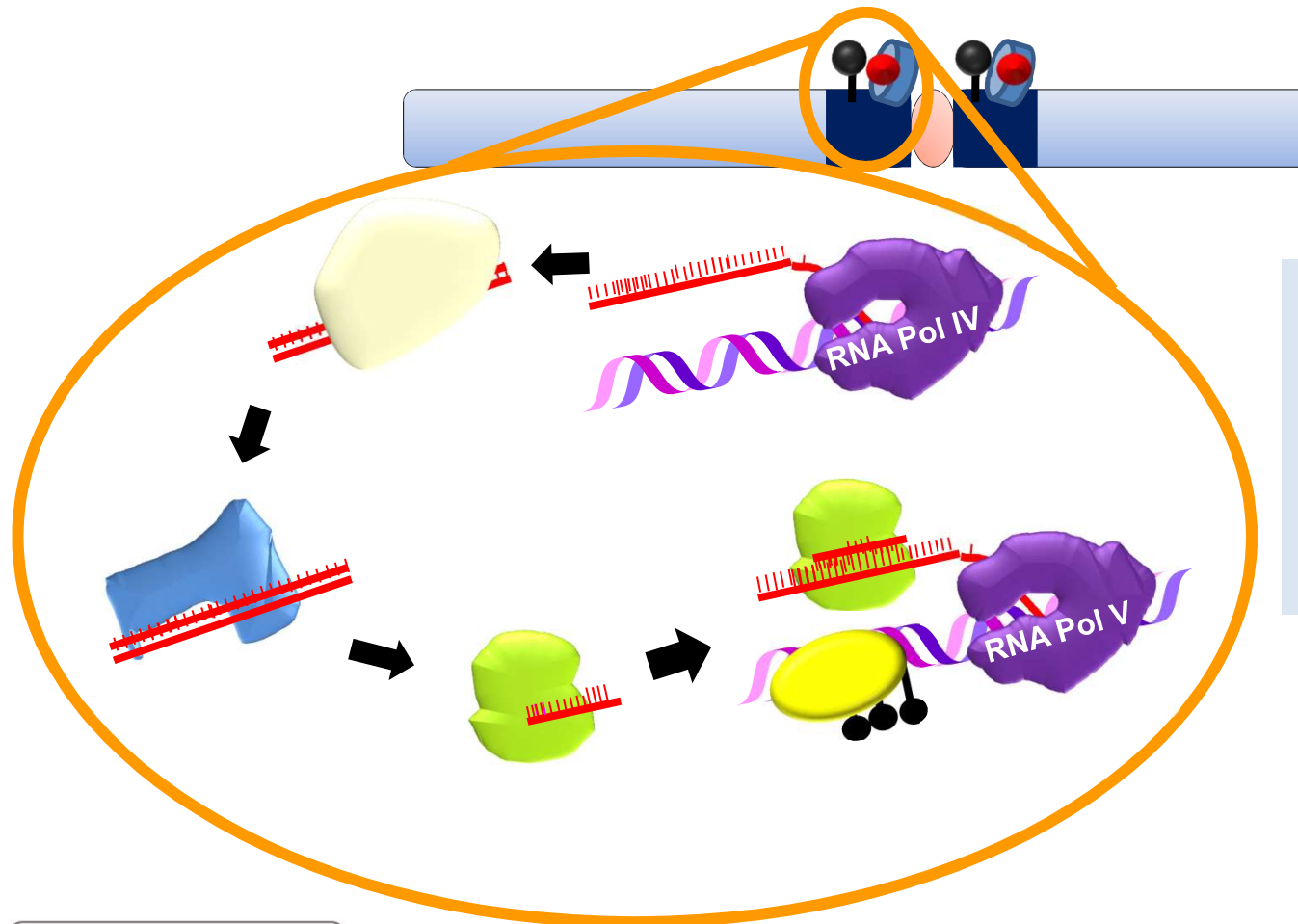
siRNAs recruit DNA methylases and histone-modifying enzymes to targets



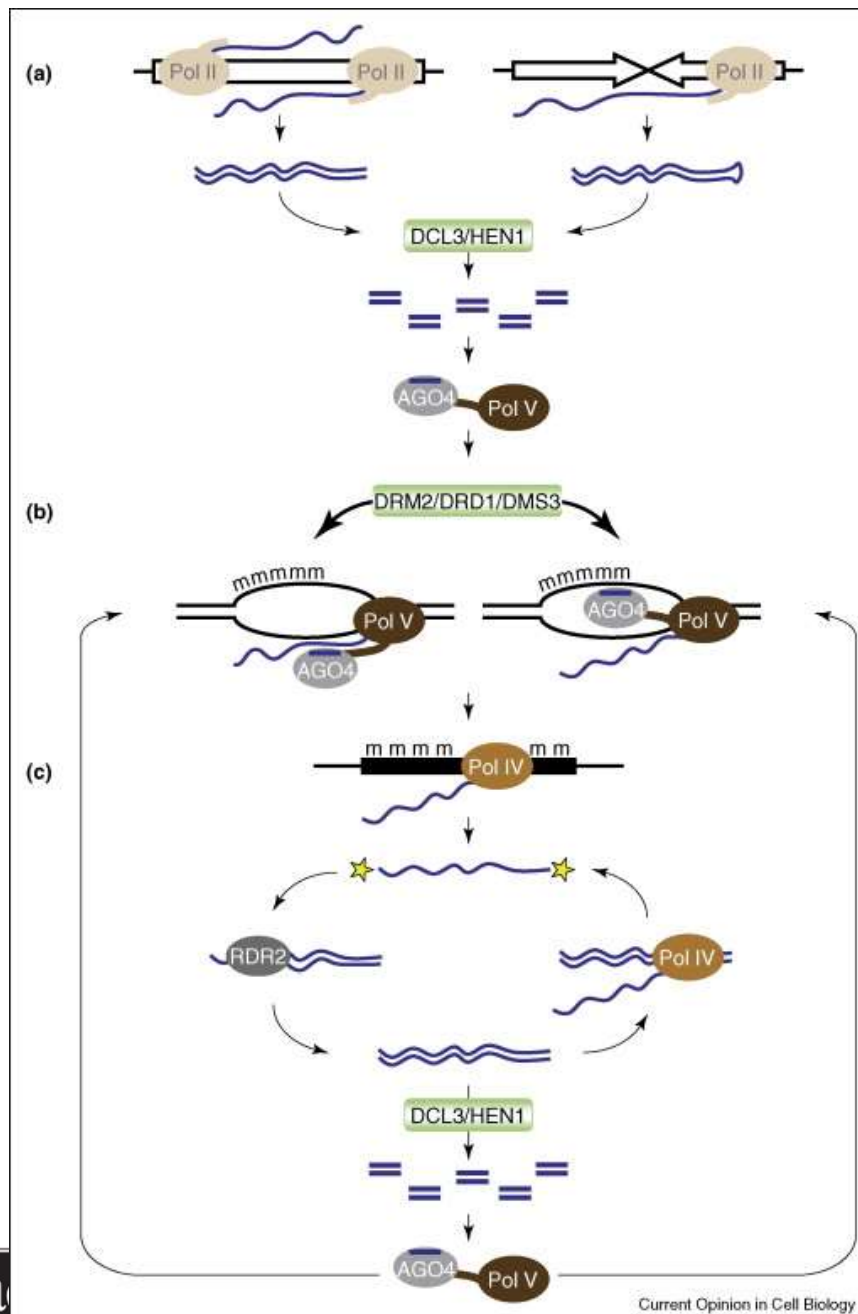
siRNAs recruit DNA methylases and histone-modifying enzymes to targets



siRNAs recruit DNA methylases and histone-modifying enzymes to targets



Two plant-specific RNA polymerases, RNA Pol IV and RNA Pol V, contribute to siRNA-mediated silencing.

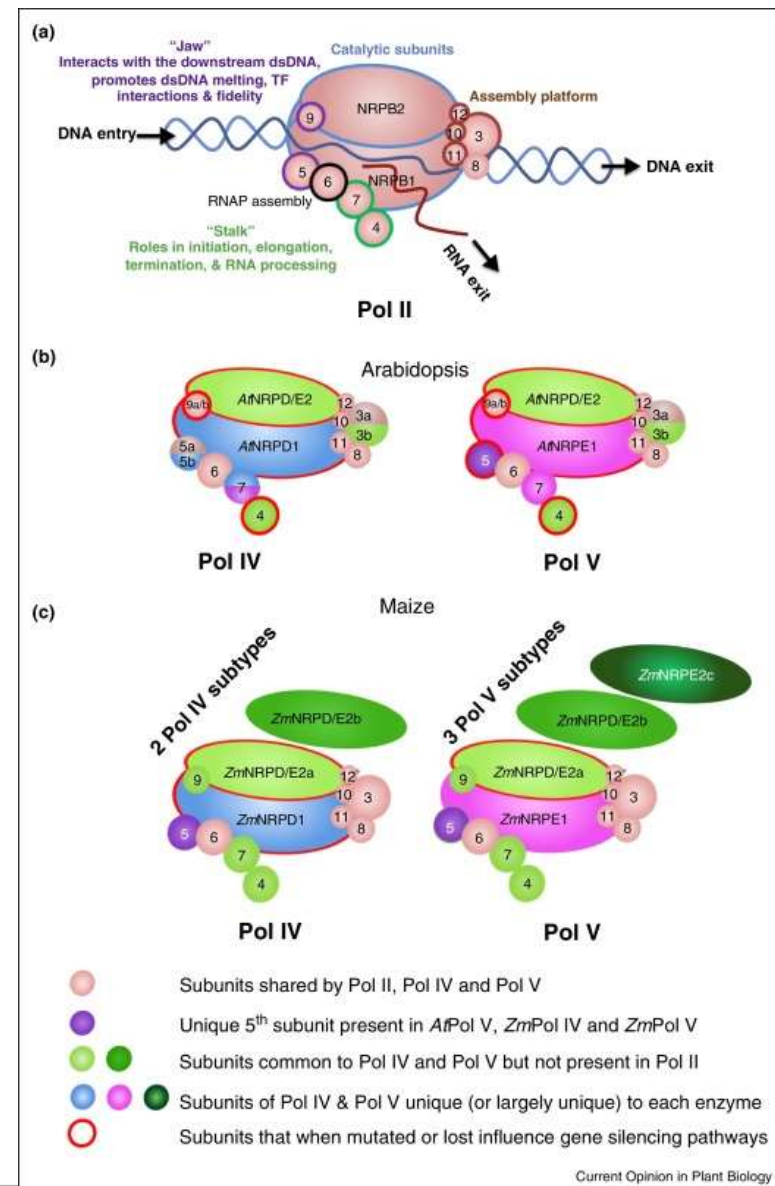


(a) dsRNA that is independent of Pol IV and Pol V can potentially result from overlapping Pol II transcription (left) or Pol II transcription of inverted repeats (right). Processing by DCL3 produces 24-nt siRNAs that are methylated at their 3' ends by HEN1. One strand is loaded onto AGO4, which interacts with **NRPE1**, the largest subunit of Pol V (b) **Pol V** transcription facilitates DNA *de novo* methylation at the siRNA-targeted site by DRM2, the major *de novo* methyltransferase.

AGO4-bound siRNAs may interact with the nascent RNA (left) or the target DNA (right) to guide methylation. (c) To amplify the siRNA trigger, **Pol IV** may directly transcribe the methylated DNA template, producing an aberrant (improperly processed or terminated) RNA (yellow stars). The aberrant RNA is copied by RDR2 to produce dsRNA precursors of siRNAs that trigger methylation (step B). Pol IV may also transcribe dsRNA in the amplification cycle.

Current data suggests that the unique subunits of Pol IV and Pol V arose from their Pol II counterparts via many independent duplication events, starting prior to the evolution of land plants, followed by ‘Escape from Adaptive Conflict’ [subfunctionalization](#)

(escape from adaptive conflict (EAC), in which a single-copy gene is selected to perform a novel function while maintaining its ancestral function. This gene is constrained from improving either novel or ancestral function because of detrimental pleiotropic effects on the other function. After duplication, one copy is free to improve novel function, whereas the other is selected to improve ancestral



Plants encode specialized RNA polymerases that act non-redundantly in the *de novo* DNA methylation pathway, differ in their subunit compositions and in the types of noncoding RNAs they generate, and employ unique machinery for their recruitment to chromatin.

Recent advances in our understanding of the evolution and composition of these polymerases has confirmed the existence of specialized Pol IV and Pol V machinery across land plants, and has revealed functional diversity both between and within Pol IV and Pol V subtypes.

The identification of Pol IV and Pol V transcripts *in vitro* and *in vivo* has provided the first clues into the activities of these polymerases and suggests that they are not playing by the same rules governing Pol II activity.

Finally, a quite detailed view of the machinery required to facilitate recruitment of these polymerases to chromatin has emerged, revealing a dependence on previously established chromatin modifications as part of an intricate network of **self-reinforcing loops**.

siRNAs - summary

The siRNA pathway silences foreign DNA, transposons and repetitive elements.

In plants, siRNAs are produced by the action of Dicer-like proteins dicing dsRNA into 24 nt siRNAs

The siRNAs associate with AGO proteins and form silencing complexes

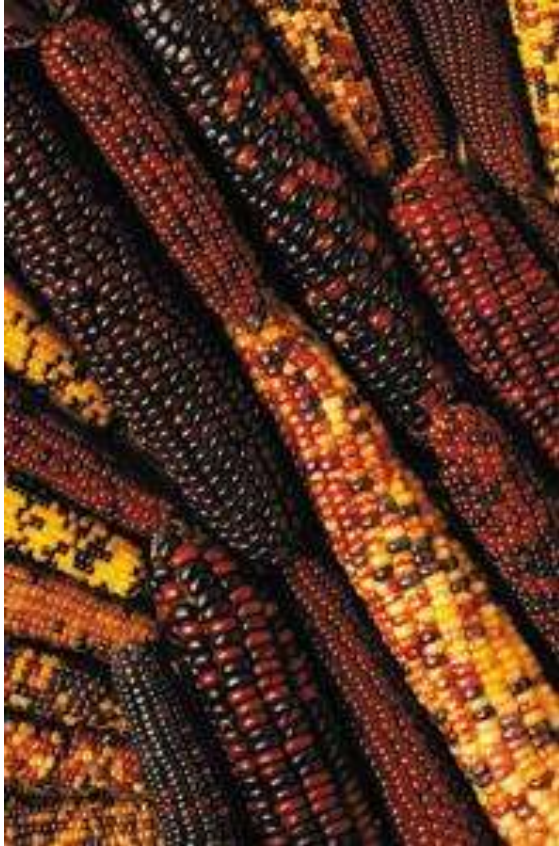
The silencing complexes can act post-transcriptionally on RNA targets, cleaving them or interfering with translation

The silencing complexes can also act on chromatin, silencing their targets by DNA methylation or histone modification

Epigenetic controls in whole-plant processes

- Transposon silencing
- Control of flowering time
- Control of imprinted genes
- Gene silencing *in trans*; paramutation
- Resetting the epigenome

Transposons



Fragments of DNA that can insert into new chromosomal locations

Some copy themselves and increase in number within the genome

Responsible for large scale chromosome rearrangements and single-gene mutagenic events

Transposons



Barbara McClintock

Transposable elements were discovered in *Zea mays* by Barbara McClintock.

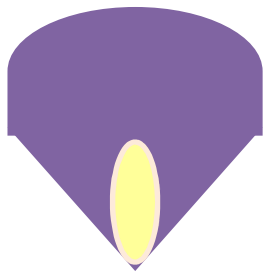
For her discovery, she was awarded the Nobel Prize in Physiology or Medicine in 1983.



Corn kernels showing transposition

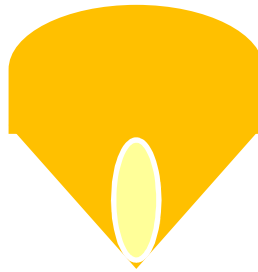
Transposons can cause inactive or unstable alleles

Gene required for pigment biosynthesis



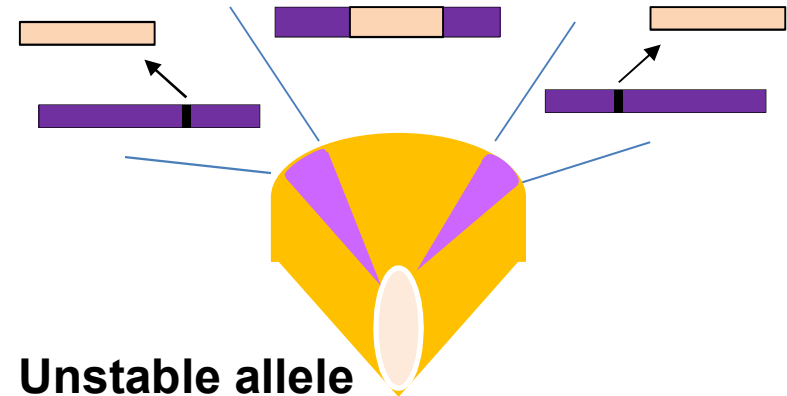
Wild-type allele
Pigmented kernel

Gene interrupted by transposon



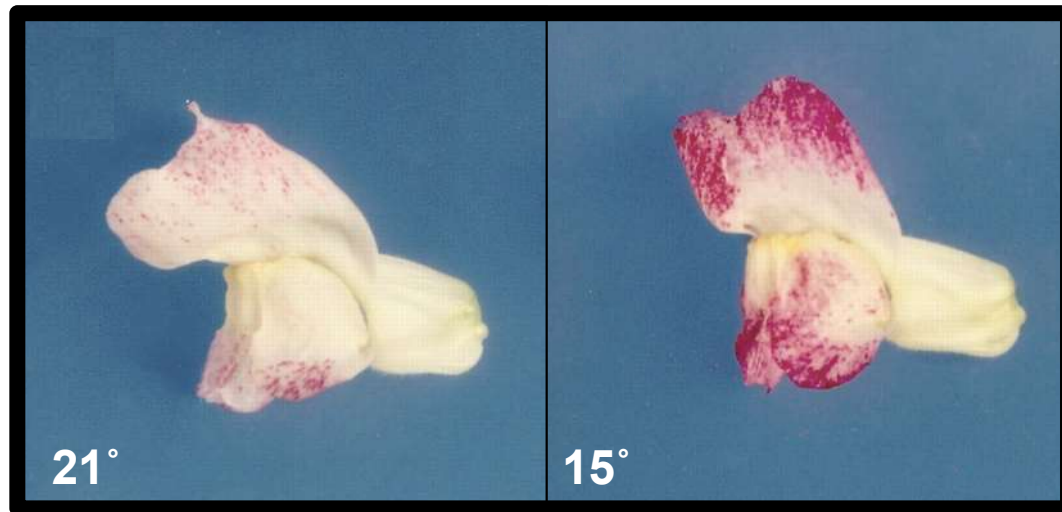
Mutant allele
Unpigmented kernel

Excision of the transposon causes unstable alleles



Unstable allele
Partially pigmented kernel

Naturally occurring transposons are a source of genetic variation



An *Antirrhinum* transposon that is only active at low temperatures.

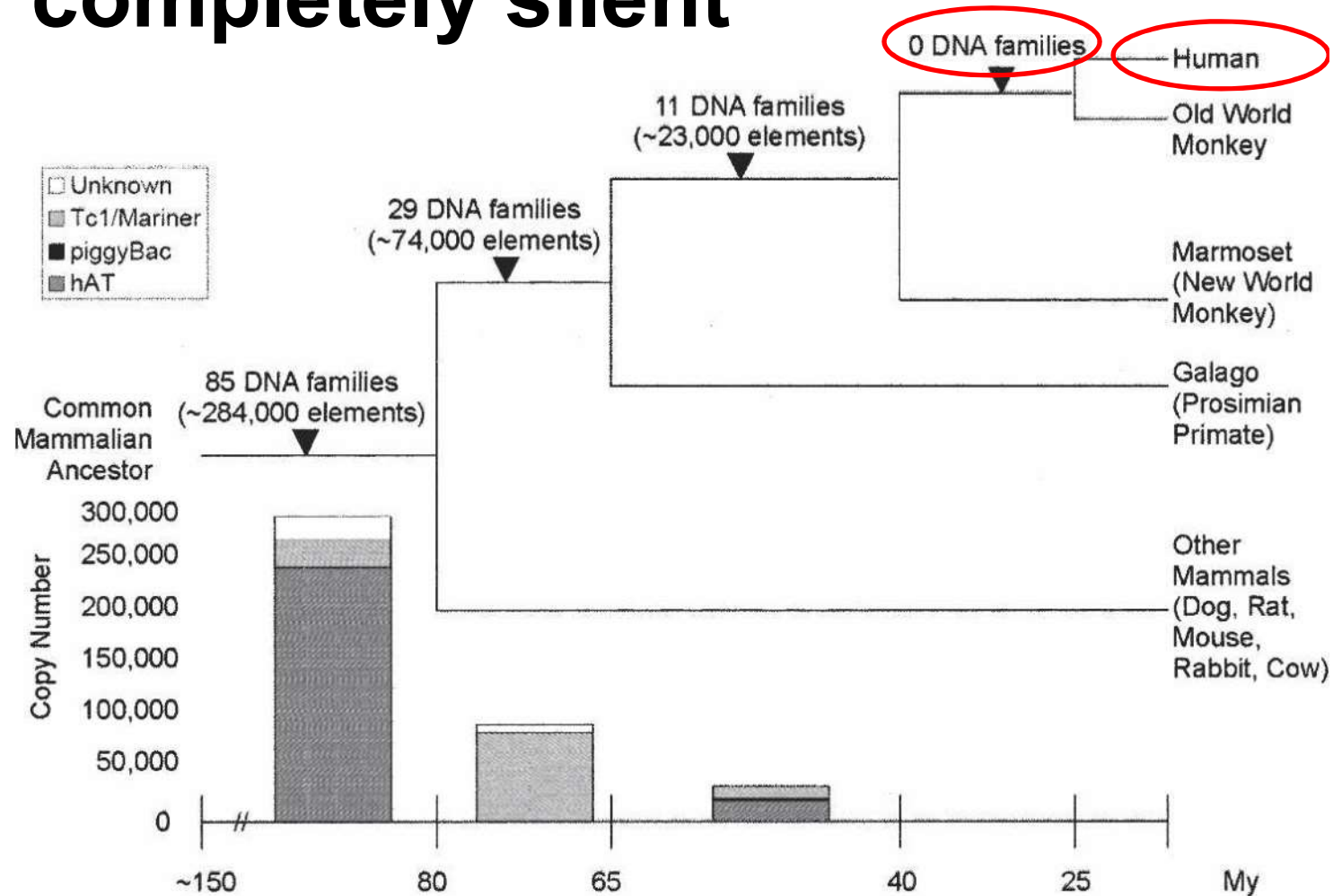
“Variation is the raw material of evolutionary change”
- Stephen Jay Gould
(1941 – 2002)

Transposons are abundant

Organism	% of genome derived from transposons
Yeast - <i>S. cerevisiae</i>	3%
Nematode - <i>C. elegans</i>	6%
<i>Arabidopsis thaliana</i>	14%
Fruitfly - <i>D. melanogaster</i>	15%
Rice - <i>Oryza sativa</i>	14%
<i>Homo sapiens</i>	44%
Corn - <i>Zea mays</i>	60%

Human transposons are almost completely silent

Number of active transposon families through evolutionary time



Pace, J.K., and Feschotte, C. (2007) The evolutionary history of human DNA transposons: Evidence for intense activity in the primate lineage. *Genome Res.* 17: 432-432

Transposon silencing

By contrast, maize has many active transposons

Epigenetic marks are thought to have evolved to silence foreign DNA (transposons, viruses)

Mutants that interfere with epigenetic silencing release transposons from silencing, and allow mutagenic transposon activity



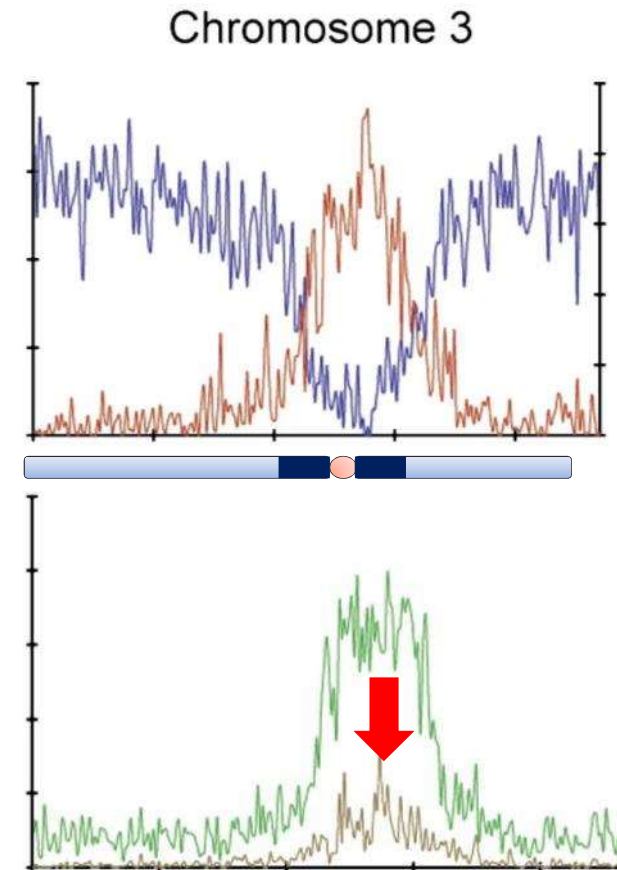
DNA methylation is necessary to silence transposons

Loss-of-function *met1* or *ddm1* (decrease in DNA methylation1) mutants have hypomethylated DNA

BLUE = Gene density
RED = Repetitive element density

GREEN = Methylated DNA

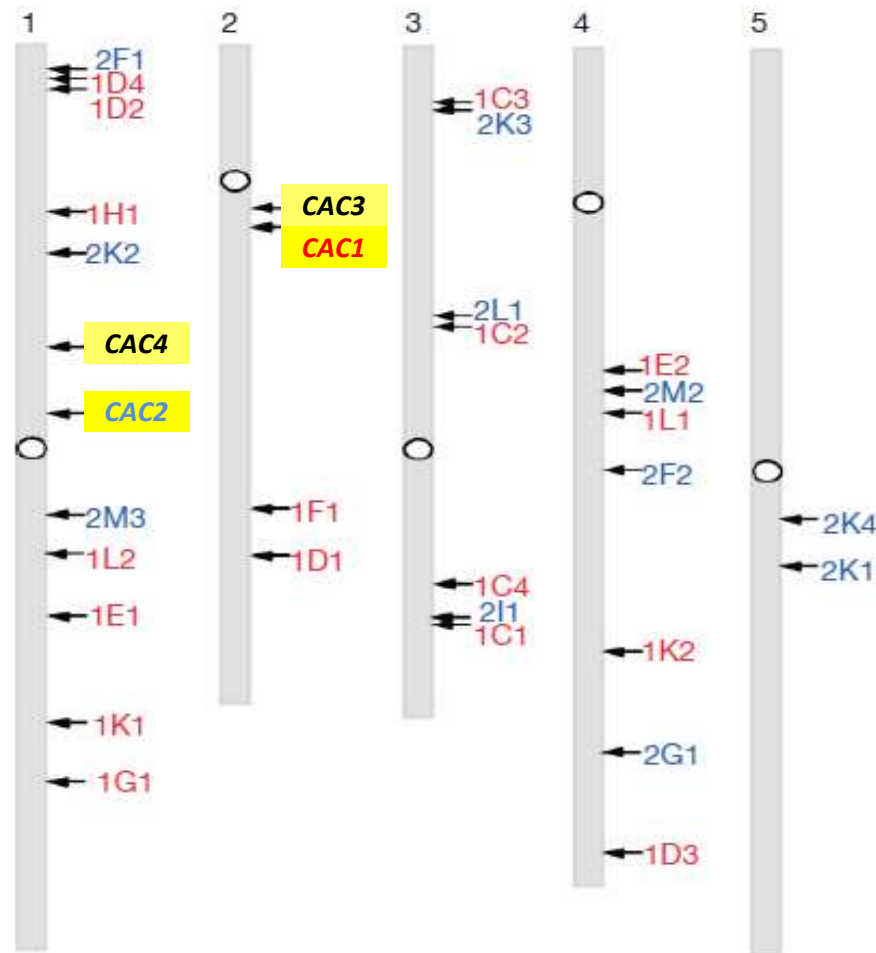
BROWN = Methylated DNA in a *met1* mutant

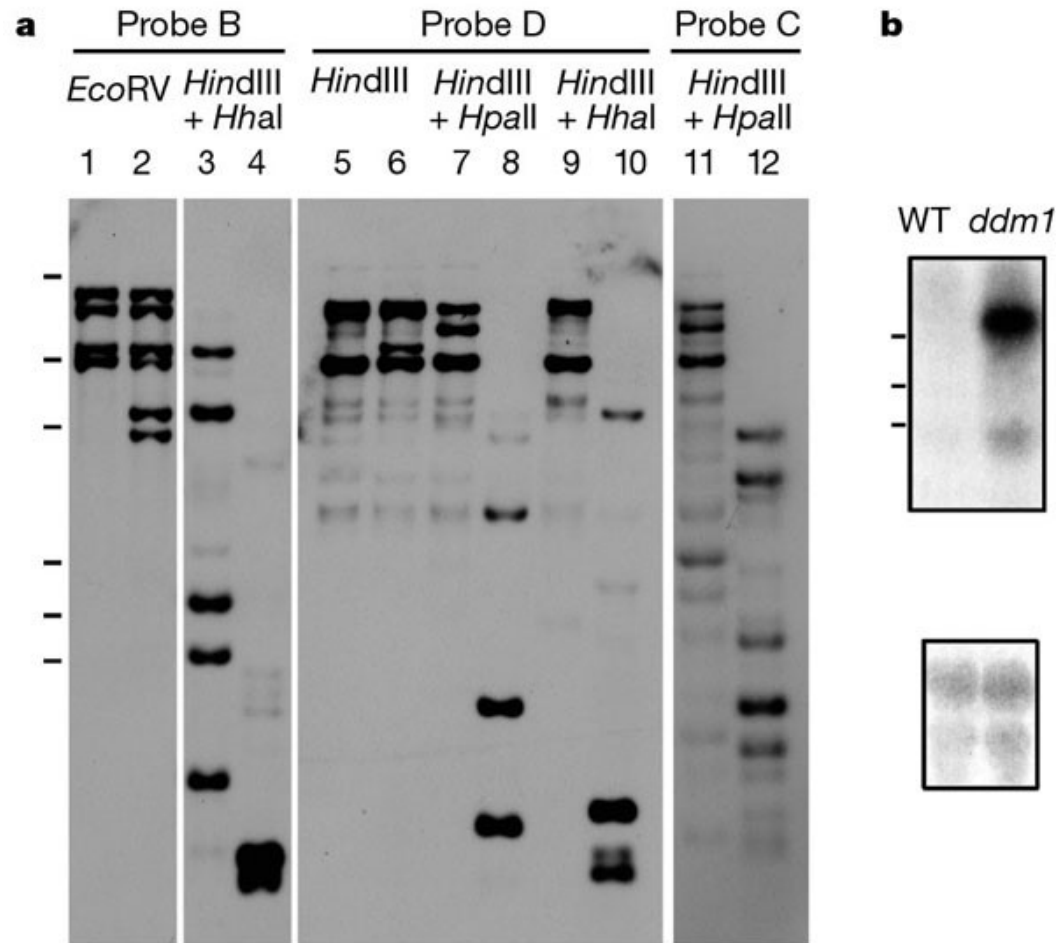


Transposons are activated in *ddm* mutants

Six generations after DNA methylation was reduced by *DDM* inactivation, newly inserted transposons were distributed throughout the genome.

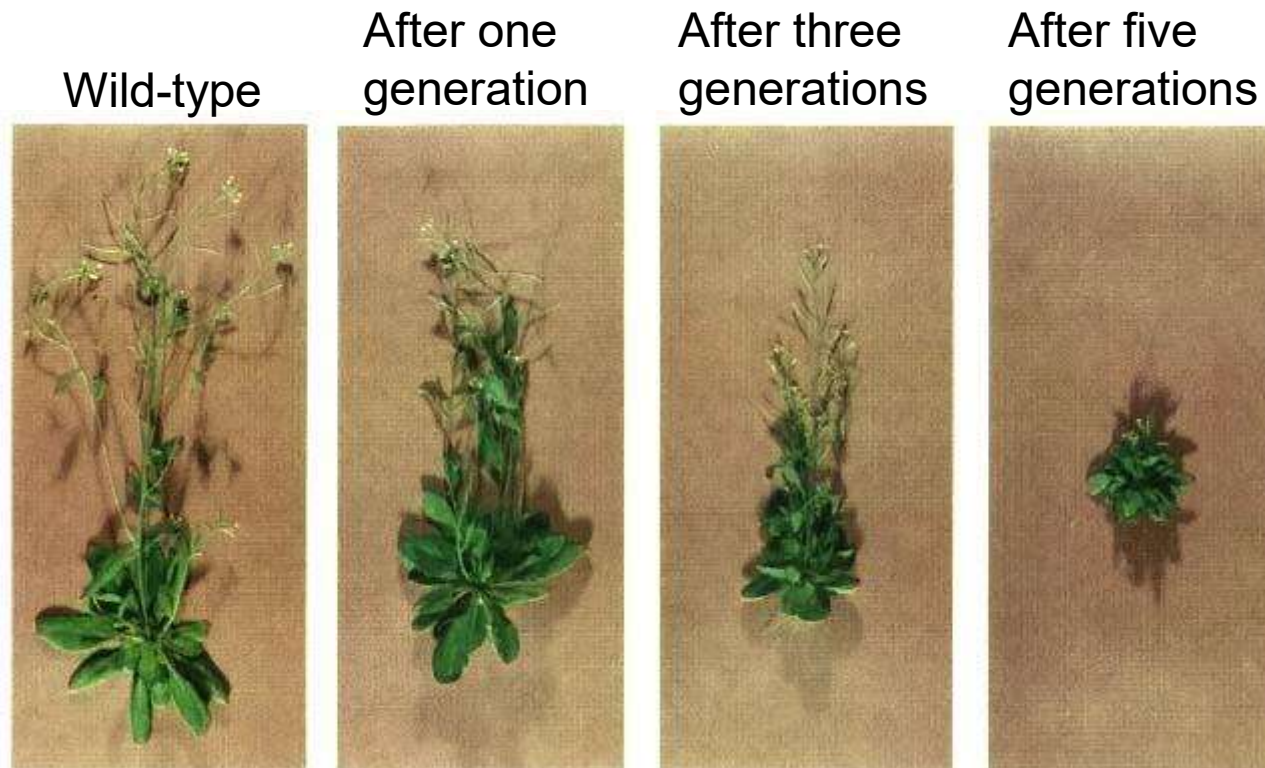
Yellow is site of original insertion, blue and red are new sites of insertion.



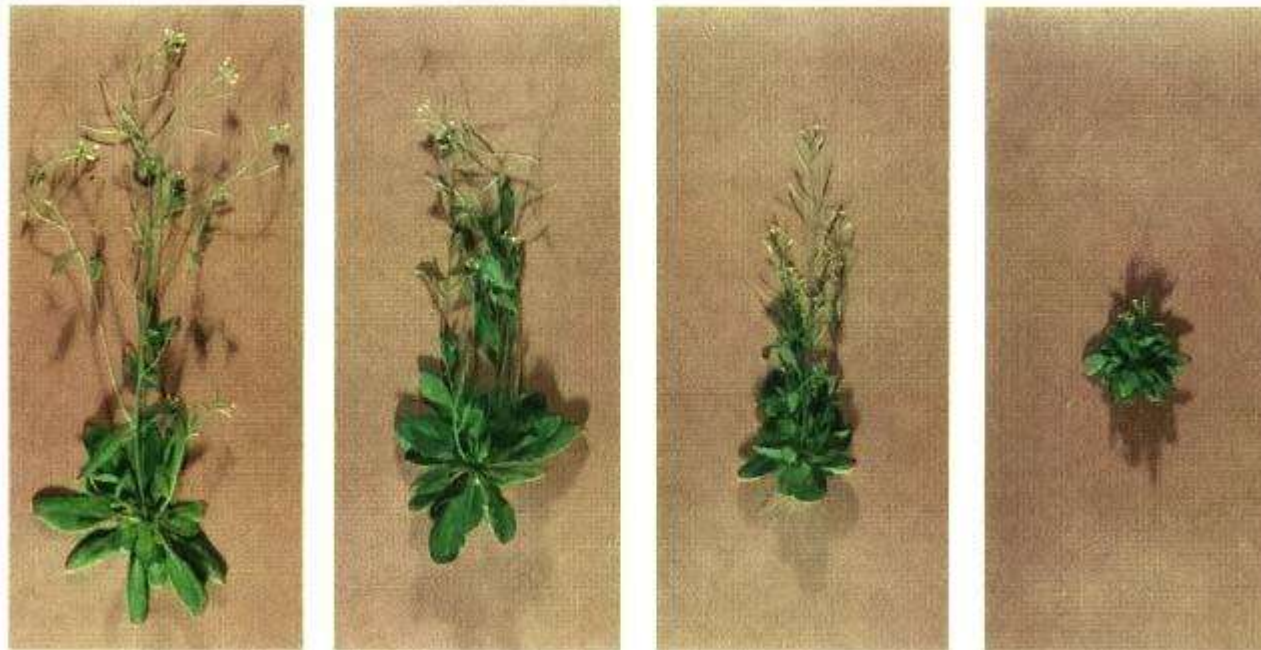


Methylation-sensitive restriction enzymes (*HpaI* or *HhaI*) and probes B, C, D (Fig. 3a) were used to compare the methylation status of *CAC* elements between *ddm1* (even lanes) and Columbia wild-type (odd lanes) plants. The *ddm1* plant is before the repeated self-pollination (four generations before the plant shown in lane 10 of Fig. 3c). It still keeps the donor copies of *CAC* elements (lane 2). The DNA length markers are 19.3, 7.74, 5.53, 3.14, 2.69 and

Activated transposons induce mutations



After *DDM* inactivation, plants become more and more abnormal as they accumulate transposon-induced mutations.



Epigenetic silencing of transposons by DNA methylation is necessary to maintain genomic integrity.

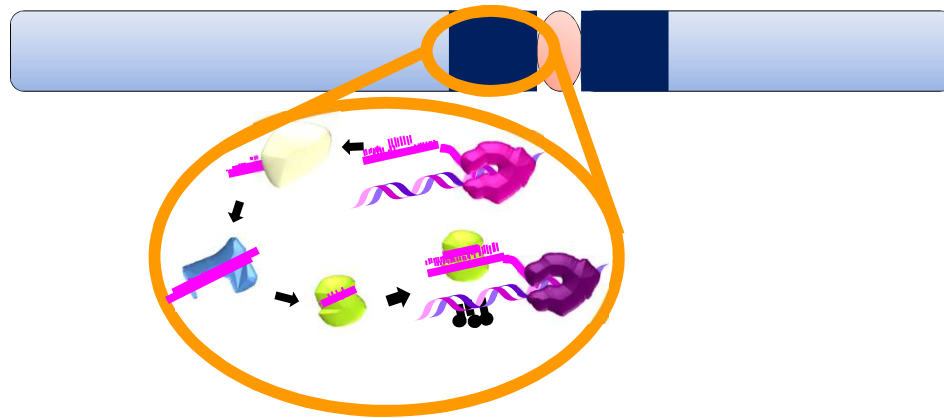
Initiating and maintaining silencing at repetitive DNA and transposons



How does the genome specifically recognize and silence repetitive elements and transposons?

In other words, how does it recognize “self” (genes) from “non-self”? What is the basis for this “genomic immune recognition system”?

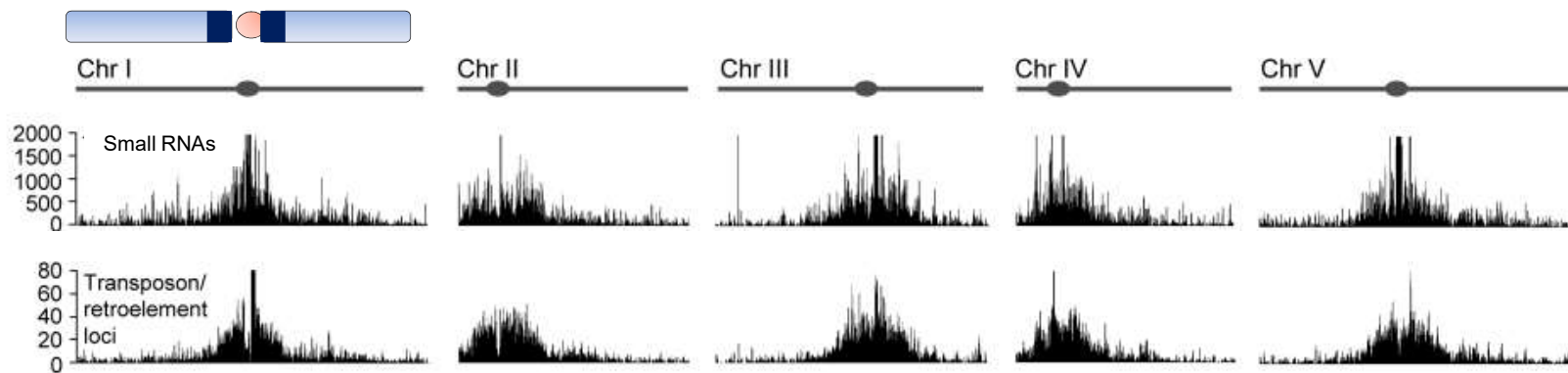
Repetitive elements and transposons are actively silenced



Maintaining transposon silencing is an active, dynamic process that requires ongoing siRNA production and epigenetic vigilance.

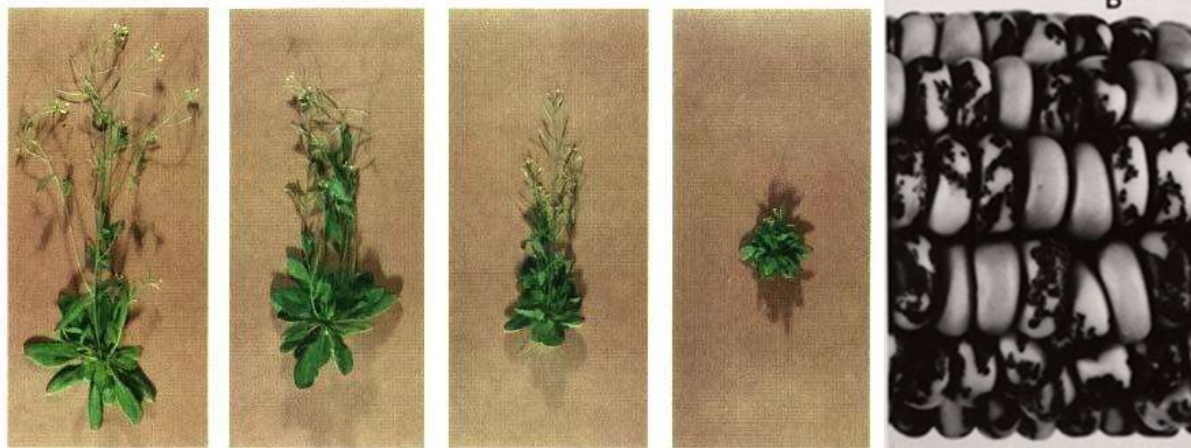


Small interfering RNAs (siRNAs) are preferentially derived from pericentromeric regions



The density of small RNA-homologous loci is highest in the centromeric and pericentromeric regions which contain a high density of repeat sequence classes, such as transposons.

Epigenetic silencing of transposons and repetitive elements



Transposons must be tightly controlled to prevent widespread mutagenic activity. Epigenetic controls to maintain silencing include DNA methylation, histone modification and siRNA production.