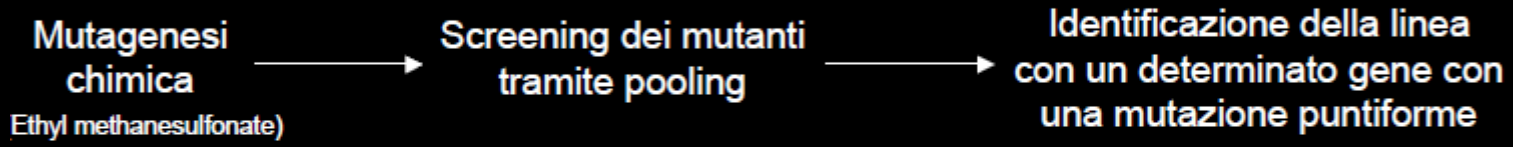
REVERSE GENETICS
ALTRI APPROCCI

1. Come faccio a studiare i fenotipi letali?
2. Come posso studiare i fenotipi associati al mio gene di interesse se per questo non è ancora disponibile un mutante inserzionale?

- TILLING
- ANTISENSO
- RNAi

Il T-DNA ed i trasposoni NON sono facilmente applicabili a tutte le specie vegetali

TILLING (Targeting Induced Local Lesions IN Genomes)

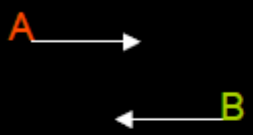


Vantaggi:

- E' una procedura semplice e più ampiamente utilizzabile
- Non necessita di colture cellulari e non genera linee transgeniche
- Produce serie alleliche con linee K.O. o solamente "attenuate"

L'endonucleasi Cel-I identifica i mismatch dell'appaiamento tra wt e mutante

Primer gene specifici

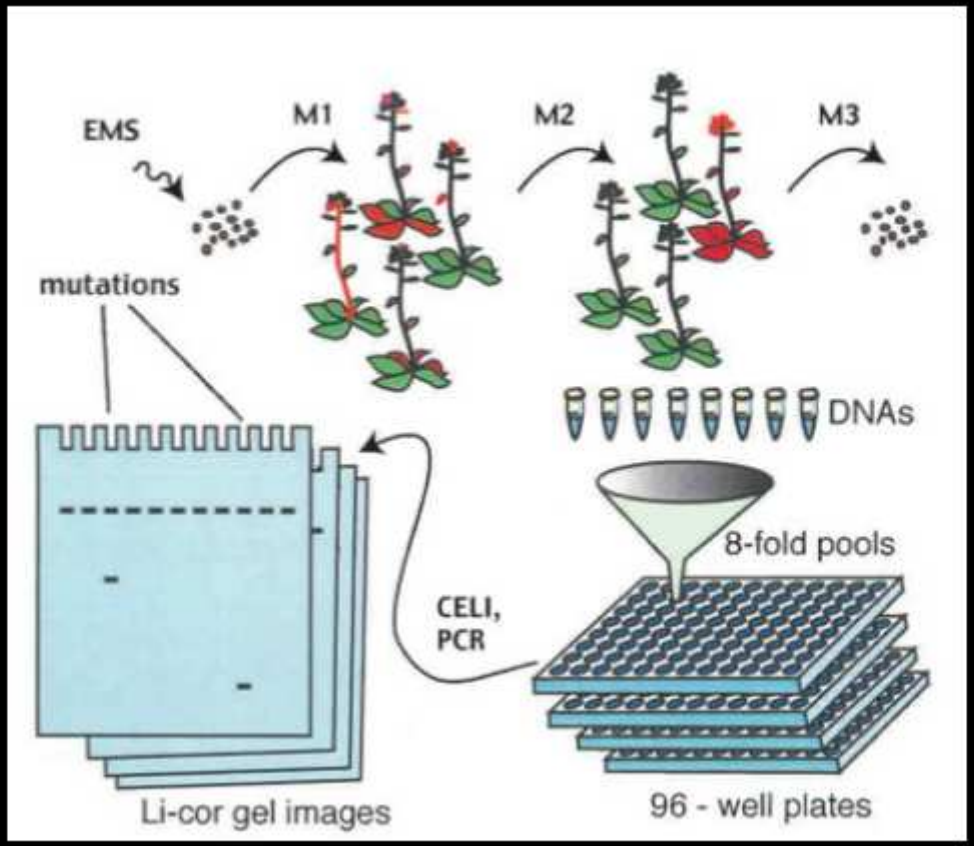


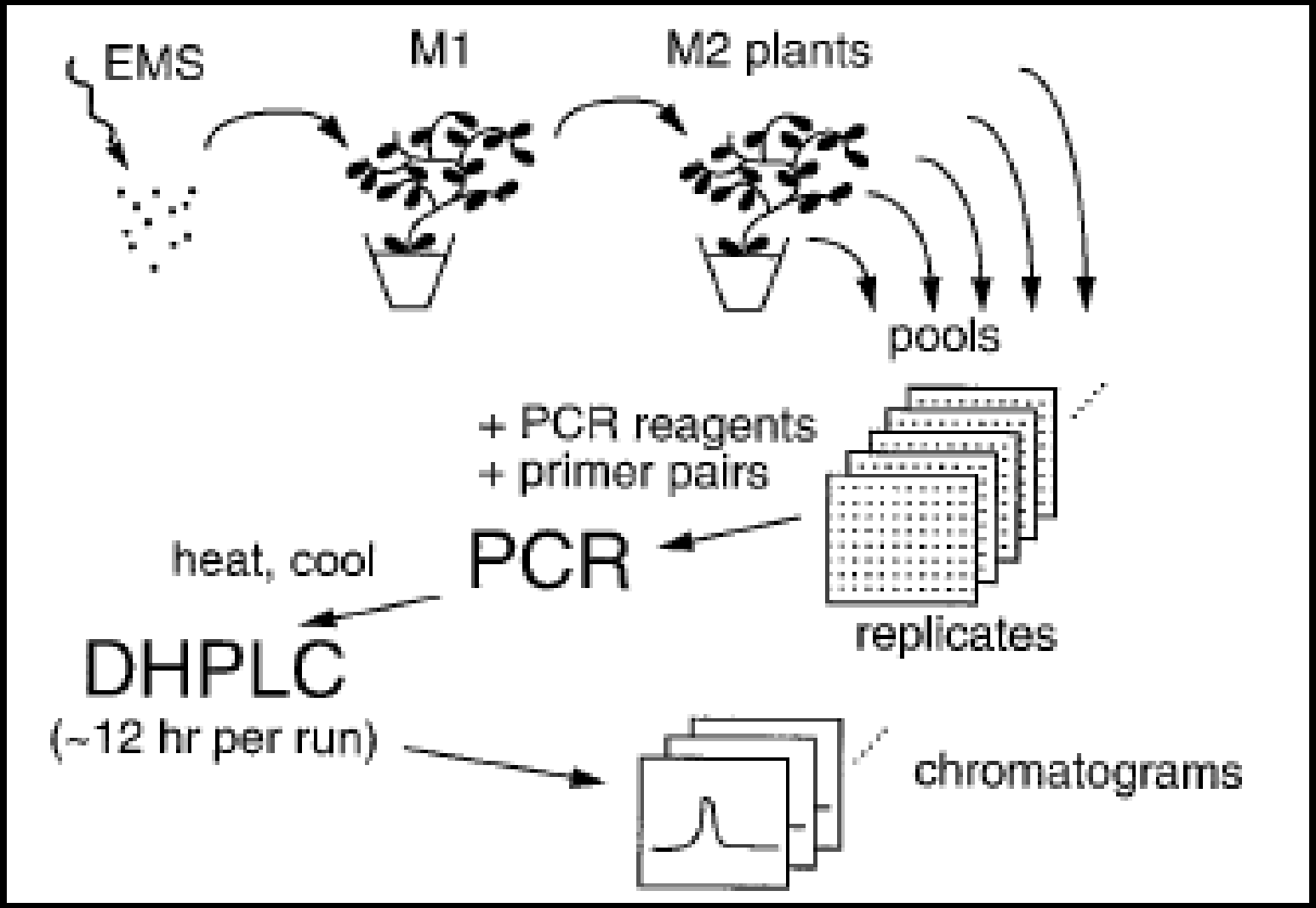
A e B due marcatori differenti

PCR sul wt e nel mutante → cicli di appaiamento dei frammenti amplificati

Heteroduplex

high-throughput TILLING - how it works



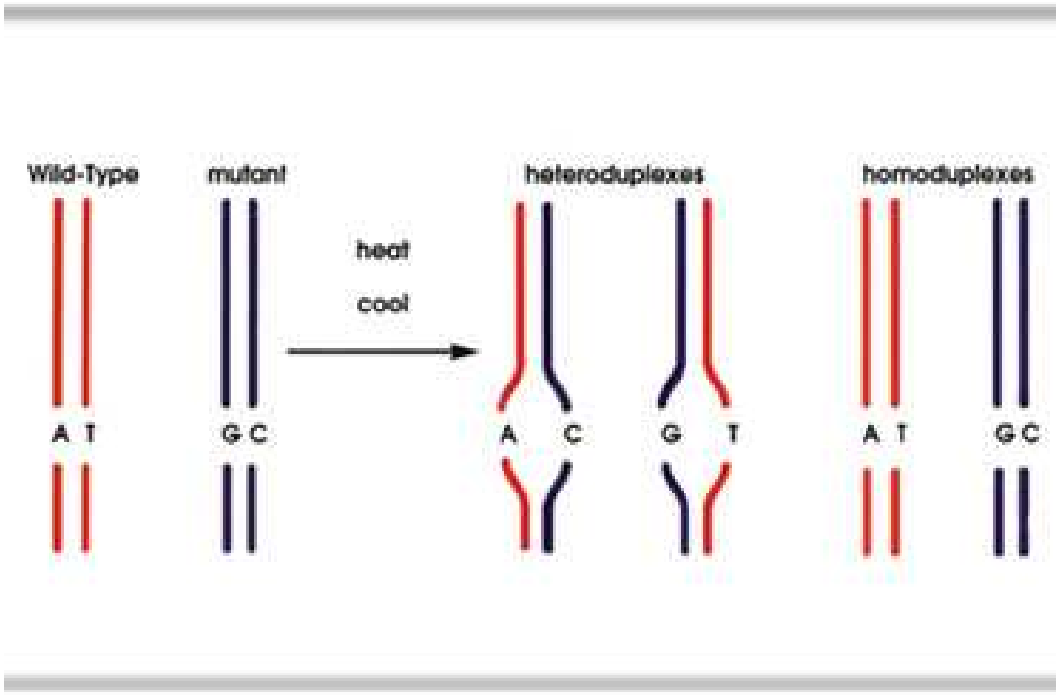

EMS
M1
M2 plants
pools
+ PCR reagents
+ primer pairs
PCR
heat, cool
DHPLC
(~12 hr per run)
replicates
chromatograms

DHPLC= HPLC denaturante

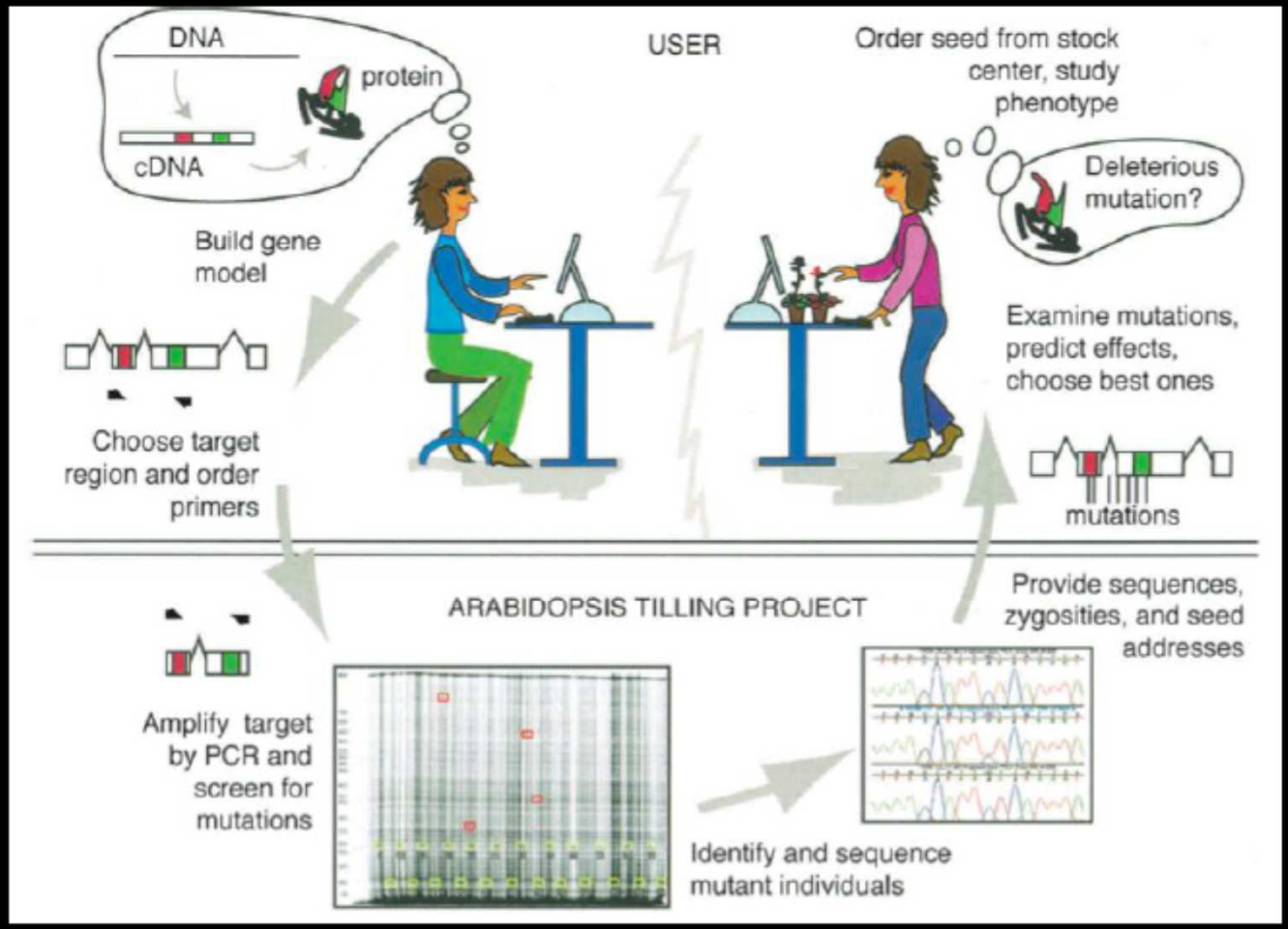
• Duplex si formano quando un frammento amplificato di DNA mutato ed uno non mutato vengono denaturati termicamente e lasciati ricombinare.

• Su una colonna cromatografica, l'eteroduplex è solitamente più veloce (meno trattenuto) dell'omoduplex

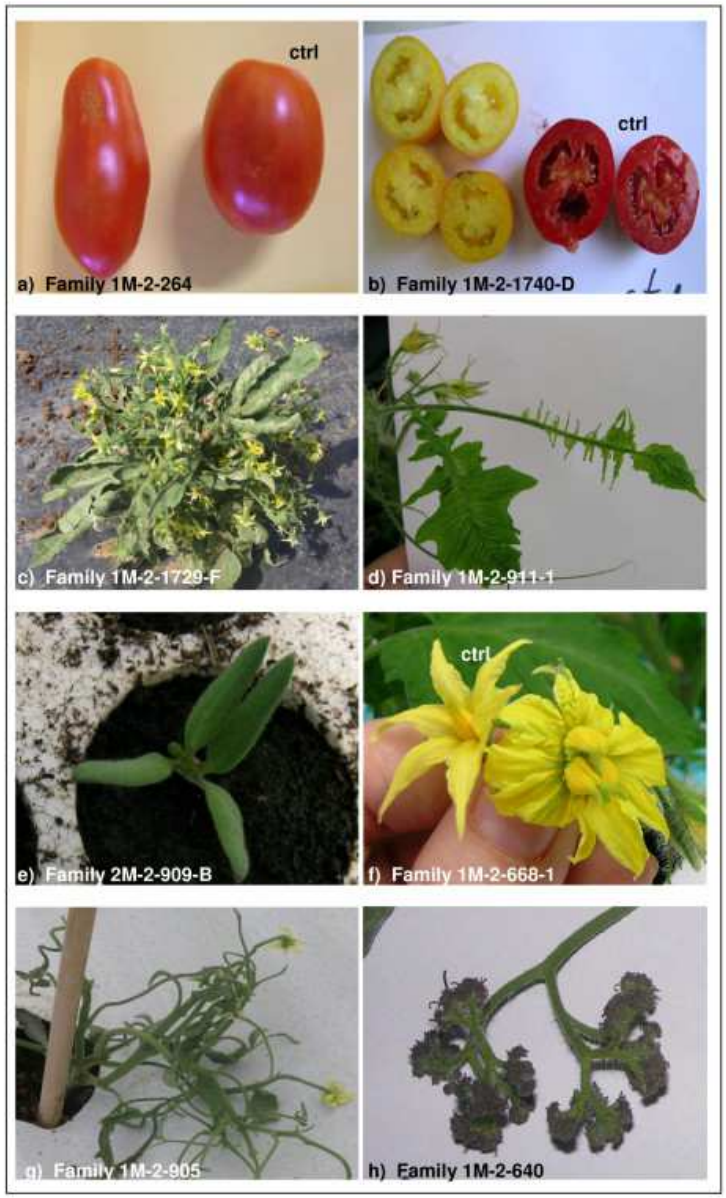
• Può essere impiegata per rilevare ogni tipo di mutazione (SNPs, inserzioni, delezioni e tandem repeat)



high-throughput TILLING - how it works



TILLING in pomodoro

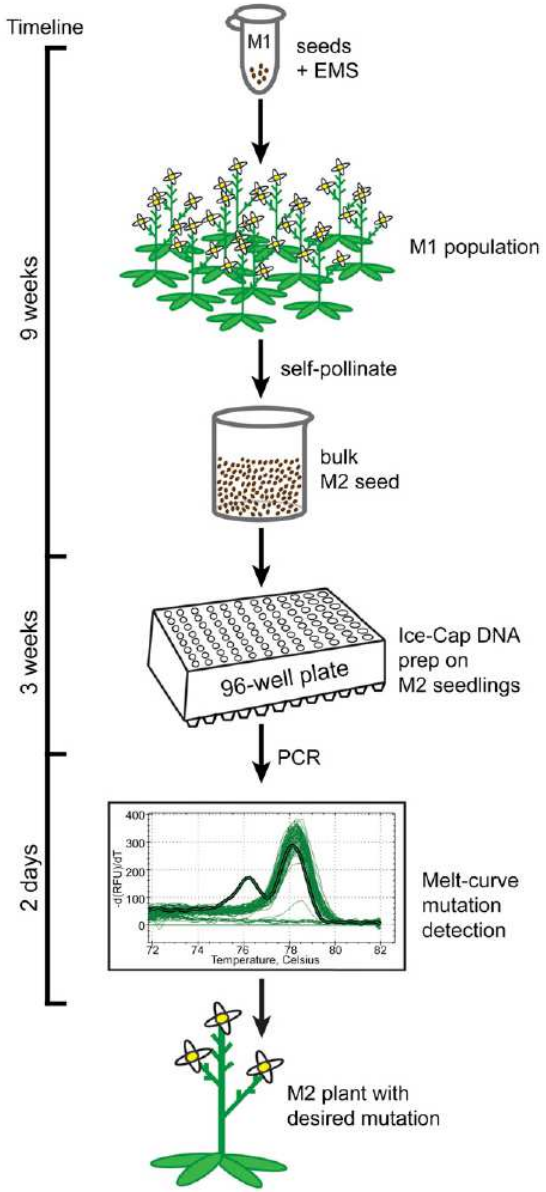


Mutation density in 0.7% EMS and 1% EMS Red Setter populations

Target gene		No. of screened M3 families		No. of identified mutations		Overall mutation density	
Name	**Amplicon size (kb)**	**0.7% EMS**	**1% EMS**	**0.7% EMS**	**1% EMS**	**0.7% EMS**	**1% EMS**
Rab11a	0.407	1,373	713	1	3	1/559 kb	1/97 kb
PG	2.587	2,791	963	7	2	1/1031 kb	1/1246 kb
Exp1	1.025	3,885	1,284	14	6	1/284 kb	1/219 kb
RIN	1.331	3,885	1,284	4	8	1/1293 kb	1/214 kb
Gr	1.409	3,885	1,284	5	3	1/1095 kb	1/603 kb
Lcy-b	1.274	3,801	1,252	4	3	1/1211 kb	1/532 kb
Lcy-e	1.414	3,630	1,185	6	0	1/855 kb	-
Total/mean	**9.447**			**41**	**25**	**1/574 kb**	**1/322 kb**

The accession numbers of the analyzed seven target genes are the following: *Rab11a* [GenBank:AJ245570], *PG* [GenBank:M37304], *Exp1* [GenBank:AF548376], *RIN* [GenBank:AF448522], *Gr* [GenBank:DQ372897], *Lcy-b* [GenBank:CQ788383], *Lcy-e* [GenBank:Y14387]. The number of screened M3 families, the number of identified mutations and the overall mutation density, estimated as described in Methods, are reported both for 0.7% and 1% EMS Red Setter populations.

Minoia *et al. BMC Research Notes* 2010 **3**:69 doi:10.1186/1756-0500-3-69

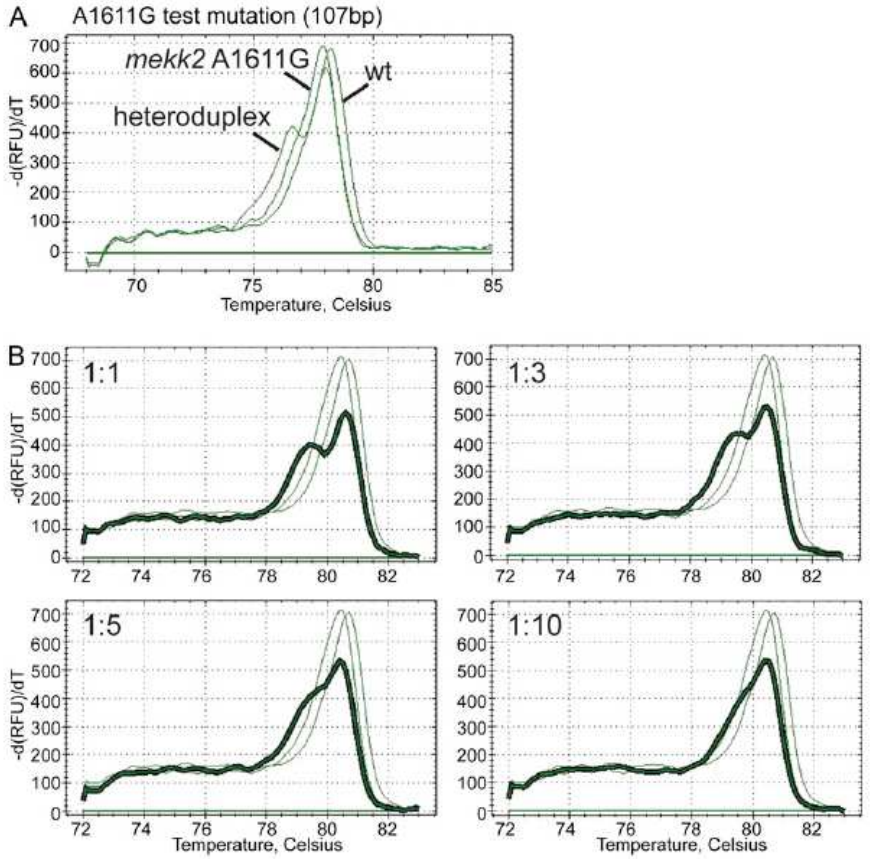


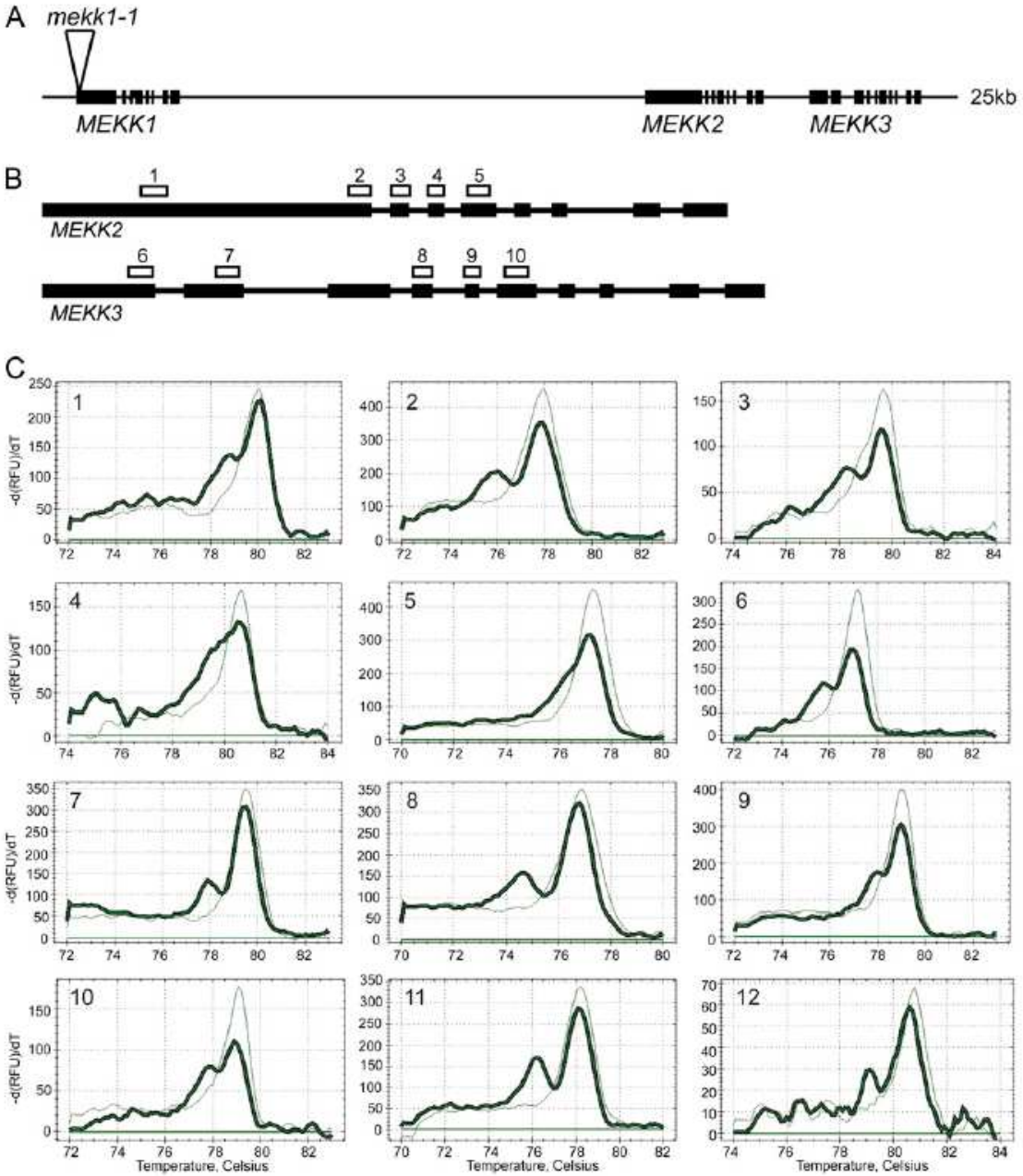
iTILLING: A Personalized Approach to the Identification of Induced Mutations in Arabidopsis[1[C][OA]]

Susan M. Bush and Patrick J. Krysan*

Department of Horticulture (S.M.B., P.J.K.) and Genome Center of Wisconsin (P.J.K.), University of Wisconsin, Madison, Wisconsin 53706

Plant Physiology®, September 2010, Vol. 154, pp. 25–35,




A
mekk1-1
25kb
MEKK1
MEKK2
MEKK3
B
1
2
3
4
5
MEKK2
6
7
8
9
10
MEKK3
C
-d(RFU)/dT
Temperature, Celsius

SILENZIAMENTO GENICO

RNAi based methods

History:

Early 1990's, phenomena first found by plant scientists: co-suppression in petunia

1998, in *C.elegans*, formally discover dsRNA as signal for RNA interference (Fire and Mello)

1999, small RNA species derived from mRNA detected

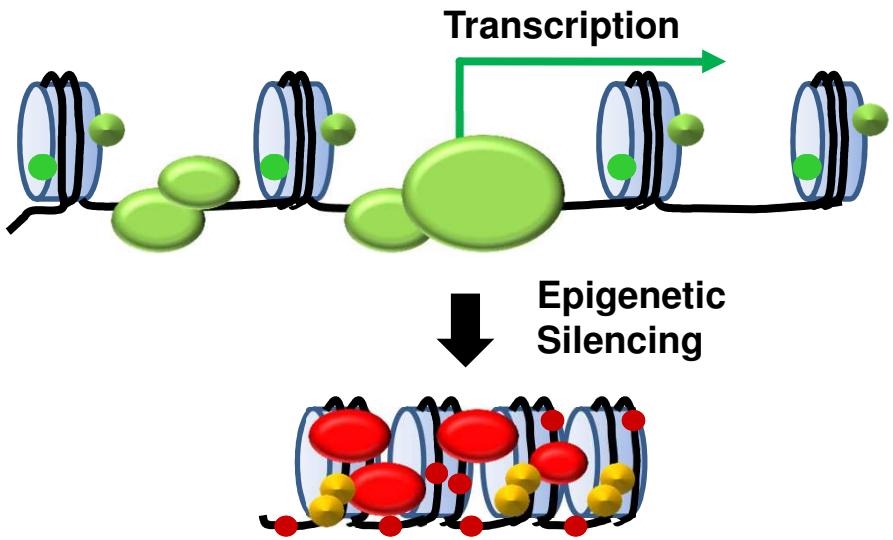
2001, discovery of dsRNA processing enzyme Dicer

More components in RISC identified

2006, A. Fire and C. Mello won Nobel prize in medicine because of their discovery of dsRNA as mediator of RNAi

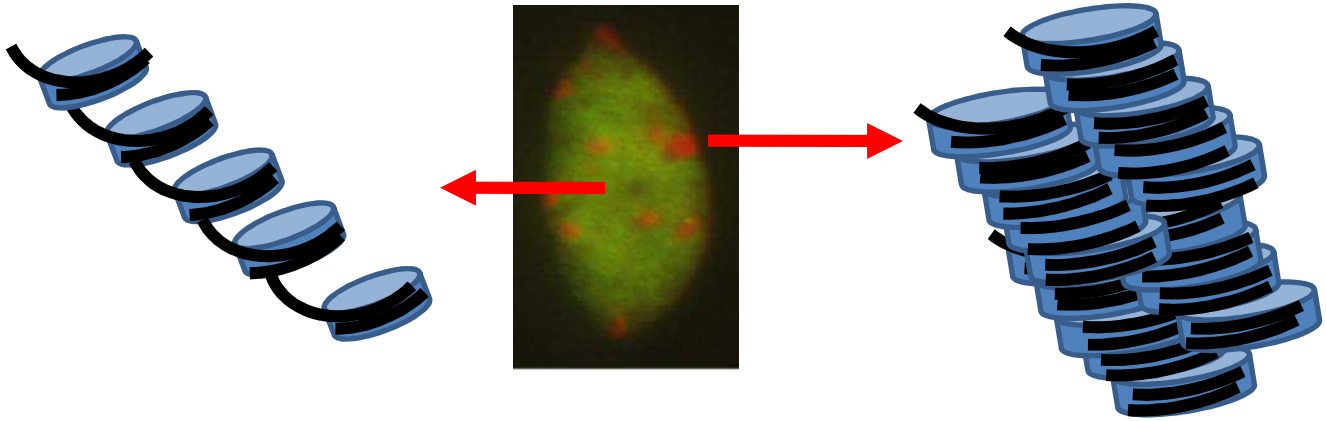
What does “epigenetics” mean?

- **Literally**, epigenetics means above, or on top of, genetics.
- Usually this means information coded beyond the DNA sequence, such as in covalent modifications to the DNA or modifications to the chromatin structure.

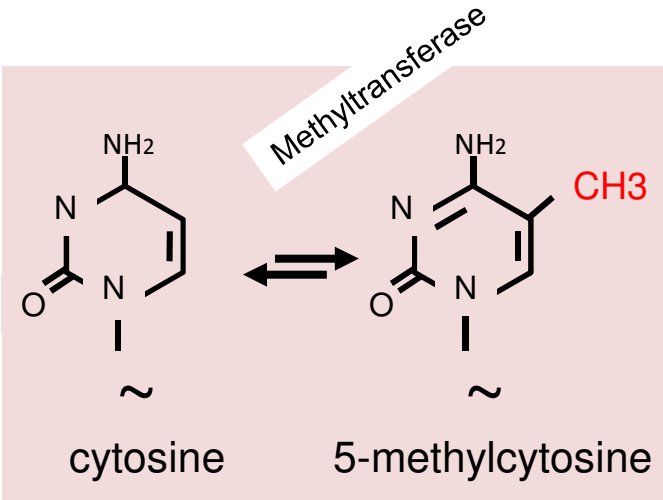


Epigenetic modifications

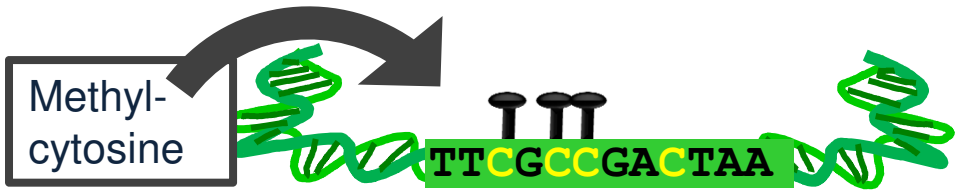
- Epigenetic modifications include:
  - Cytosine methylation of DNA
  - Histone modifications
- Collectively, these changes contribute to the distribution of DNA into silent, heterochromatin and active euchromatin



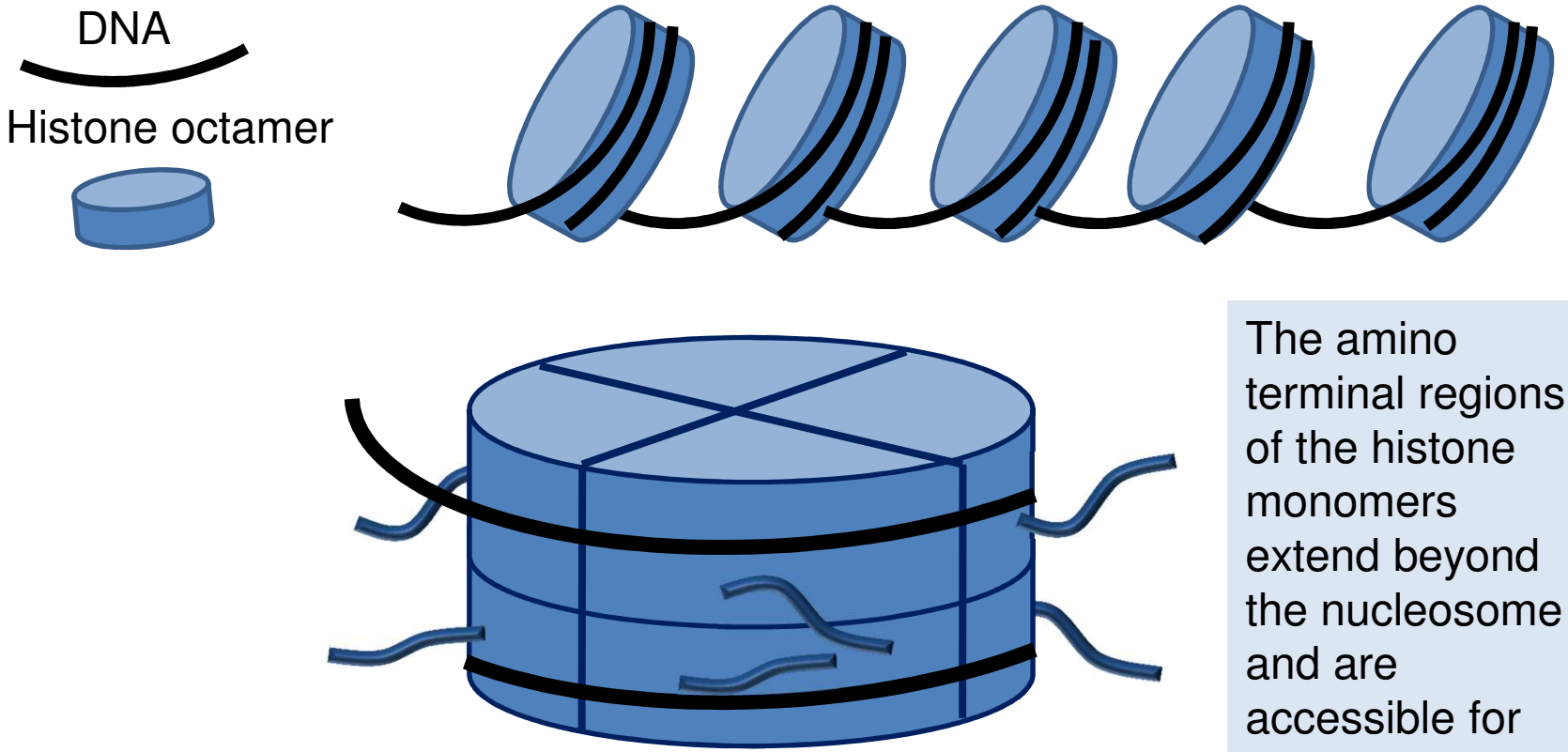
DNA methylation



DNA can be covalently modified by cytosine methylation.



Histone proteins can be modified to affect chromatin structure



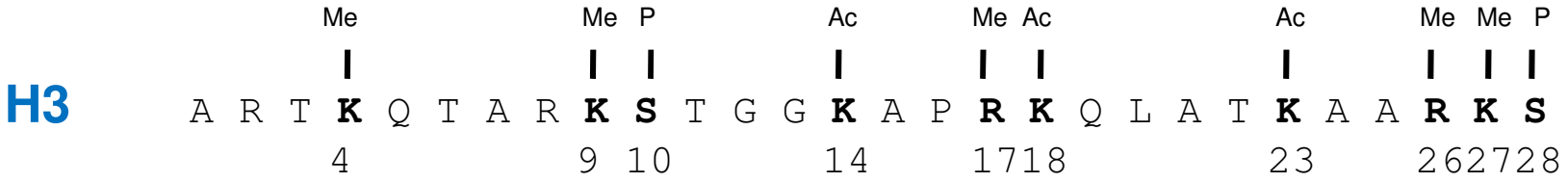
NUCLEOSOME

The amino terminal regions of the histone monomers extend beyond the nucleosome and are accessible for modification.

The Histone Code

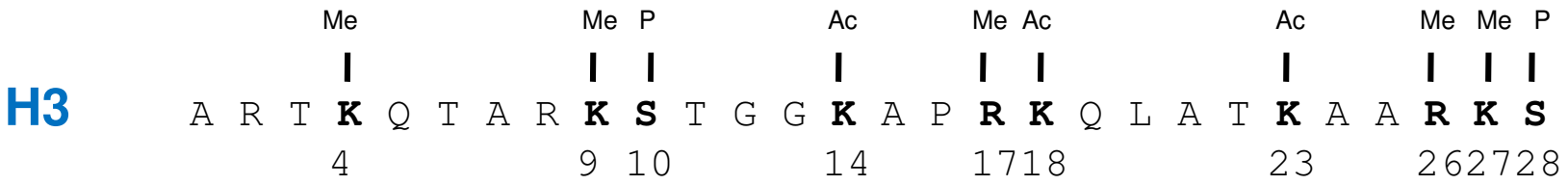
- Histones can be modified by
  - Acetylation (Ac)
  - Ubiquitination (Ub)
  - Methylation (Me)
  - Phosphorylation (P)
  - Sumoylation (Su)
- Depending on their position, these can contribute to transcriptional activation or inactivation.

Example – H3 modifications



The amino terminus of H3 is often modified at one or more positions, which can contribute to an activation or inhibition of transcription.

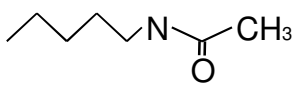
Example – H3 modifications



The amino terminus of H3 is often modified at one or more positions, which can contribute to an activation or inhibition of transcription.

Lysine can be acetylated, or mono-, di-, or tri-methylated

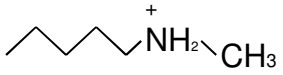
Lysine (K)



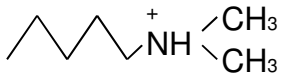
Acetylated lysine (KAc)

Methylated lysine

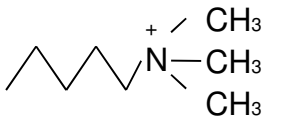
Mono (Kme1)



Di (Kme2)



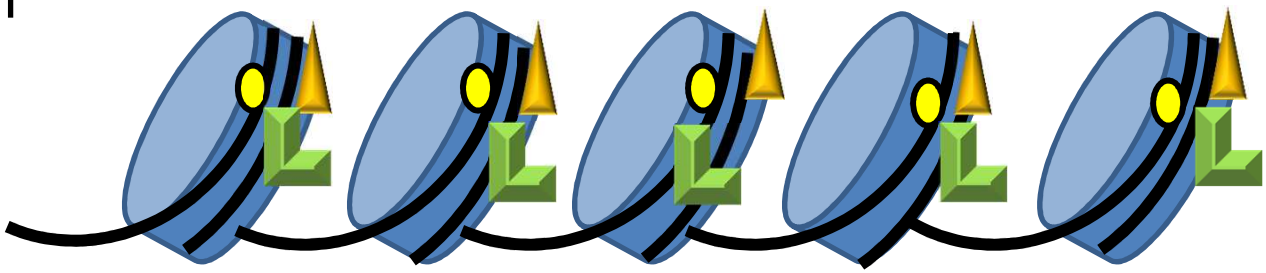
Tri (Kme3)



Histone modification affects chromatin structure

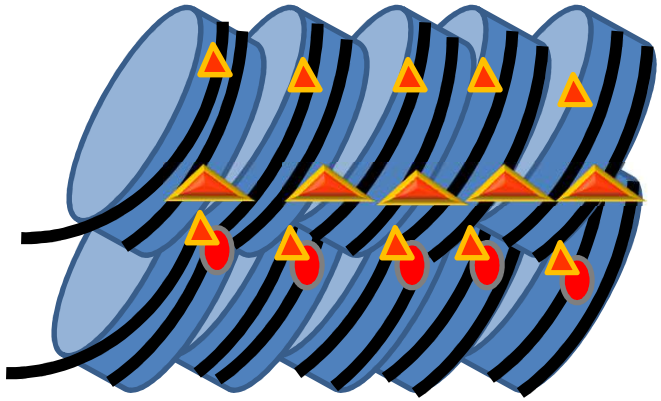
Open configuration

	Me	P	Ac
H3	K4	S10	K14

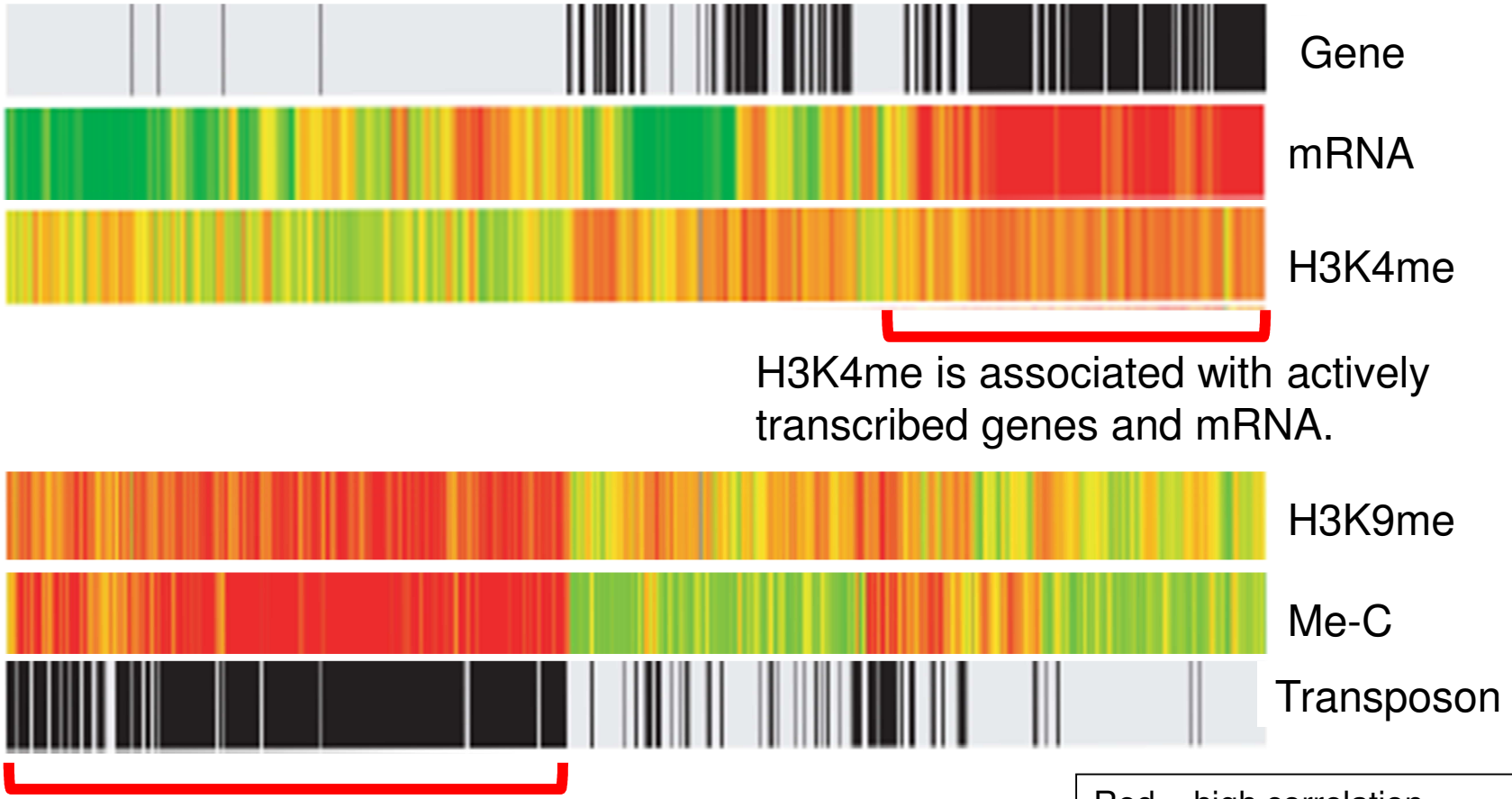


Closed configuration

	Me	Me P
H3	K9	K27 S28



Different histone modifications are associated with genes and transposons

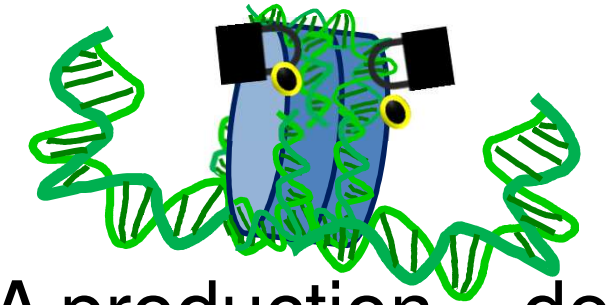


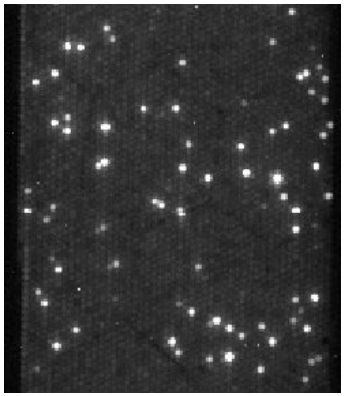
Methods for studying epigenetic modifications

Methods for studying epigenetic modifications

- DNA methylation– bisulfite sequencing

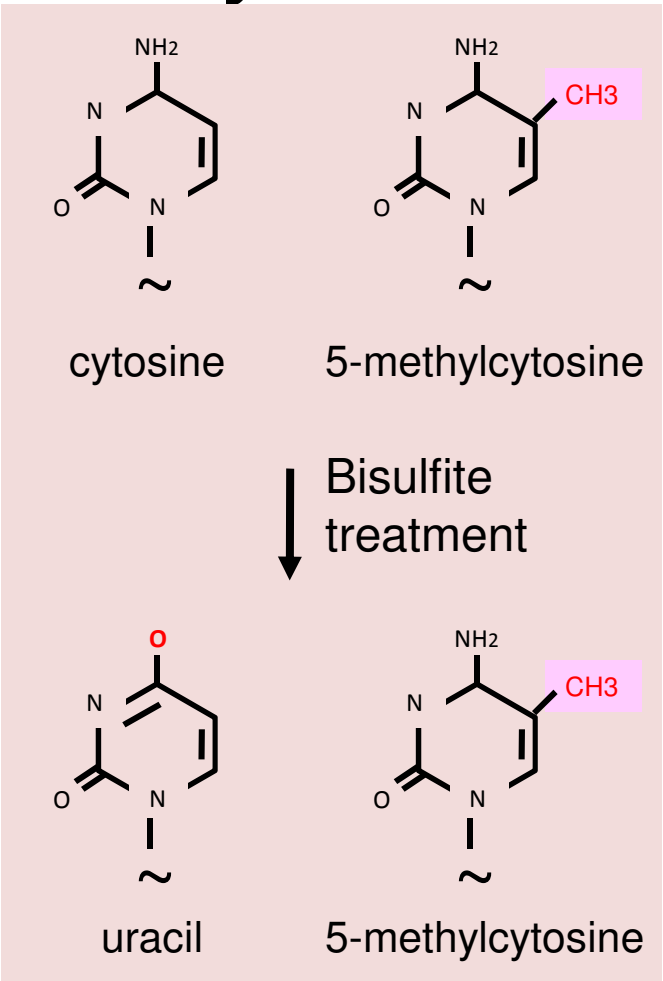
- Histone modification
  - chromatin immunoprecipitation (ChIP)
  - DNA adenosine methylation identification (DamID)

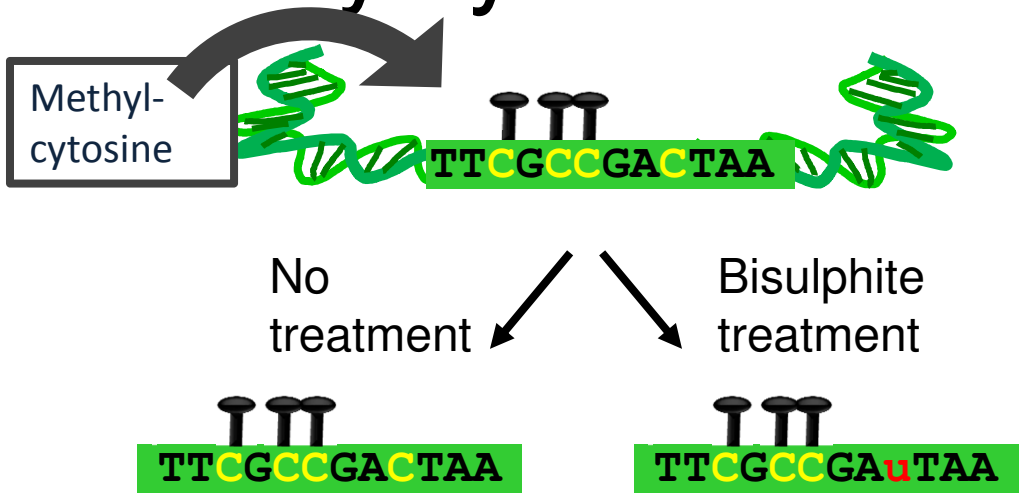




- siRNA production – deep sequencing

Bisulfite treatment differentiates cytosine and methylcytosine



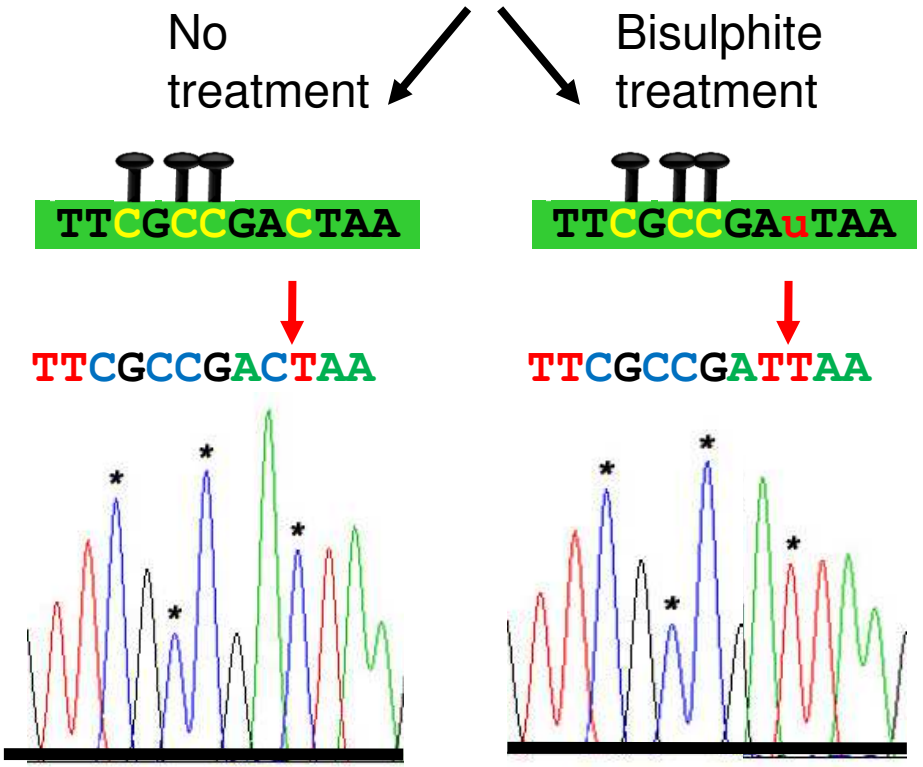


When DNA is bisulfite treated, unmethylated cytosine is converted to uracil. Methylcytosine is not affected.

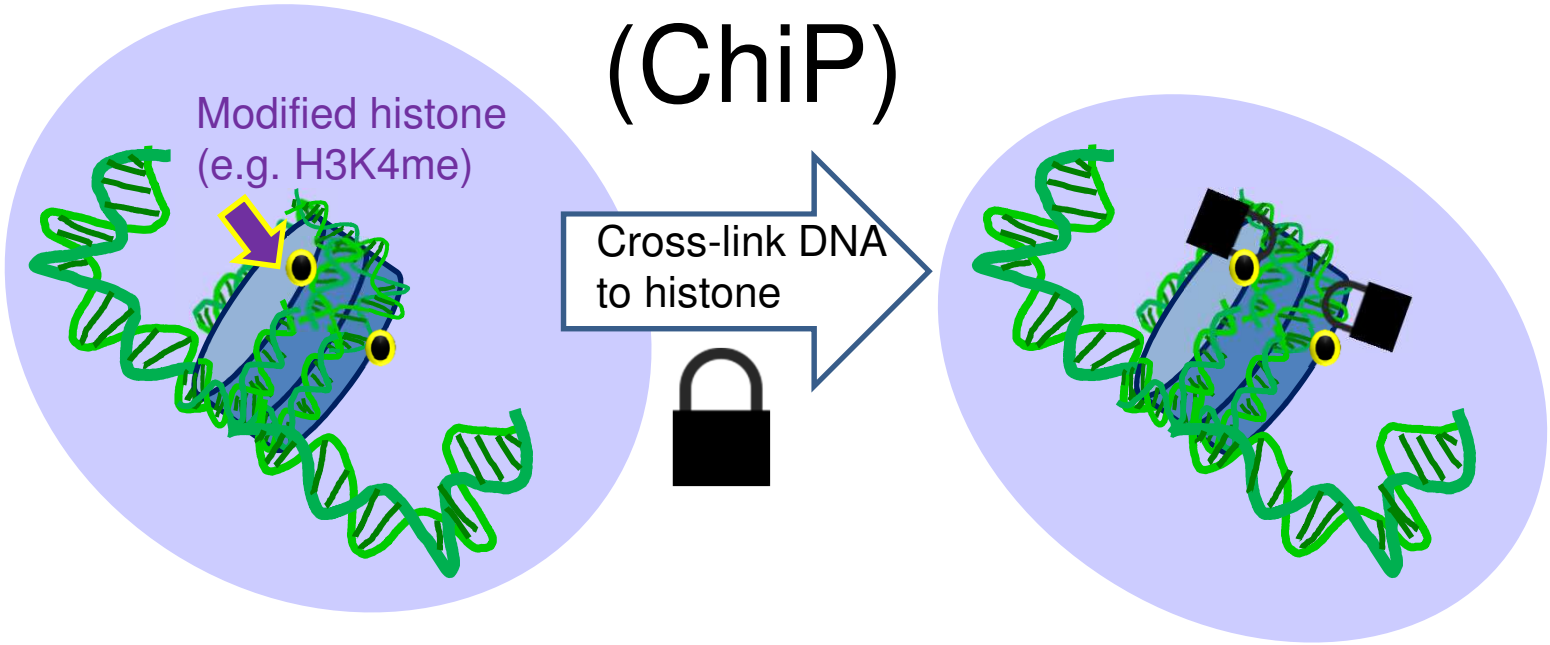
Bisulfite treatment differentiates cytosine and methylcytosine

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

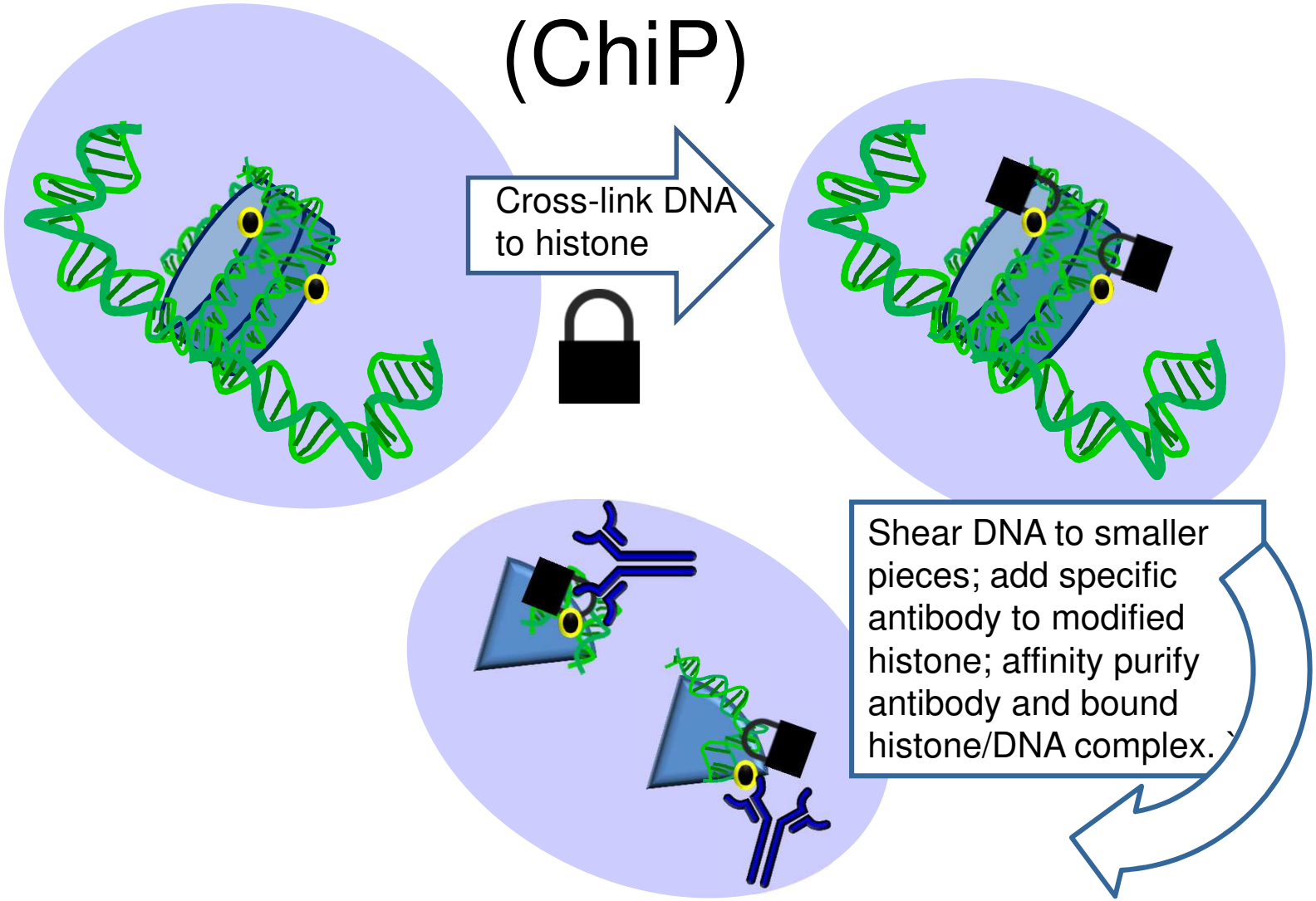
By contrast, **methyl-C** is read as **C** and is the same as the reference sequence.



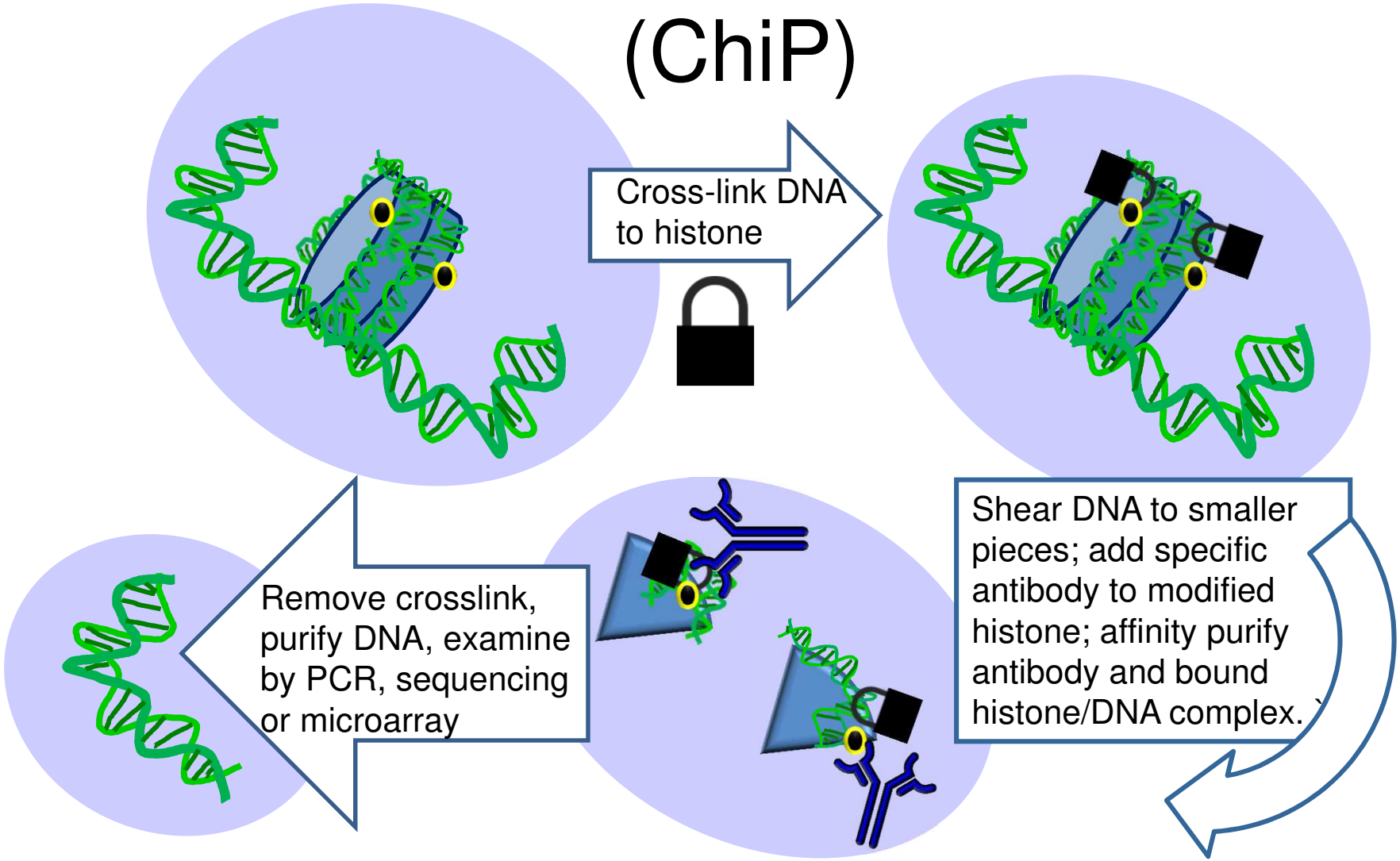
Chromatin Immunoprecipitation (ChiP)



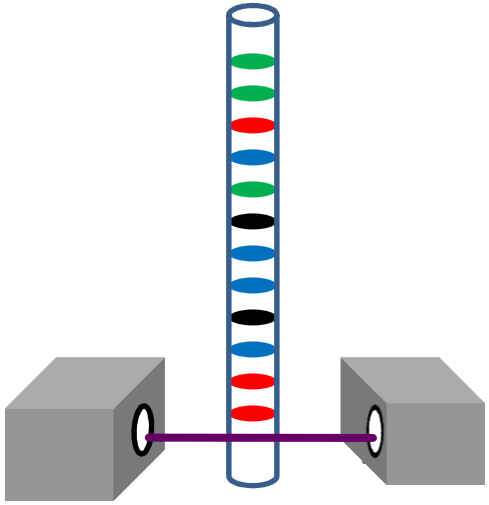
Chromatin Immunoprecipitation (ChiP)



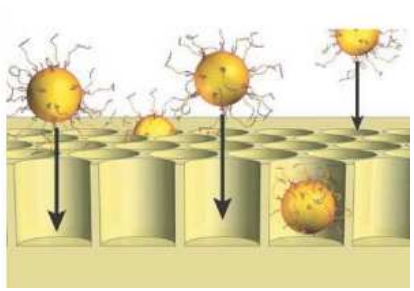
Chromatin Immunoprecipitation (ChiP)

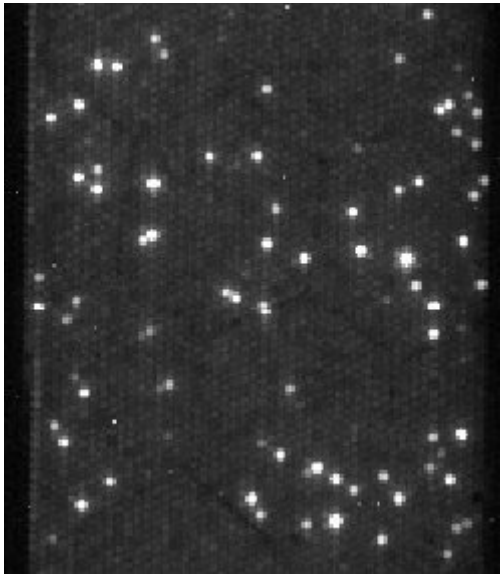


Deep sequencing by "next generation" DNA sequencing methods



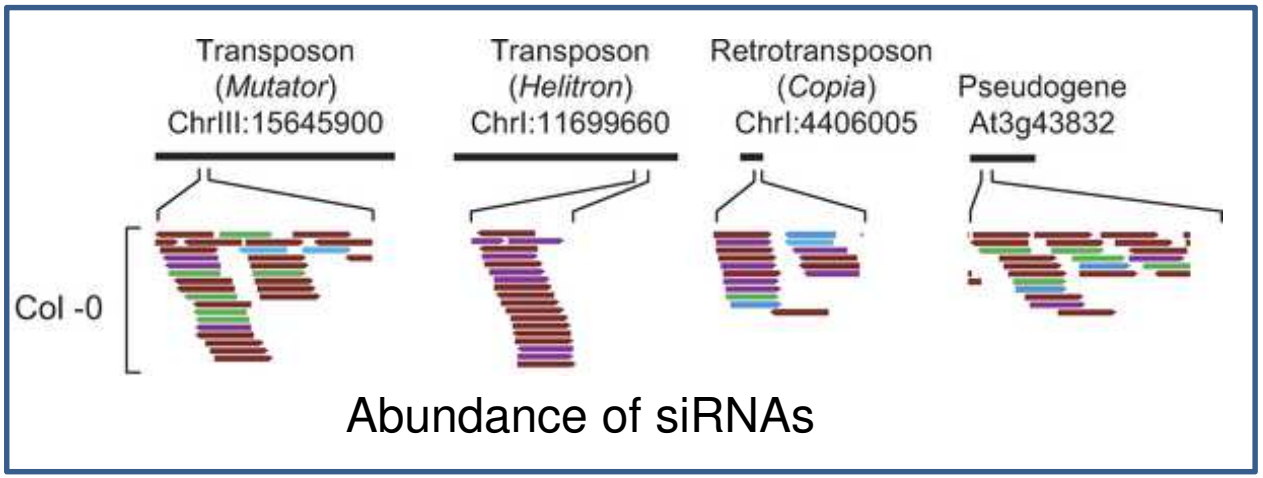
"Classical" DNA sequencing – one molecule examined at a time

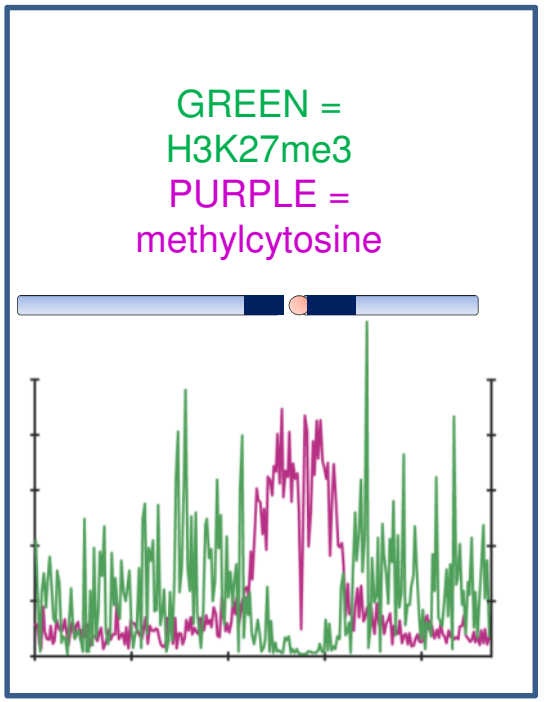




"Next generation" DNA sequencing – one million molecules examined at a time

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



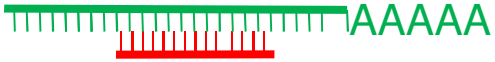


Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, et al. 2007 Genome-Wide Profiling and Analysis of *Arabidopsis* siRNAs. PLoS Biol 5(3): e57.
Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y.V., Pellegrini, M., Goodrich, J., Jacobsen, S.E. (2007) Whole-genome analysis of histone H3 lysine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

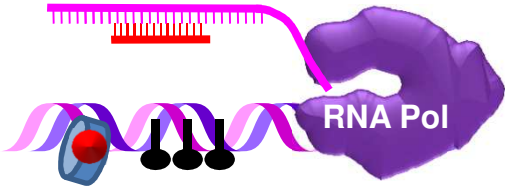
What are small RNAs?

•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

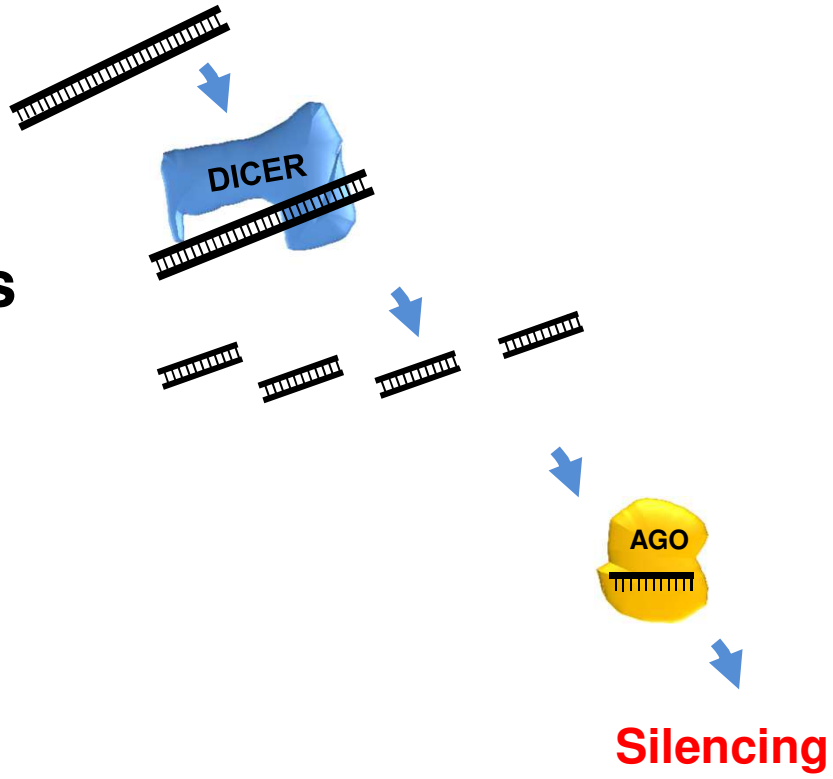


Histone modification, DNA methylation

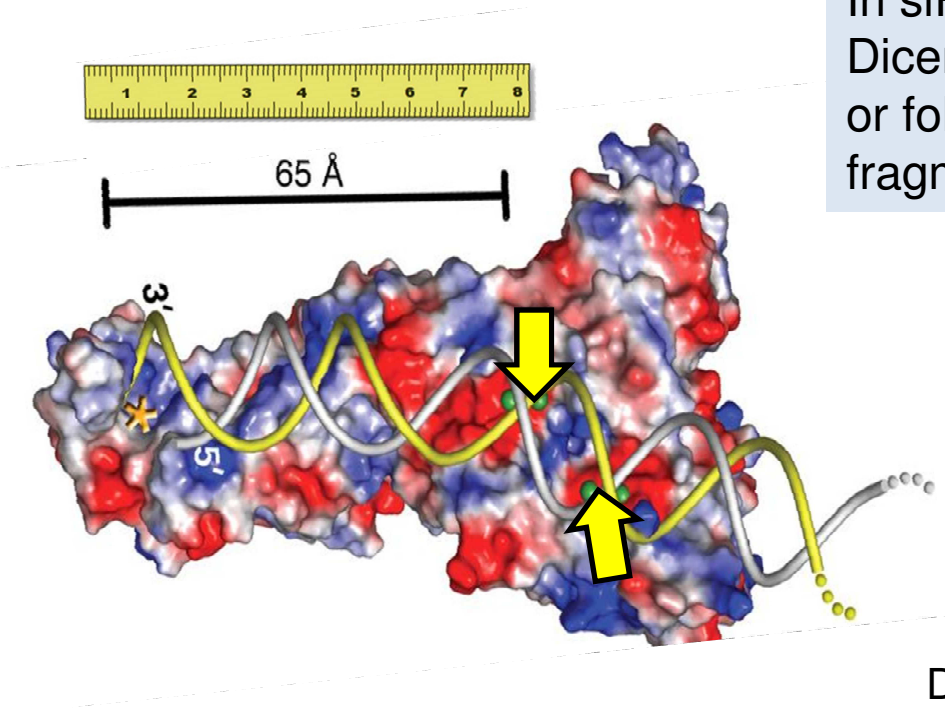
The core of RNA silencing: Dicers and Argonautes

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



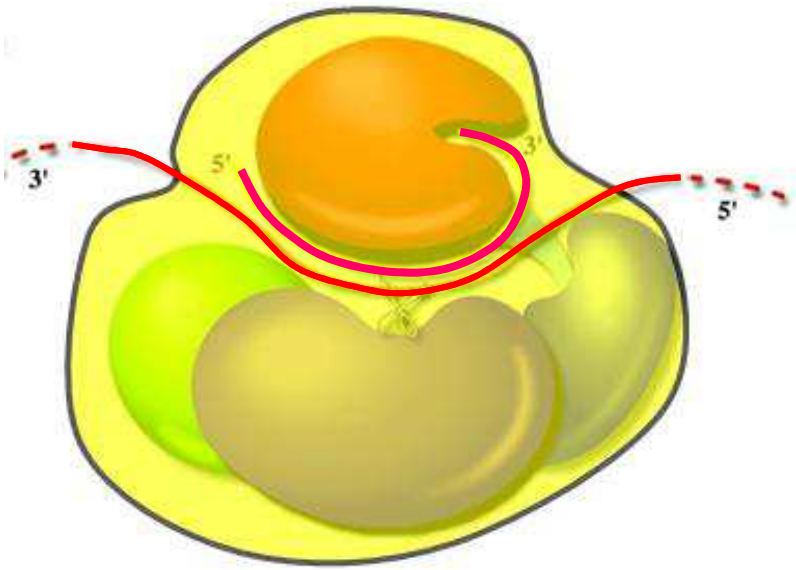
In siRNA and miRNA biogenesis, Dicer or Dicer-like (DCL) proteins cleave long dsRNA or foldback (hairpin) RNA into ~ 21 – 25 nt fragments.

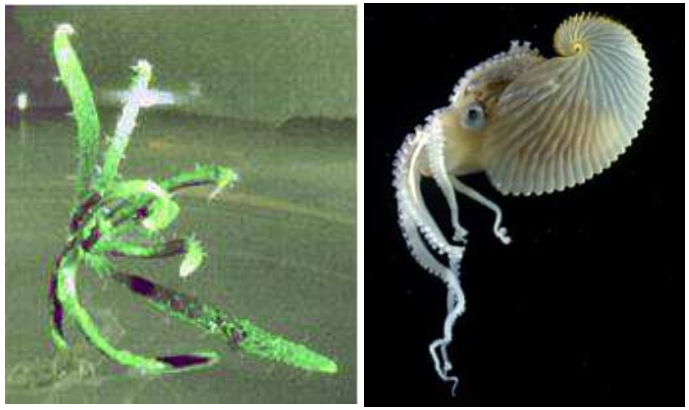
Dicer's structure allows it to measure the RNA it is cleaving. Like a cook who "dices" a carrot, Dicer chops RNA into uniformly-sized pieces.

From MacRae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.., Adams, P.D., and Doudna, J.A. (2006) Structural basis for double-stranded RNA processing by Dicer. Science 311: 195 -198. Reprinted with permission from AAAS. Photo credit: Heidi

Argonaute proteins

ARGONAUTE proteins bind small RNAs and their targets.



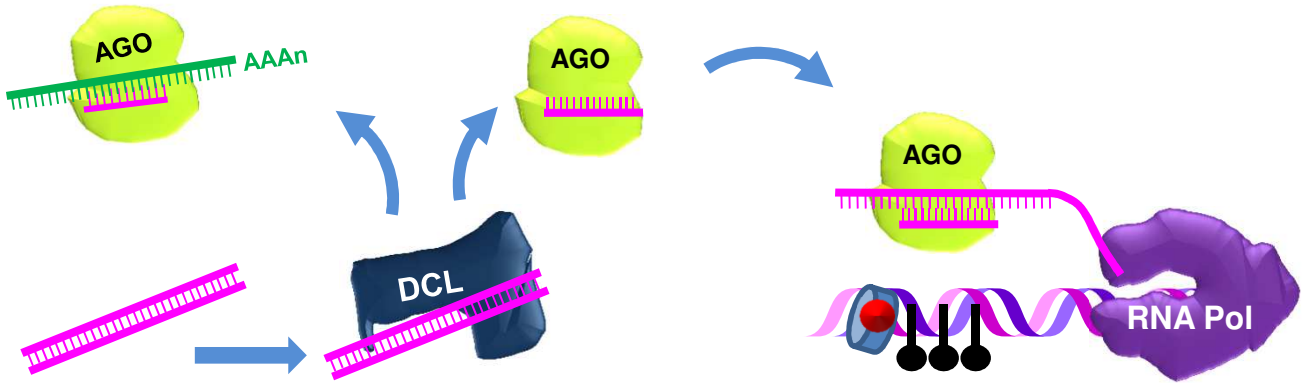


The Arabidopsis *ago1* mutant and the octopus *Argonauta argo*

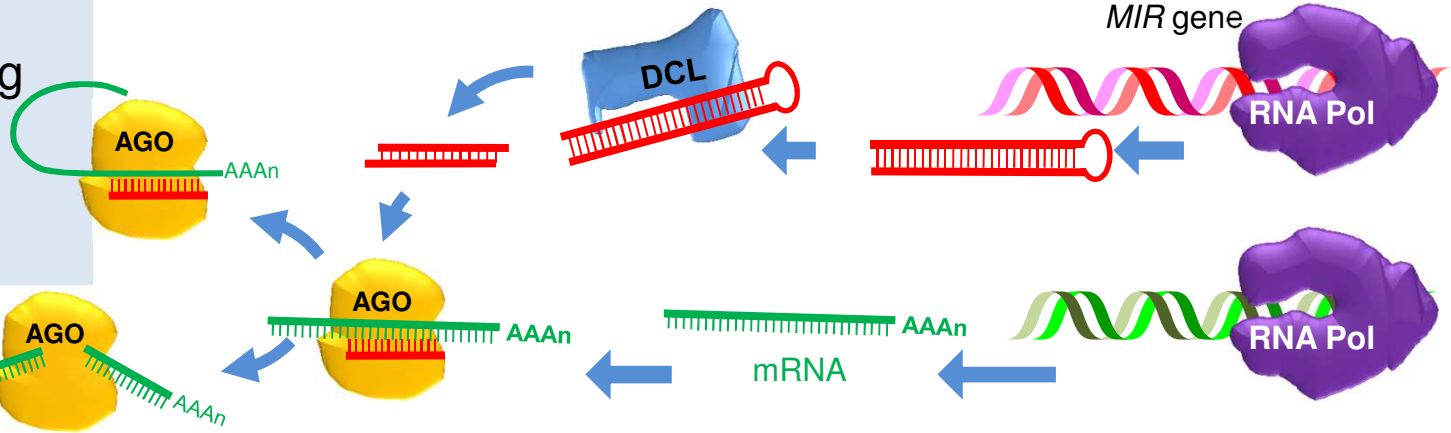
ARGONAUTE proteins are named after the *argonaute1* mutant of Arabidopsis; *ago1* has thin radial leaves and was named for the octopus *Argonauta* which it resembles.

RNA silencing - overview

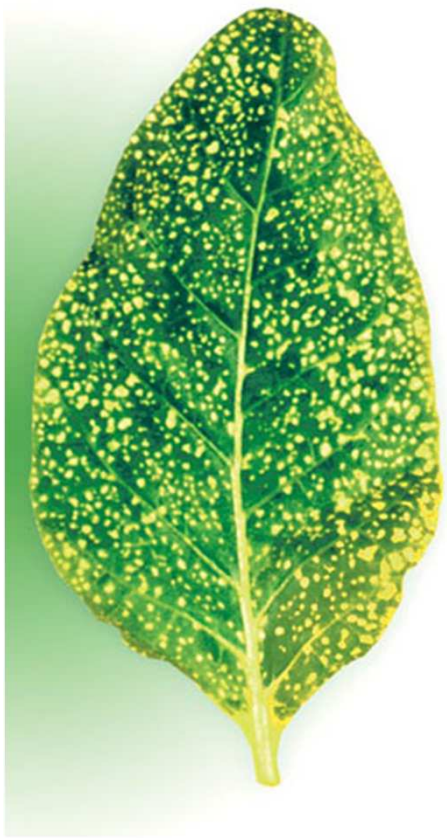
siRNA-mediated silencing via post-transcriptional and transcriptional gene silencing



miRNA - mediated slicing of mRNA and translational repression

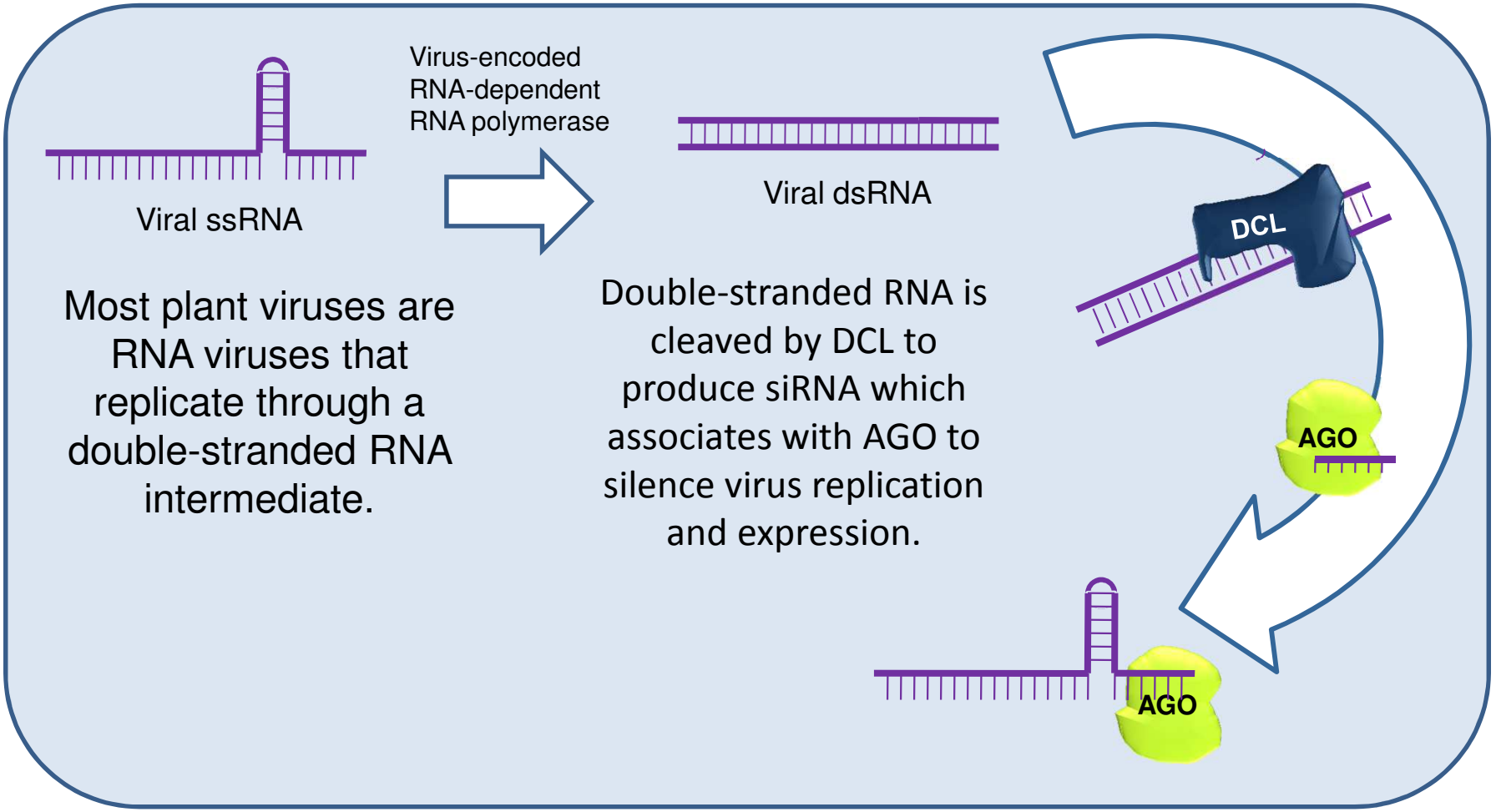


siRNAs – Genomic Defenders

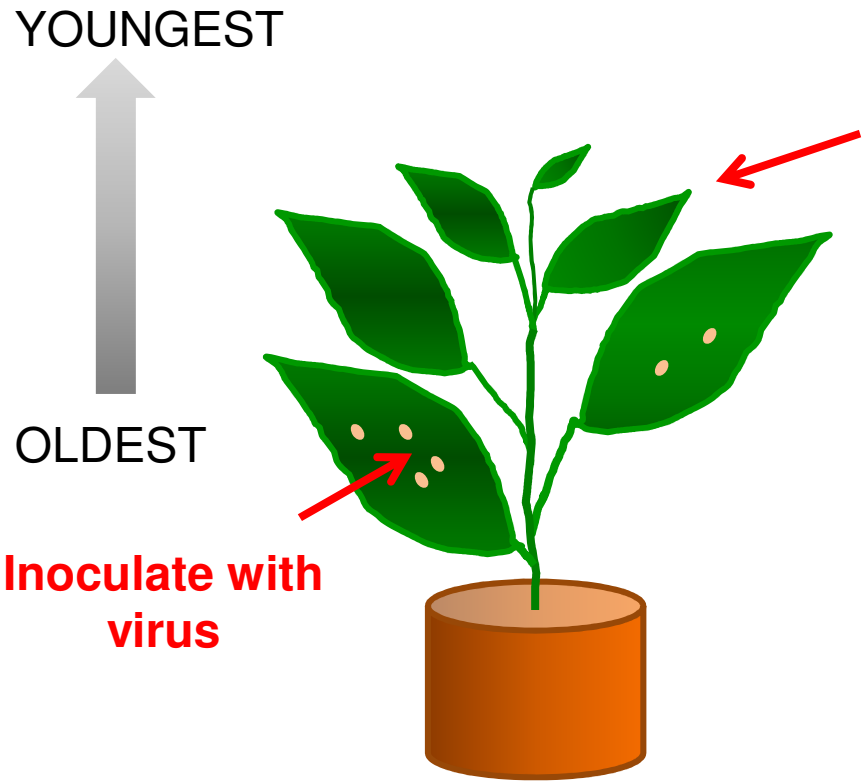


- siRNAs protect the genome by
- Suppressing invading viruses
- Silencing sources of aberrant transcripts
- Silencing transposons and repetitive elements
- siRNAs also maintain some genes in an epigenetically silent state

Viral induced gene silencing - overview



Plants can recover from viral infection and become resistant



Younger leaves produced on a virus-infected plant can be symptom-free, indicating that the plant has **recovered** from the infection.

Plants can recover from viral infection and become resistant

OLDEST → YOUNGEST

Leaf inoculated with virus

Leaf inoculated with virus

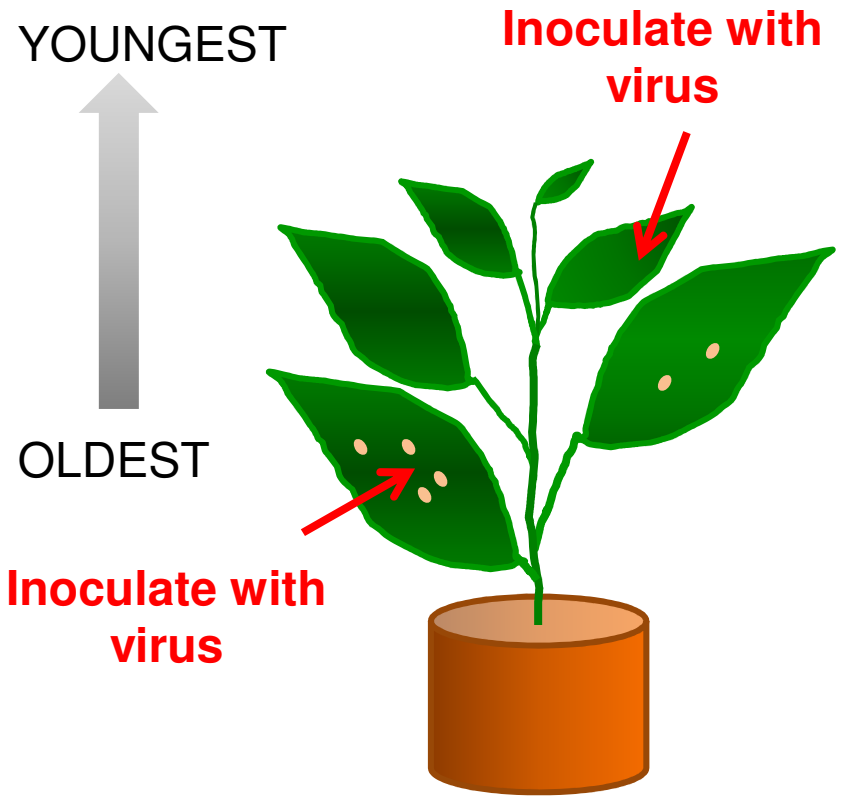
Virus-induced cell death

No symptoms: **recovery**

No symptoms: **resistance**

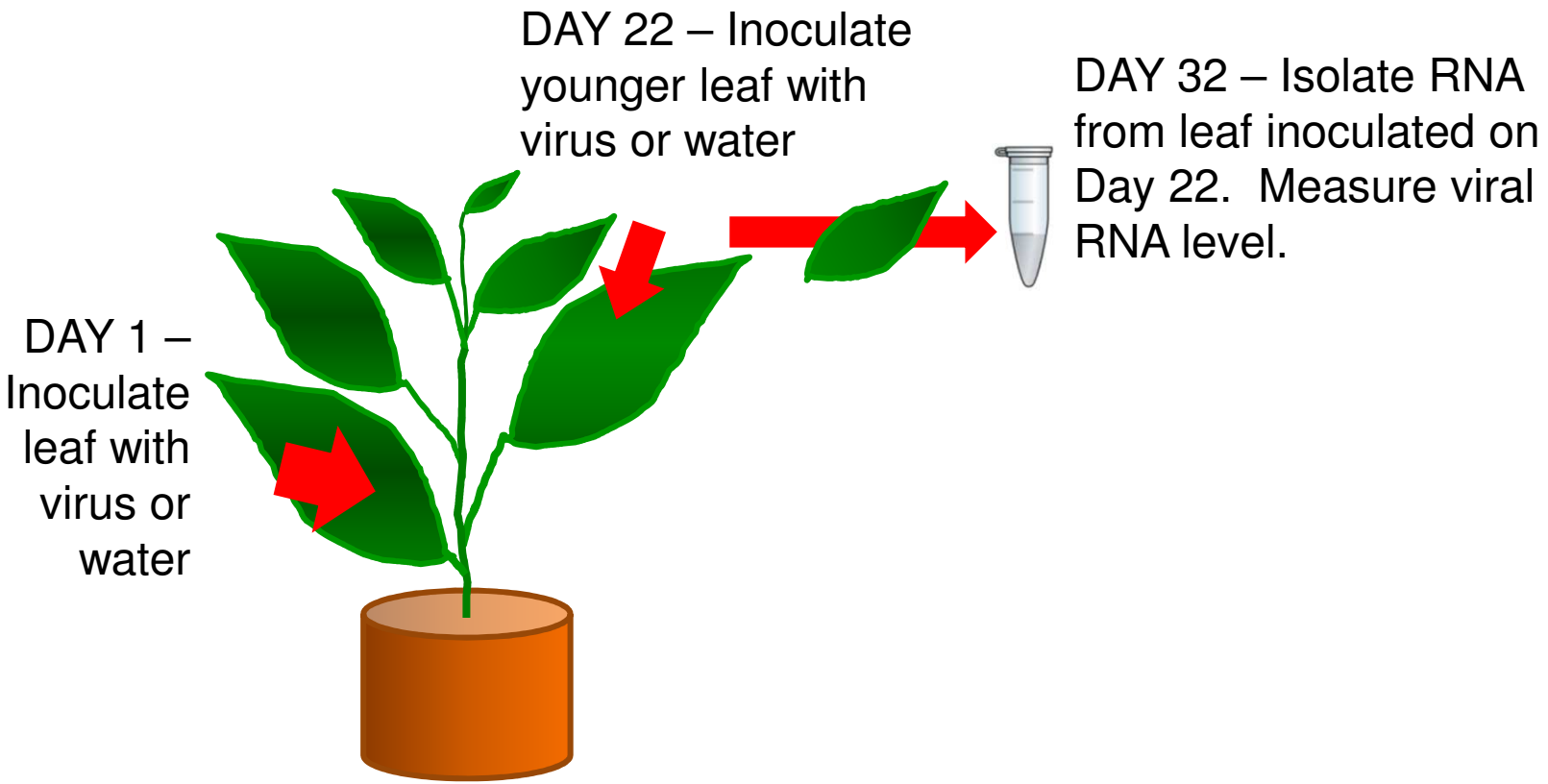
From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral and gene silencing in plants. Science 276: 1558–1560. Reprinted with permission from AAAS.

Plants can recover from viral infection and become resistant



These leaves are also **resistant** to subsequent infection by the same virus.

Viral resistance involves siRNA-mediated silencing

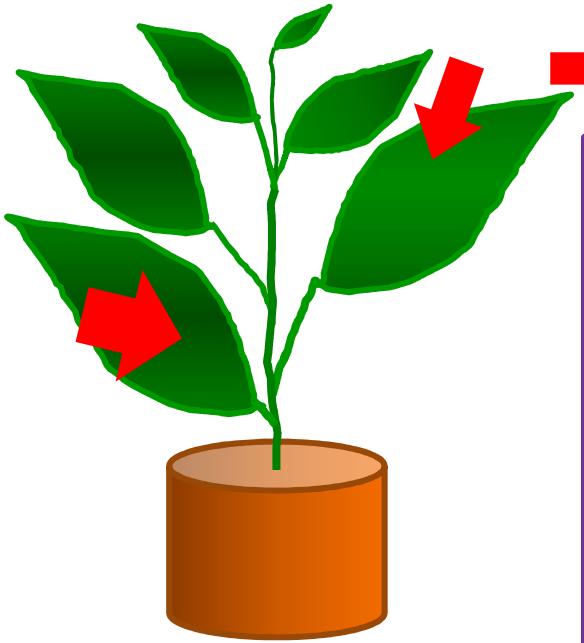


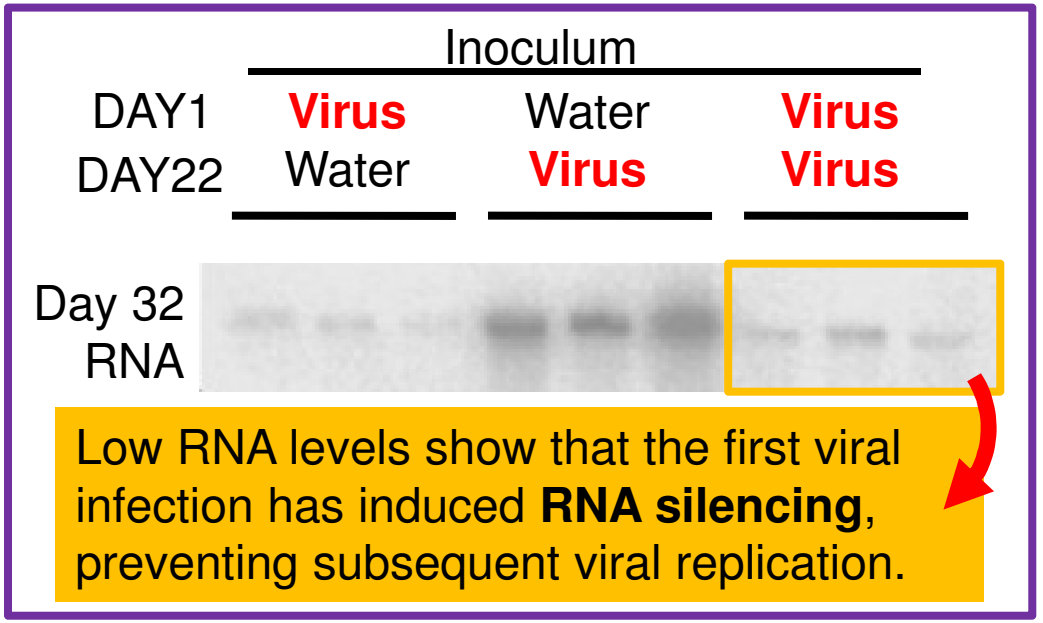
Viral resistance involves siRNA-mediated silencing

DAY 22 – Inoculate younger leaf with virus or water

DAY 32 – Isolate RNA from leaf inoculated on Day 22. Measure viral RNA level.

DAY 1 – Inoculate leaf with virus or water

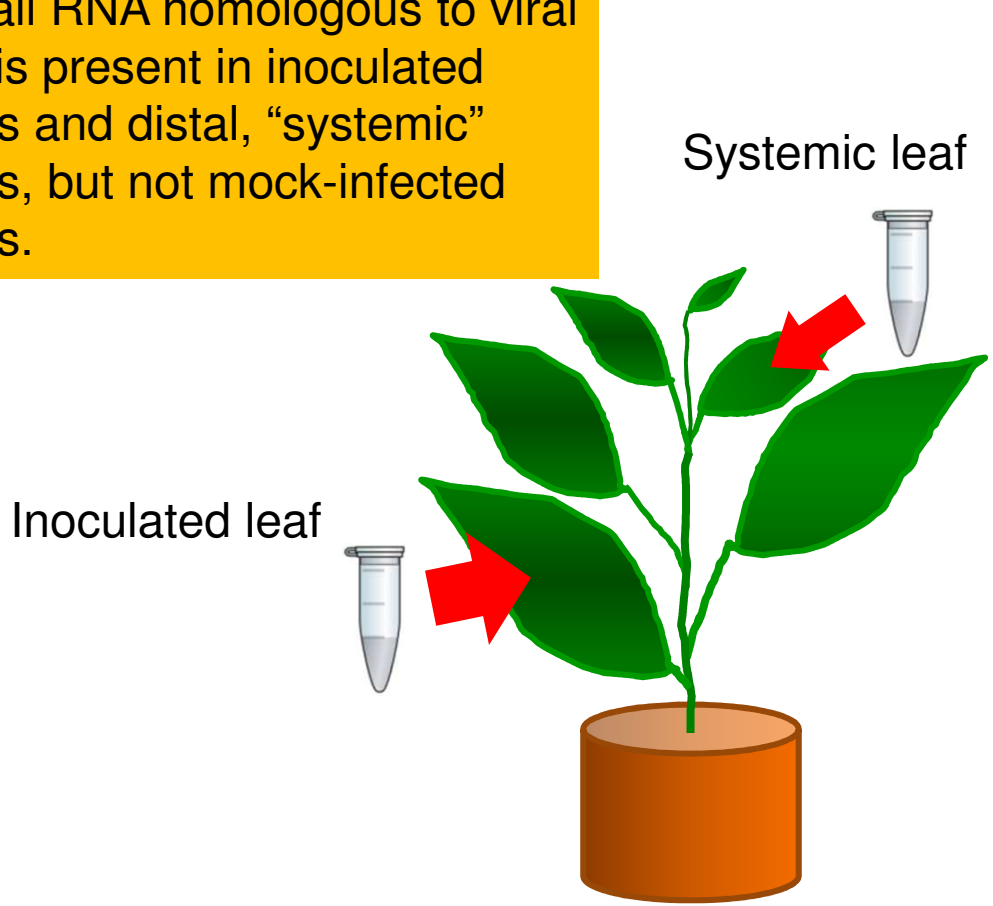


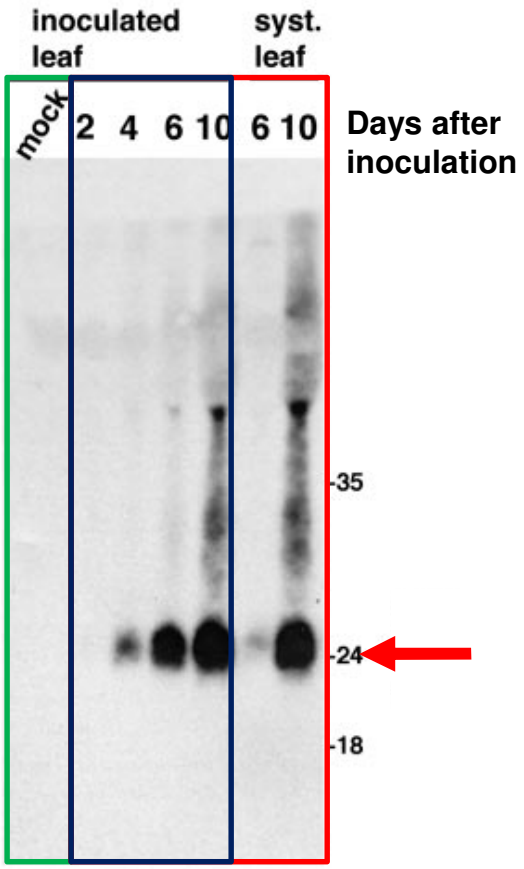


From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral defense and gene silencing in plants. Science 276: 1558–1560. Reprinted with permission from AAAS.

Small RNAs are correlated with viral-induced gene silencing

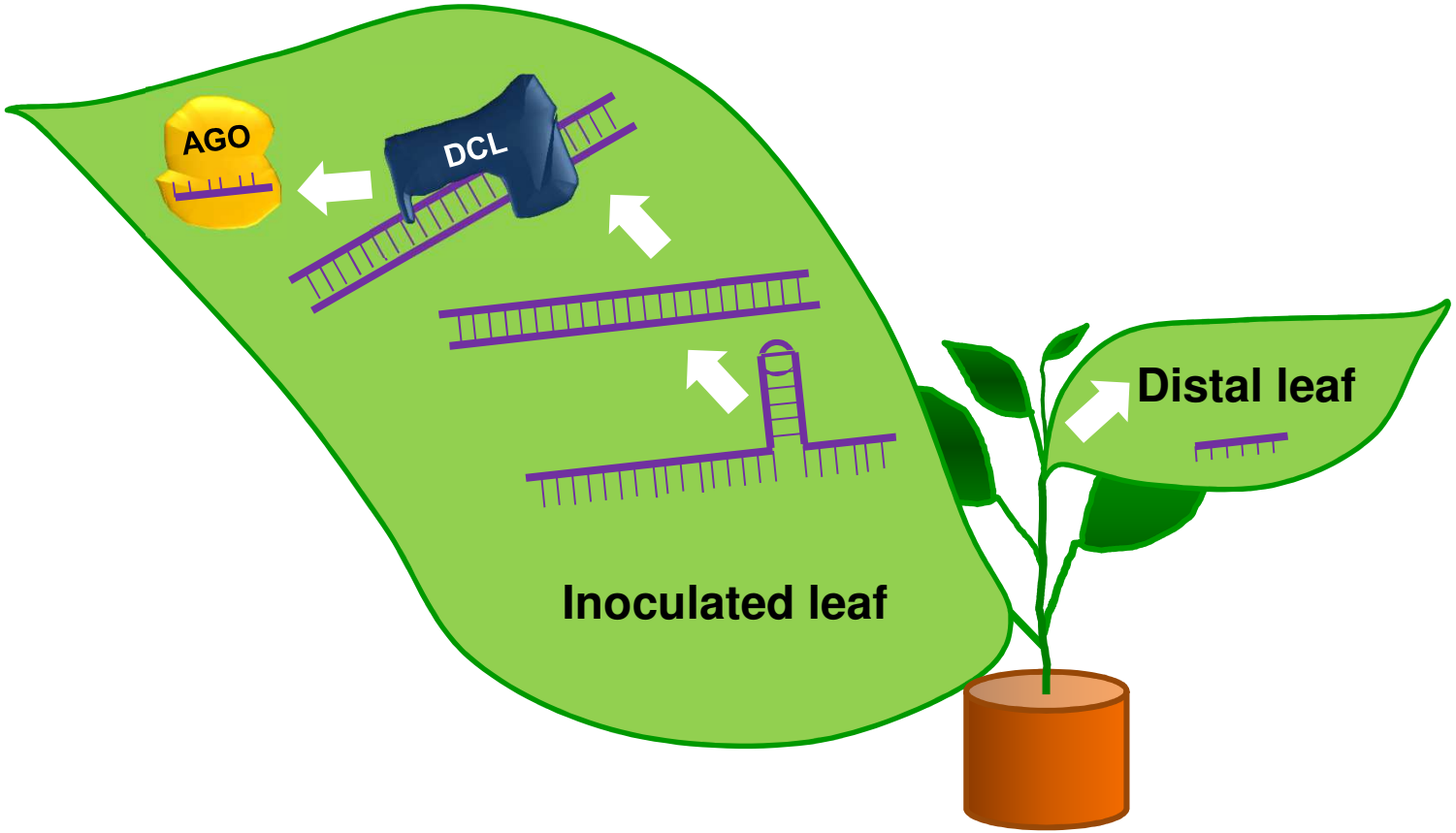
A small RNA homologous to viral RNA is present in inoculated leaves and distal, “systemic” leaves, but not mock-infected leaves.



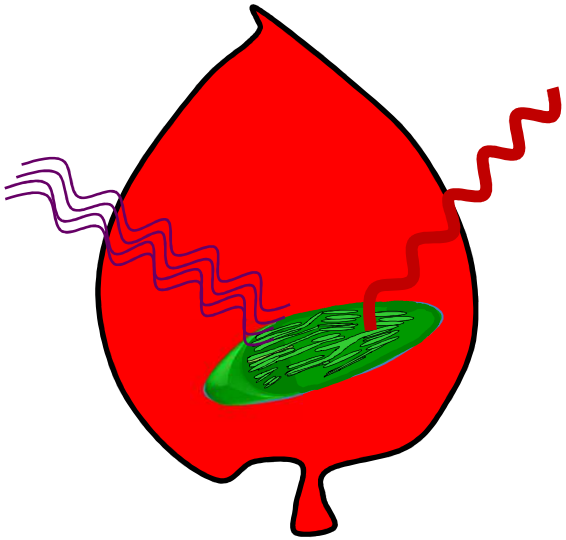


From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral defense and gene silencing in plants. Science 276: 1558–1560. Reprinted with permission from AAAS.

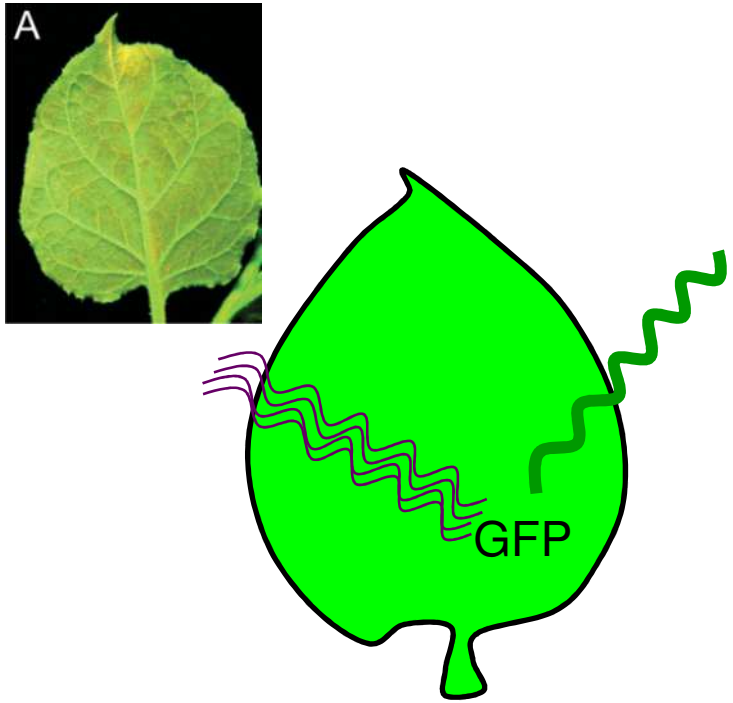
Virus infection causes systemic siRNA accumulation



How does RNA silencing spread systemically???

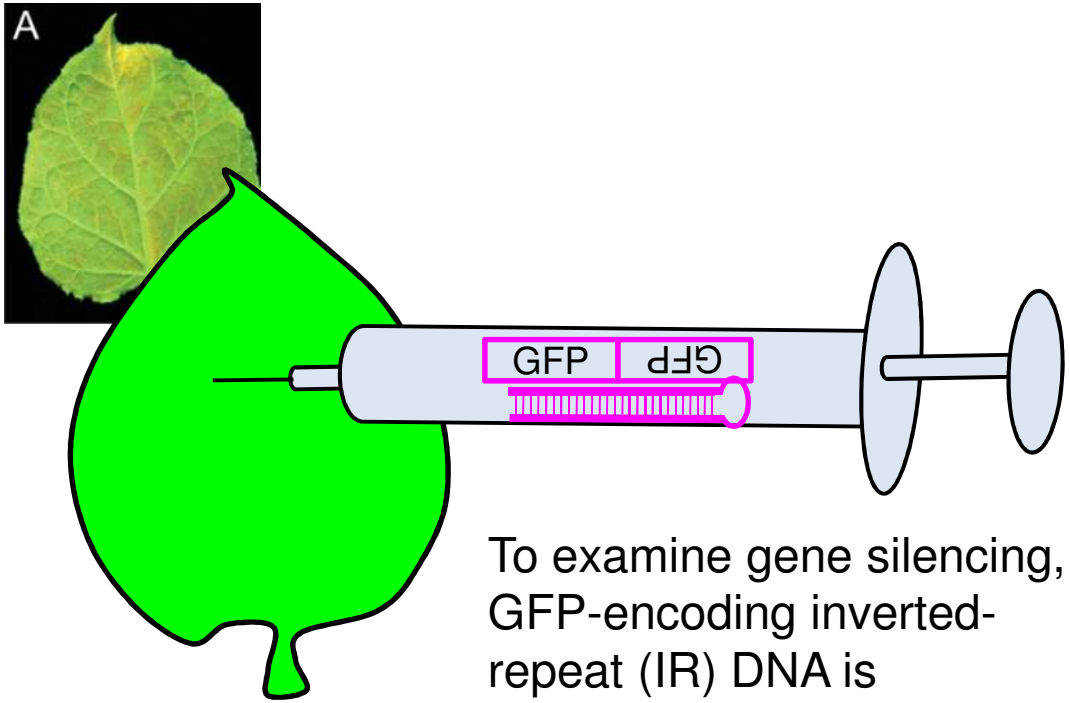


Under UV light, wild-type leaves fluoresce **red**, from chlorophyll in the chloroplasts.



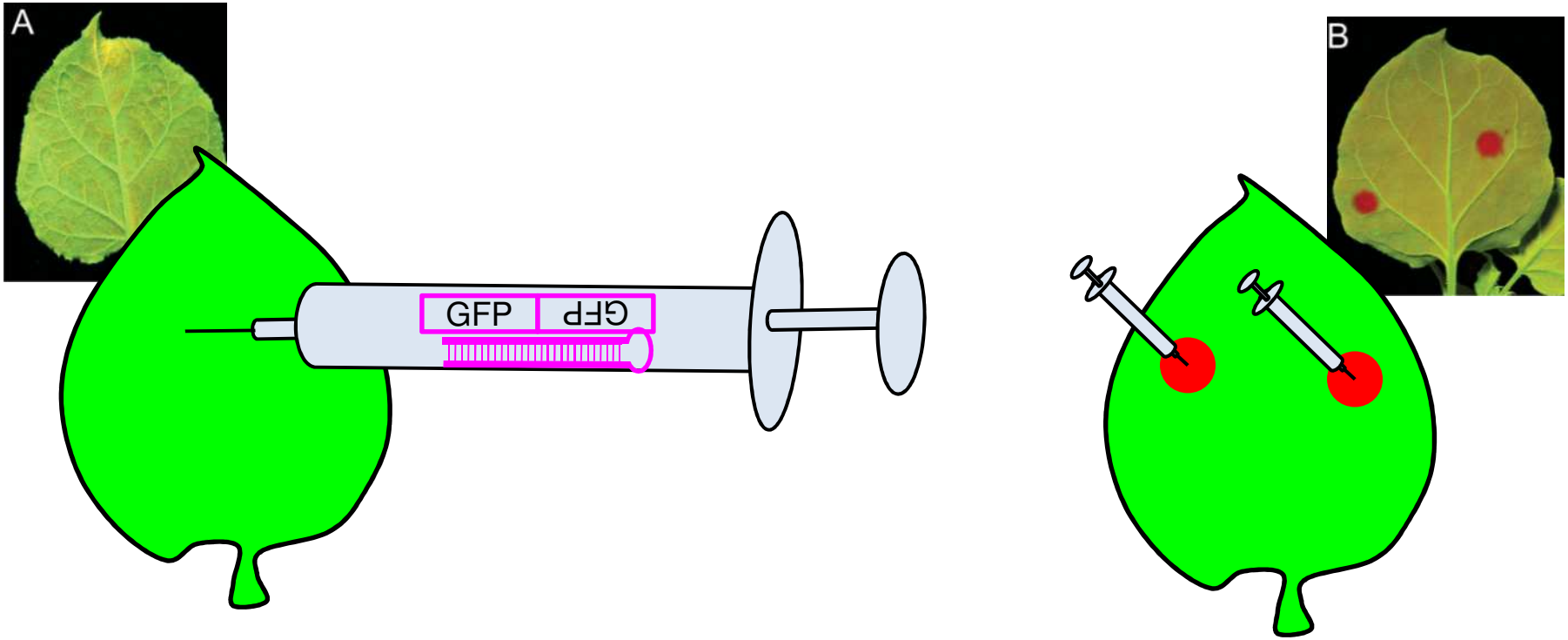
A plant expressing GFP fluoresces **green** under UV light.

Spreading of RNA silencing



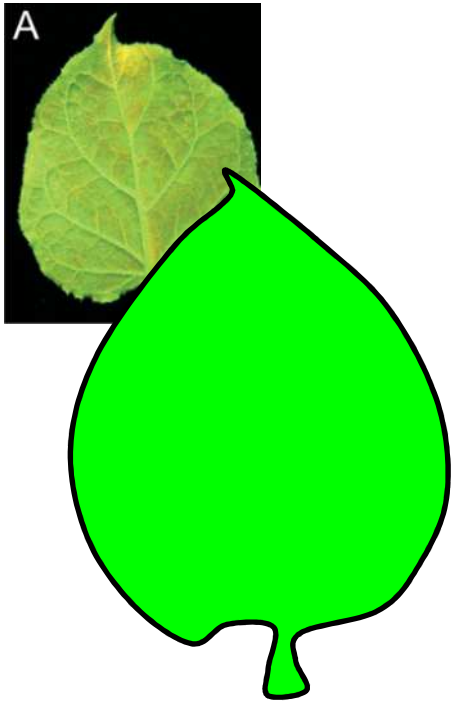
To examine gene silencing, GFP-encoding inverted-repeat (IR) DNA is introduced into the GFP-expressing cells.

Spreading of RNA silencing

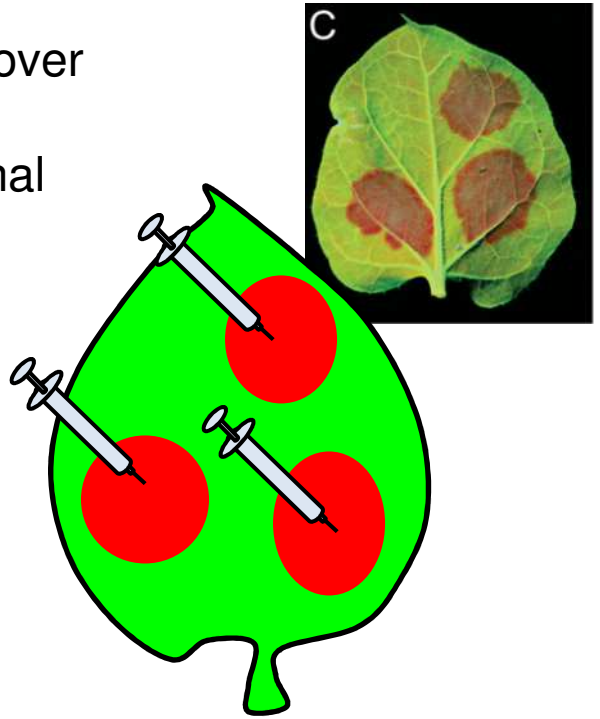


When GFP is silenced, the red chlorophyll fluorescence becomes visible.

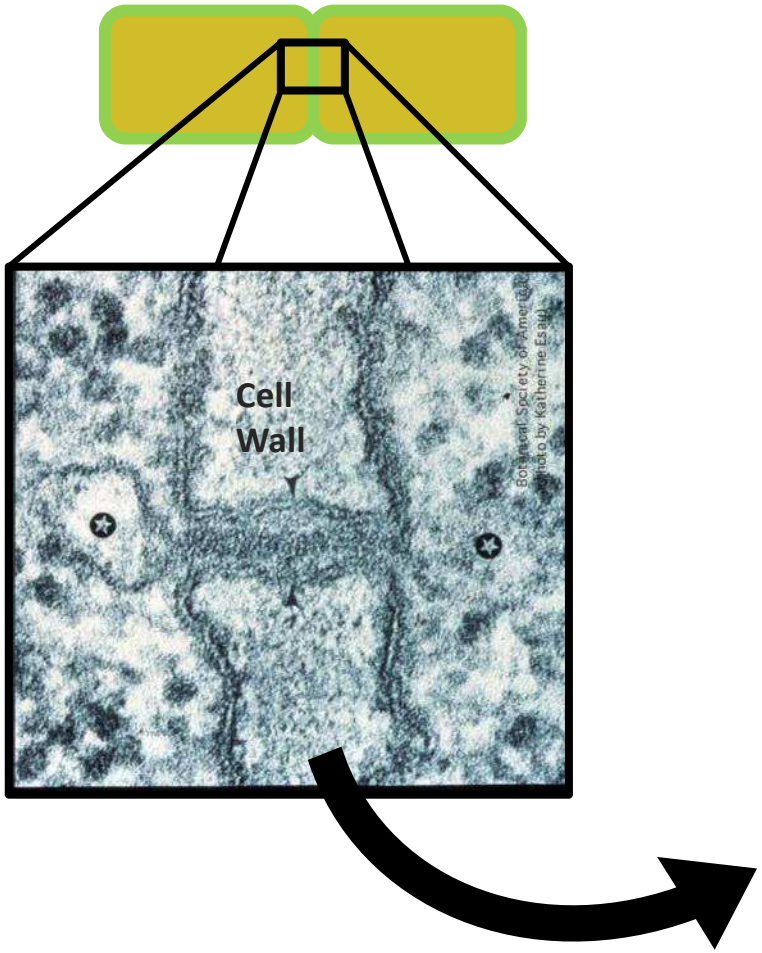
Silencing can spread locally

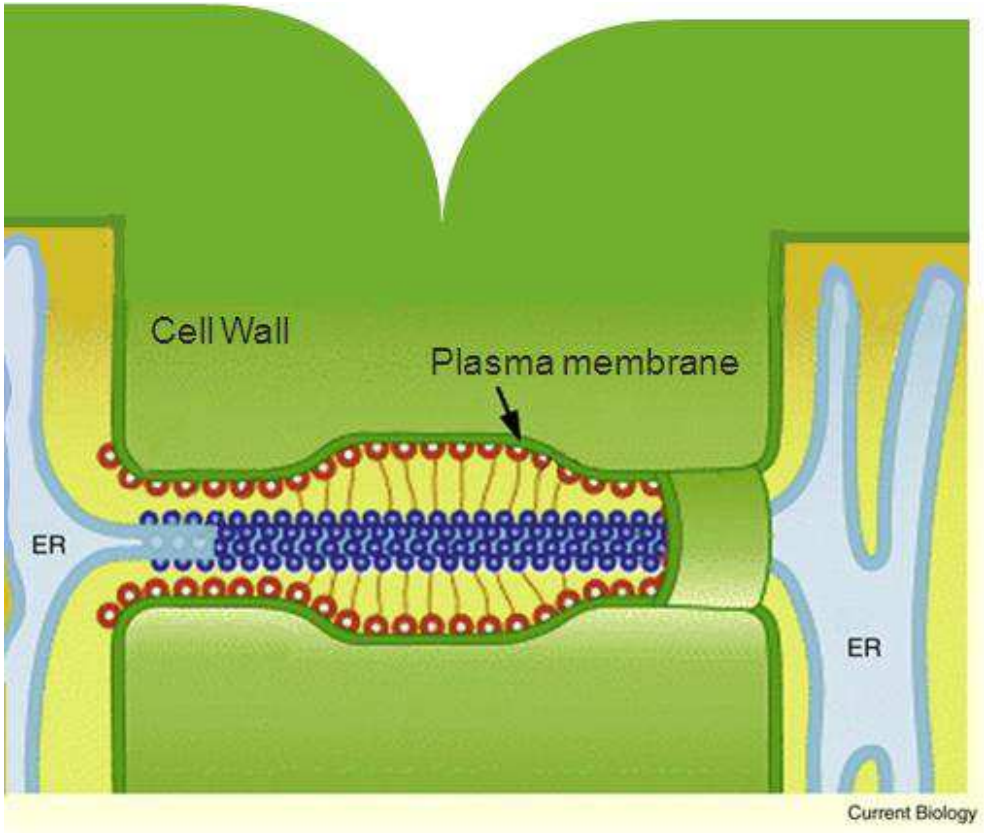


Often the silencing spreads over up to 15 cells, probably by diffusion of the silencing signal through the plasmodesmata.



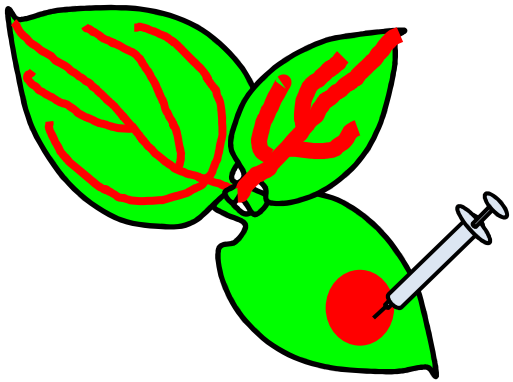
Plasmodesmata are regulated connections between plant cells



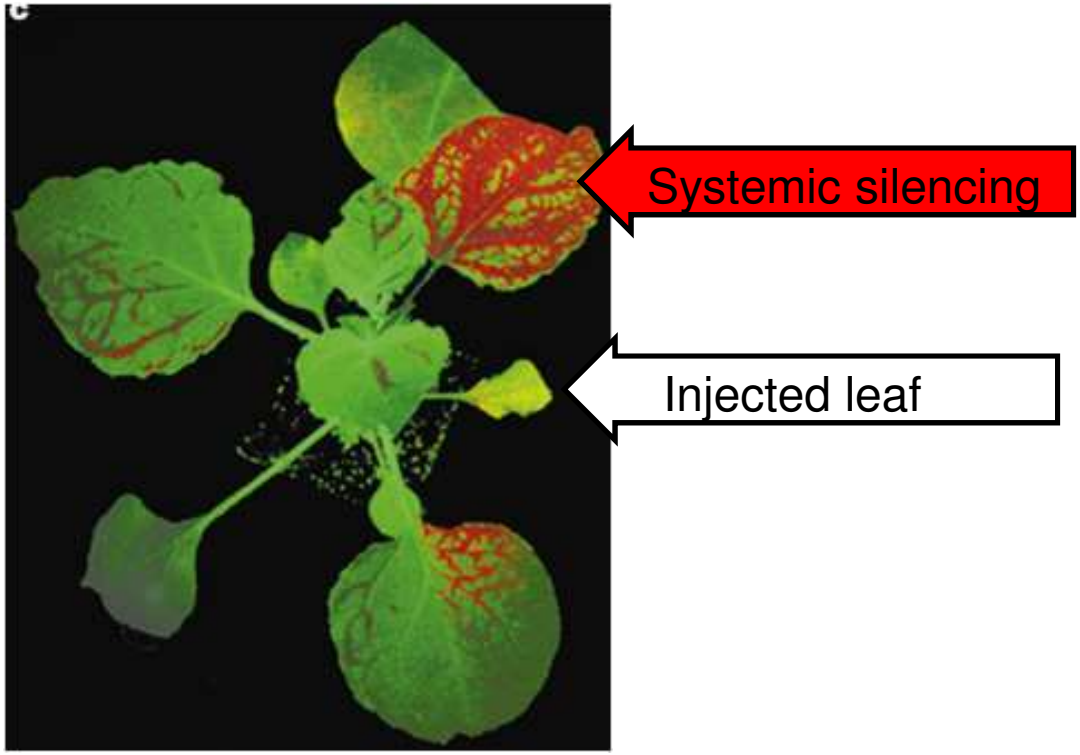


Reprinted from Zambryski, P. (2008) Plasmodesmata. Curr. Biol. 18: R324-325 with permission from Elsevier. TEM image credit BSA Photo by Katherine Esau;

Silencing can spread systemically through the phloem

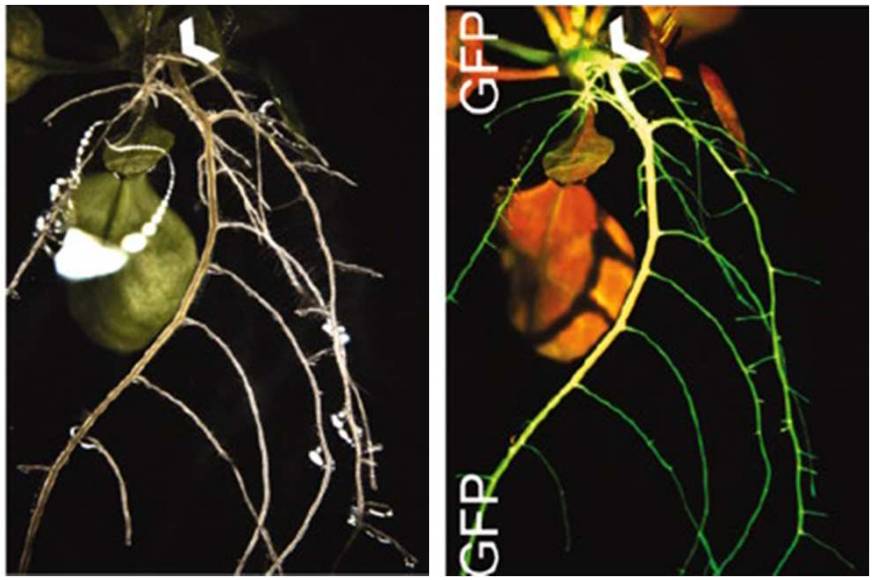


Recent experiments have shed light on the identity of the silencing signal…



Small RNAs can move from shoot to root in Arabidopsis

Control GFP expressing plant showing GFP in shoot and root



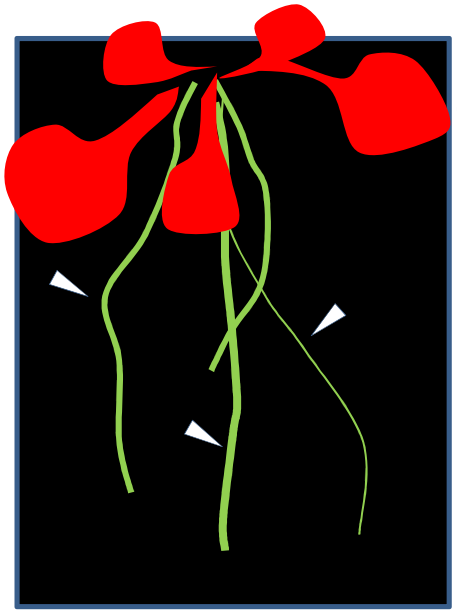
White light Fluorescence

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.

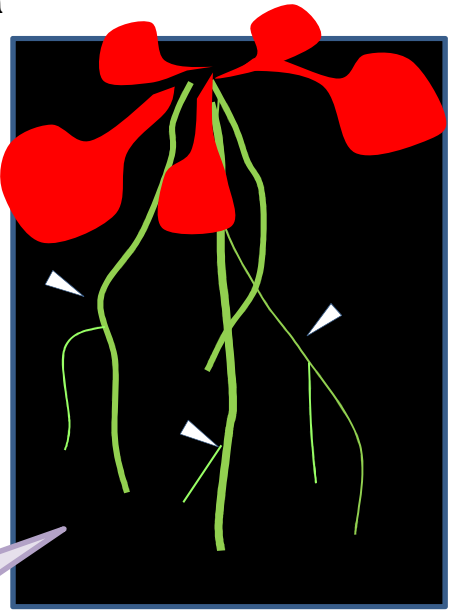
Dicer activity for sRNA production in the shoot is sufficient



GFP-IR

GFP

GFP silenced



GFP-IR

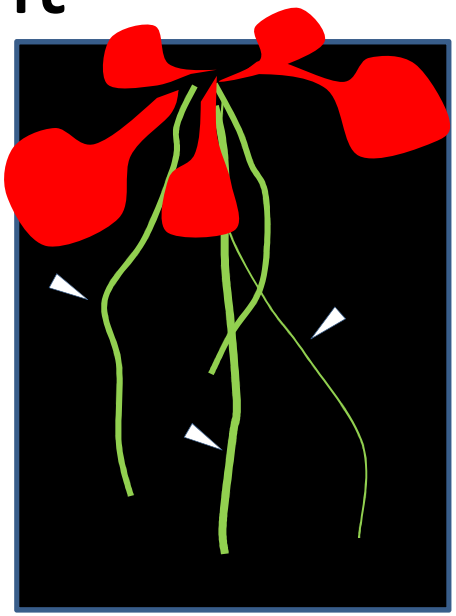
dcl2,3,4

GFP

dcl2,3,4

No silencing; no sRNAs in root

The *dcl2,3,4* mutant cannot make sRNA from dsRNA precursors



GFP-IR

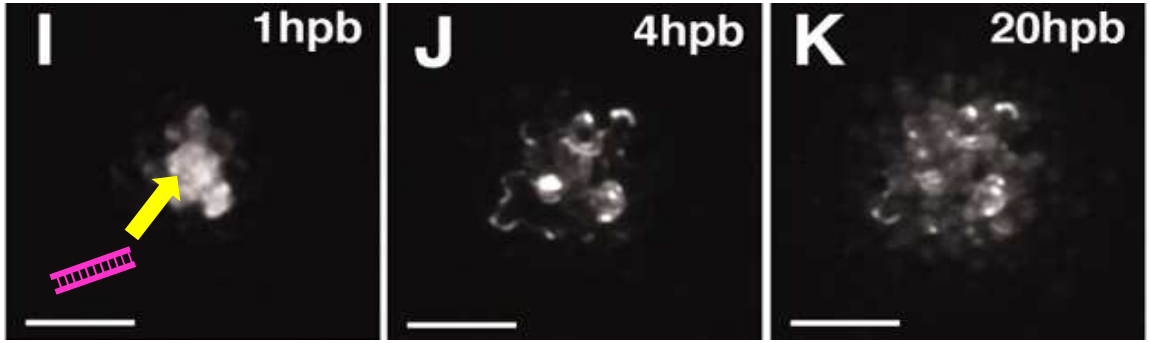
GFP

dcl2,3,4

GFP silenced; sRNAs in root are derived from shoot

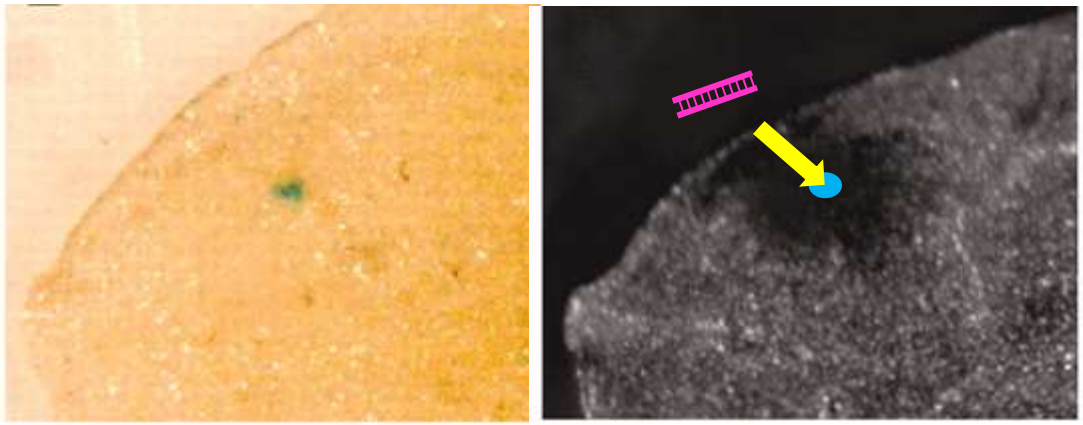
From Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science **328:** 872-875; reprinted with permission from AAAS.

siRNA duplexes move between cells and are sufficient to confer silencing



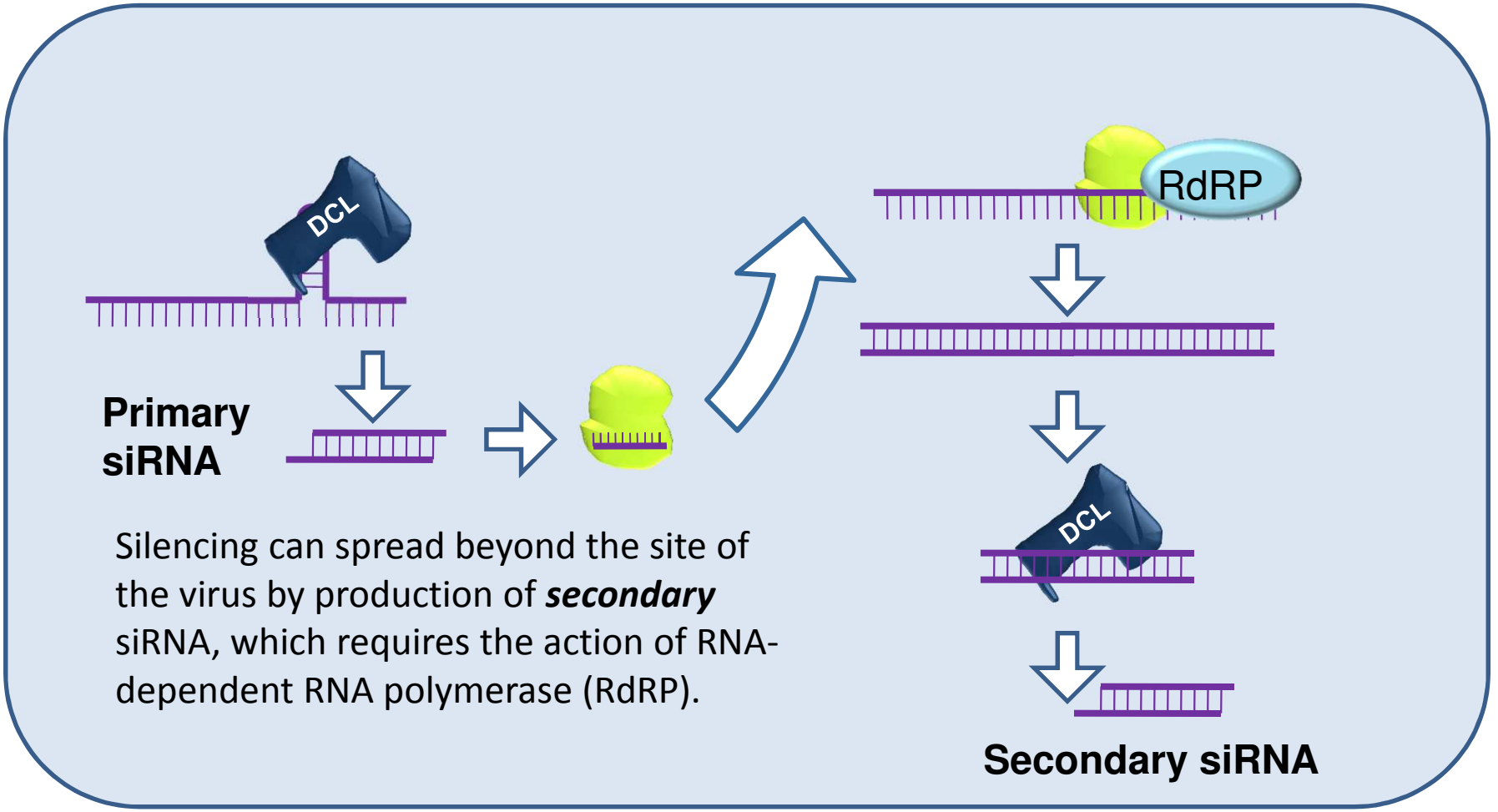
Fluorescently labeled duplex siRNA was bombarded into a single cell. Spreading to adjacent cells is visible at 20 hours post-bombardment.

The duplex siRNA spread from the site of entry (blue dot) and silenced GFP expression in the adjoining cells: it is ***sufficient*** for 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



Silencing can spread beyond the site of the virus by production of ***secondary*** siRNA, which requires the action of RNA-dependent RNA polymerase (RdRP).

siRNA production mutants are more susceptible to viral disease

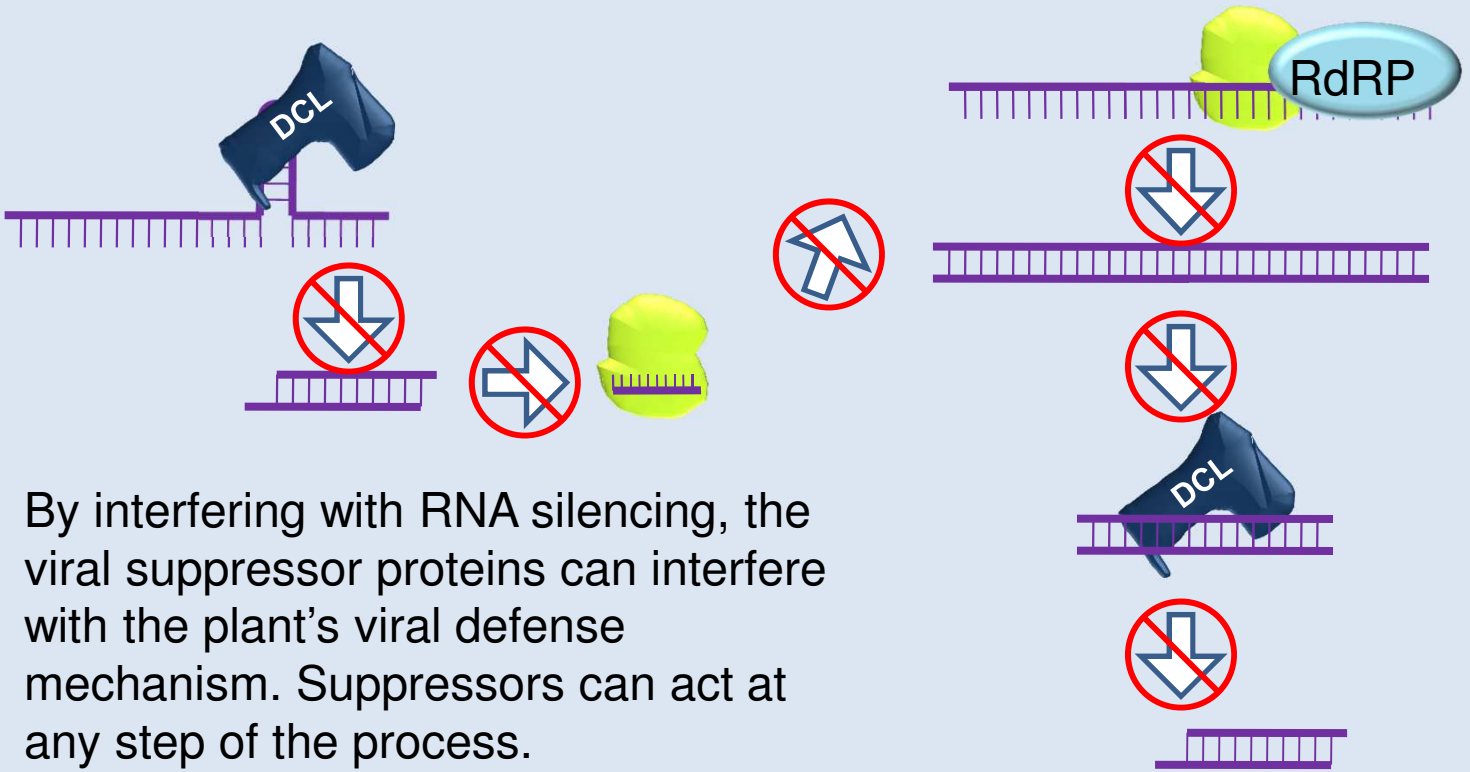
Tobacco Rattle Virus (TRV) silencing in wild-type Arabidopsis plants prevents disease symptoms. Mutants deficient in Dicer activity are unable to suppress viral infection.

WT Arabidopsis inoculated with TRV

Double mutant of *dcl2-dcl4* inoculated with TRV

From Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K., Carrington, J.C., and Voinnet, O. (2006) Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. Science 313: 68–71. Reprinted with permission from AAAS.

Viruses have suppressor proteins that interfere with RNA silencing



By interfering with RNA silencing, the viral suppressor proteins can interfere with the plant's viral defense mechanism. Suppressors can act at any step of the process.

A viral suppressor protein in action

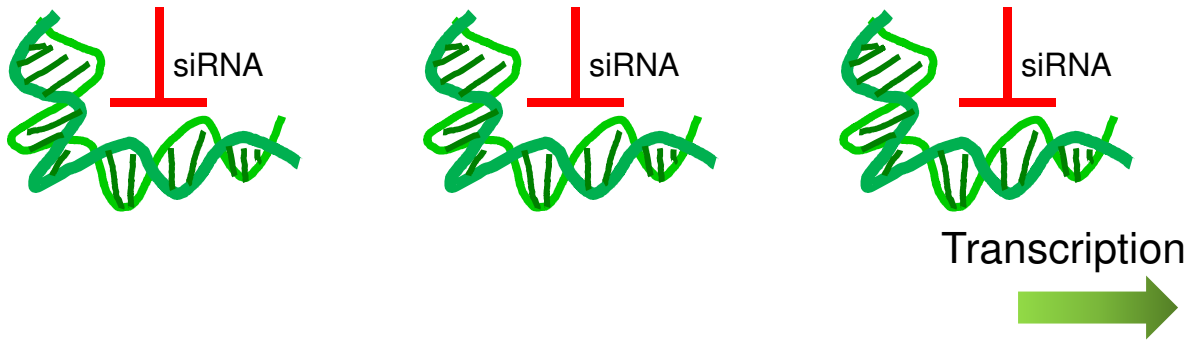
Genes encoding functional, mutant, or no viral suppressor proteins were introduced into plants carrying a silenced GUS gene. The plants were inoculated with a virus expressing GUS. Blue spots indicate GUS expression.

No viral Suppressor: GUS gene silent

Mutant viral suppressor: GUS gene silent

Functional viral suppressor: GUS gene expressed

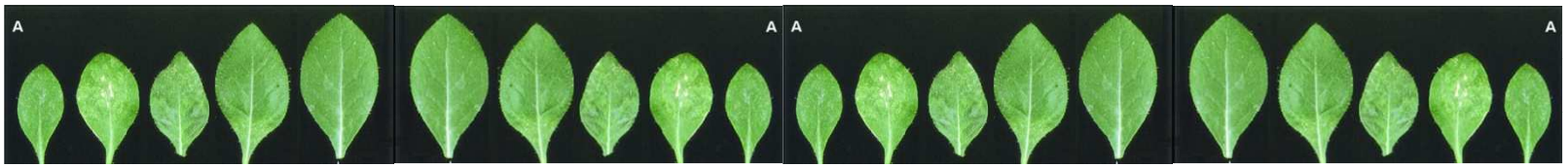
The plant's RNA silencing efforts are suppressed by the viral protein.



Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., and Vance, V.B. (1998). A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA 95: 13079–13084.

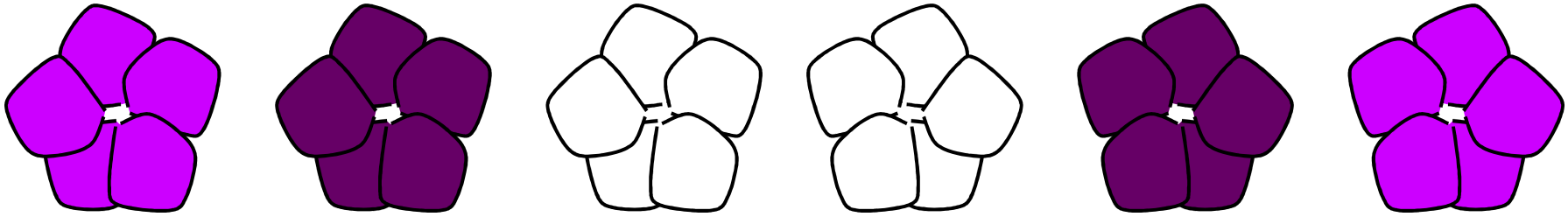
Viral-induced gene silencing summary

- 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



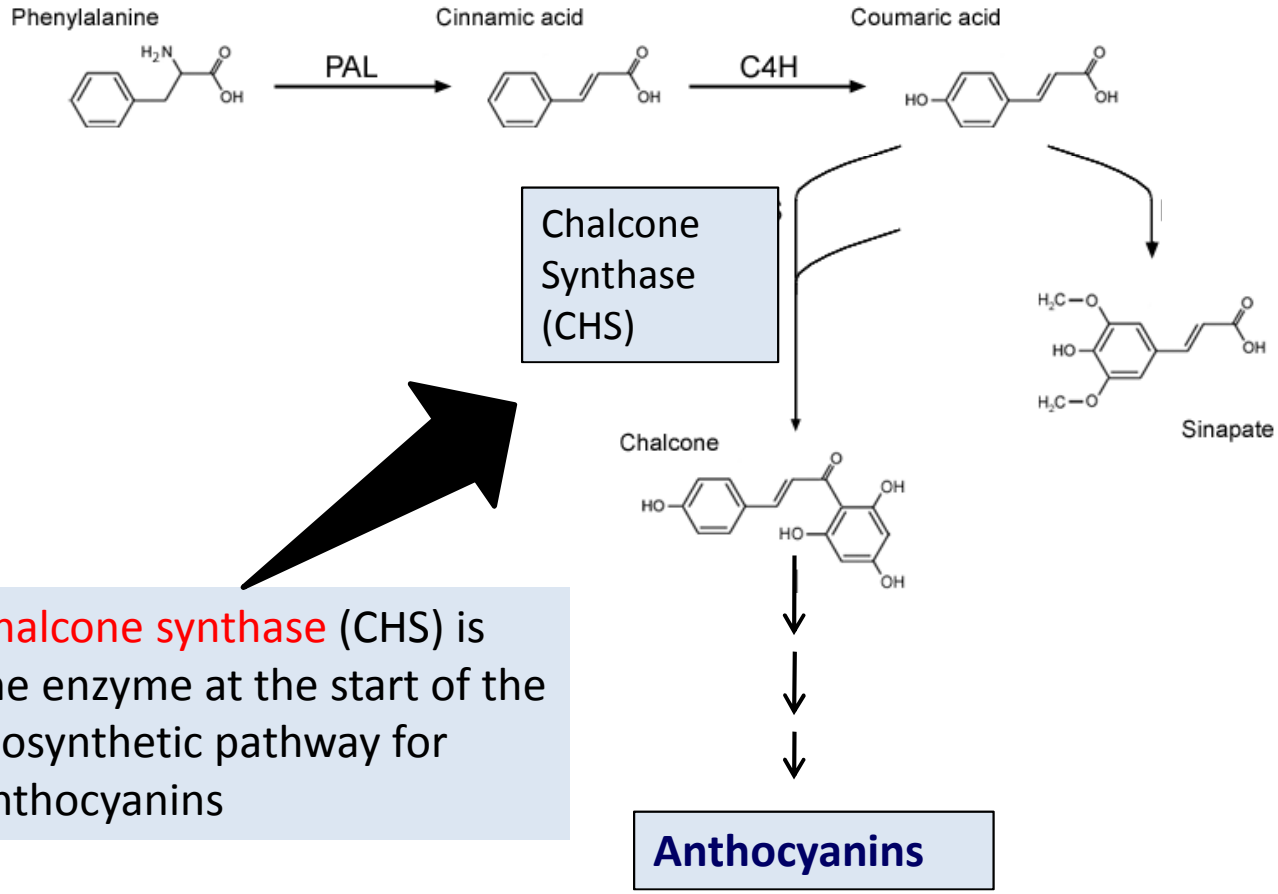
Silencing of transgenes

- 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**



Manipulation of chalcone synthase expression to modify pigmentation

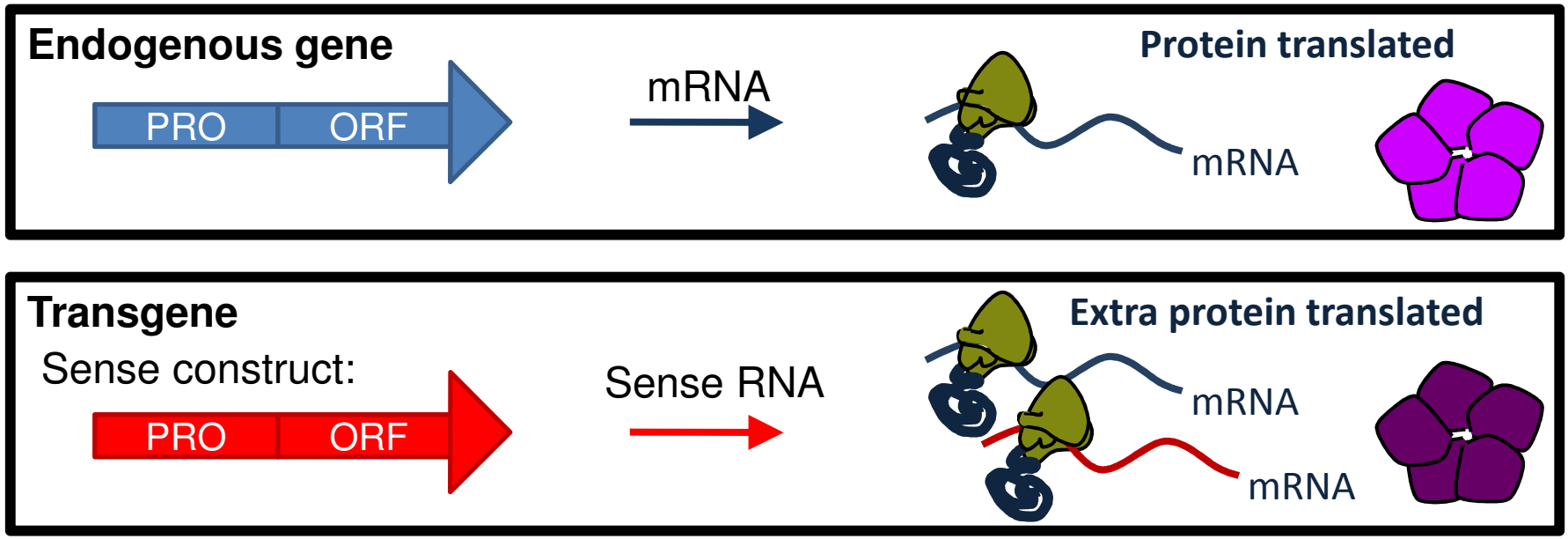
Wild-type petunia producing purple anthocyanin pigments



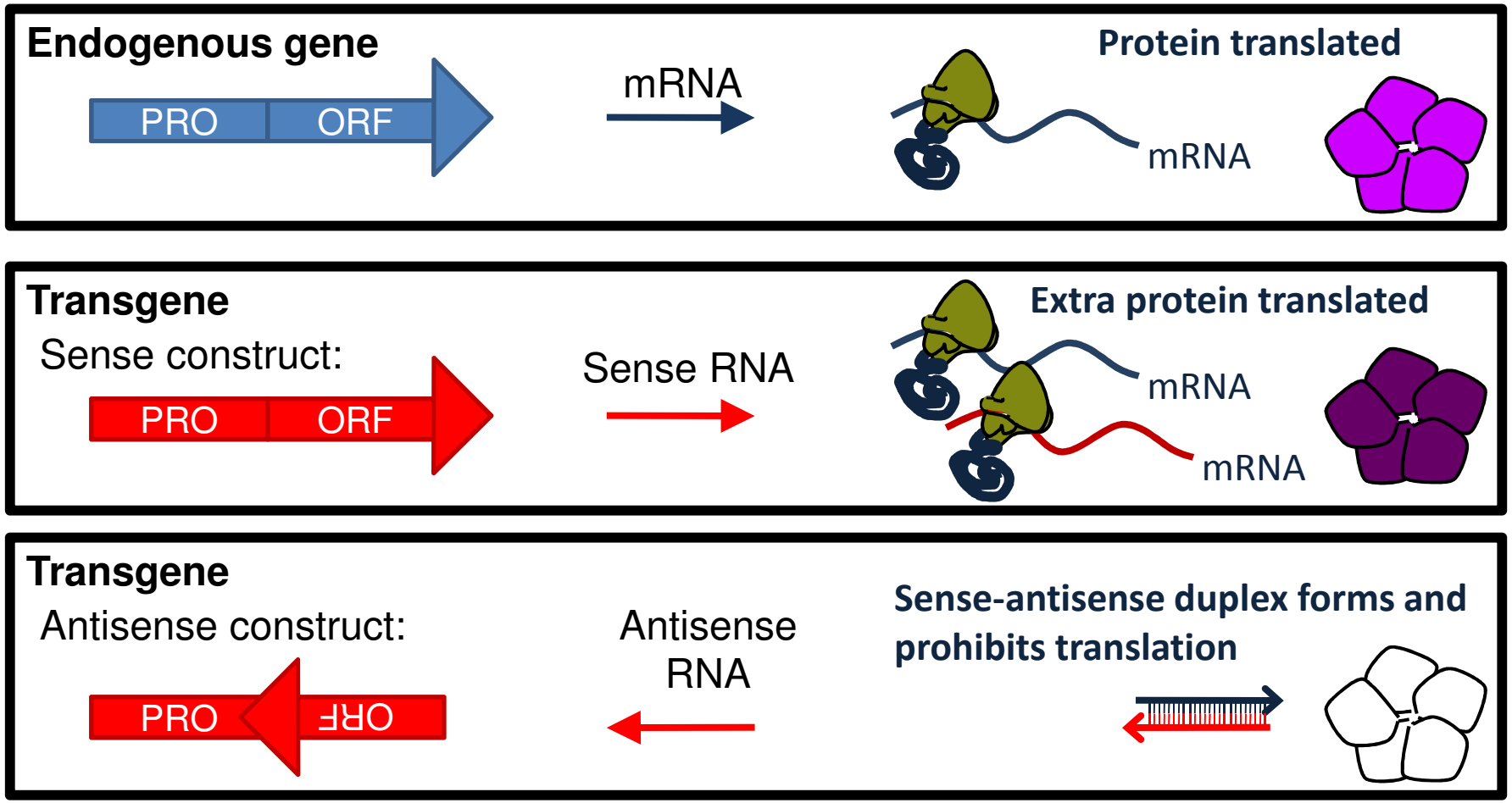
Chalcone synthase (CHS) is the enzyme at the start of the biosynthetic pathway for anthocyanins

Photo credit Richard Jorgensen; Aksamit-Stachurska *et al.* (2008) BMC Biotechnology 8: 25.

Expectation – sense RNA production would enhance pigmentation...



..and antisense RNA production would block pigmentation



Surprisingly, *both* antisense and sense gene constructs can inhibit pigment production

Plants carrying CHS transgene

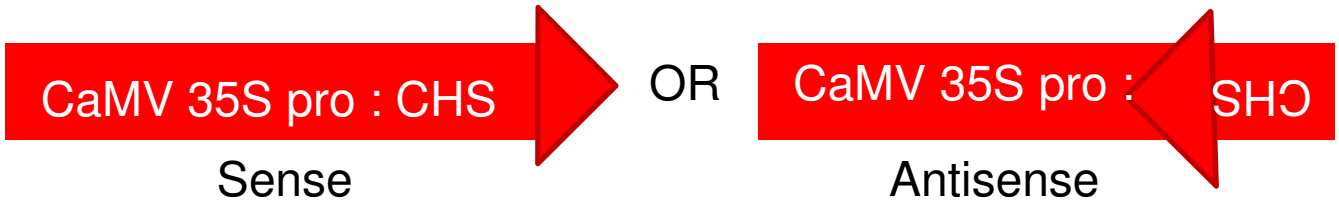
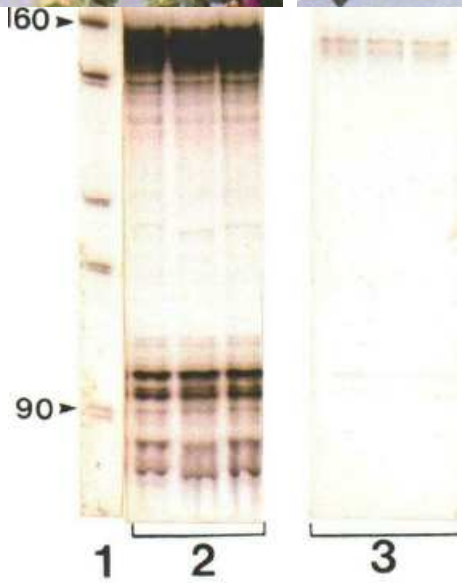


Photo credit Richard Jorgense

Silenced tissues do not express endogenous or introduced CHS

Purple flowers

White flowers



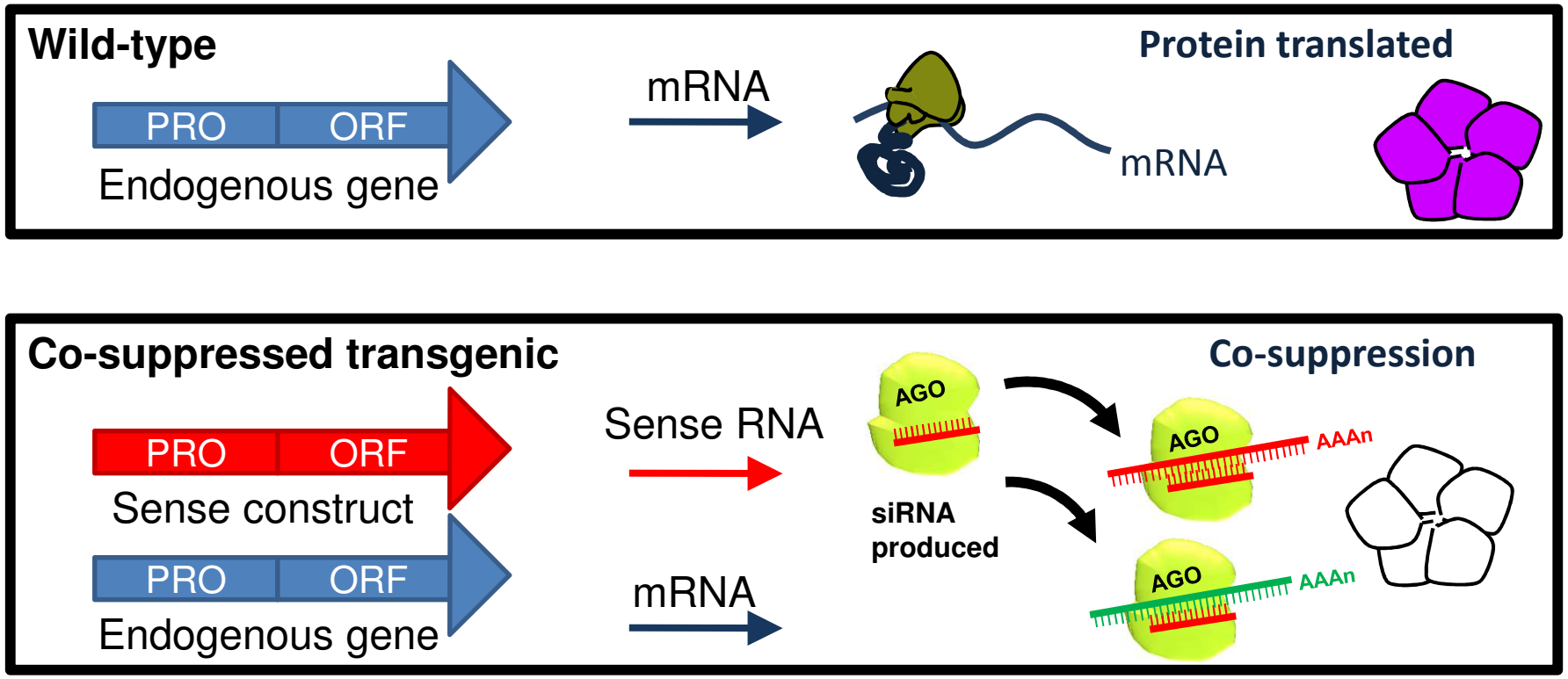
Transgene RNA

Endogenous gene RNA

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

Napoli, C., Lemieux, C., and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell **2**: 279–289.

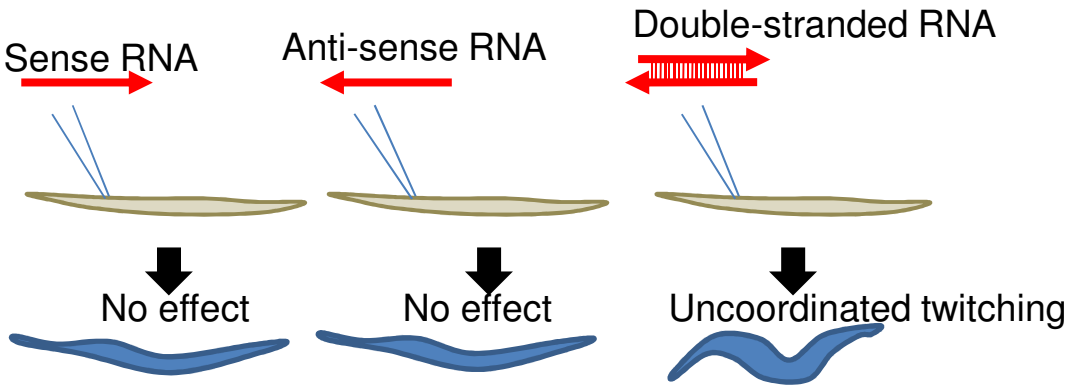
Co-suppression is a consequence of siRNA production



De Paoli, E., Dorantes-Acosta, A., Zhai, J., Accerbi, M., Jeong, D.-H., Park, S., Meyers, B.C., Jorgensen, R.A., and Green, P.J. (2009). Distinct extremely abundant siRNAs associated with cosuppression in petunia. RNA 15: 1965–1970.

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

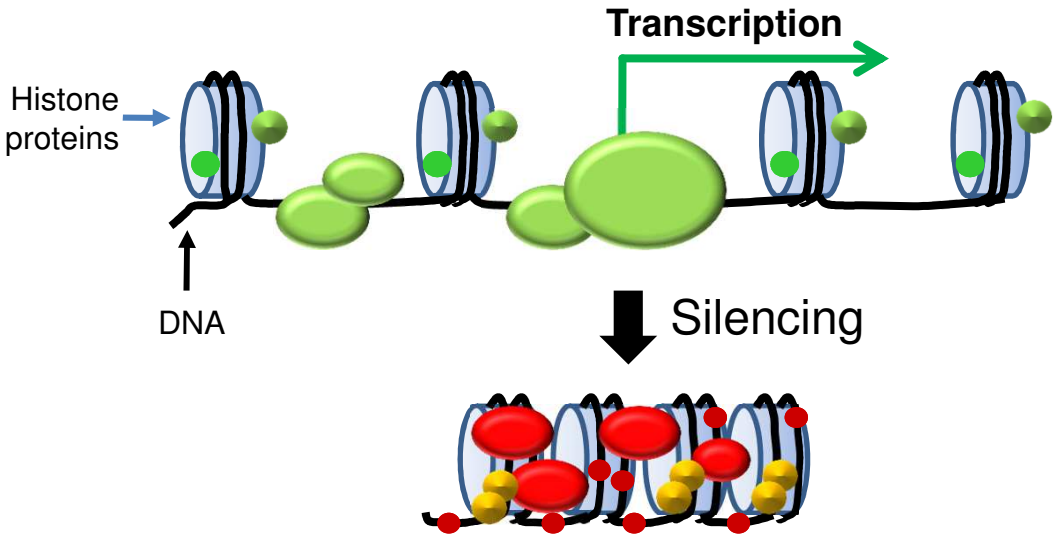
Sense, antisense or double-stranded RNAs homologous to the *unc-22* gene were introduced into worms. Silencing of *unc-22* causes loss of muscle control – hence its name, "*uncoordinated*".



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.

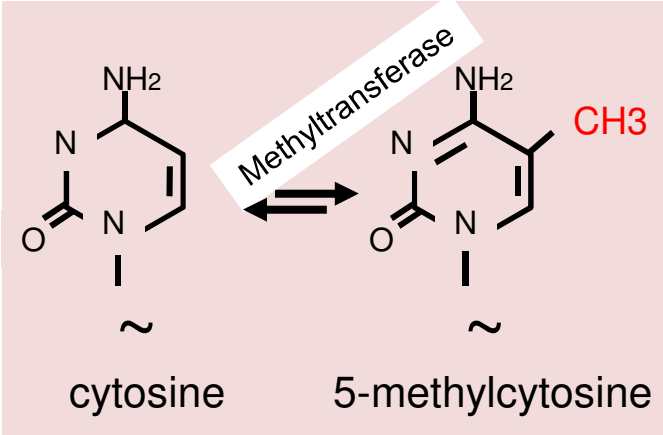
Transcriptional gene silencing

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



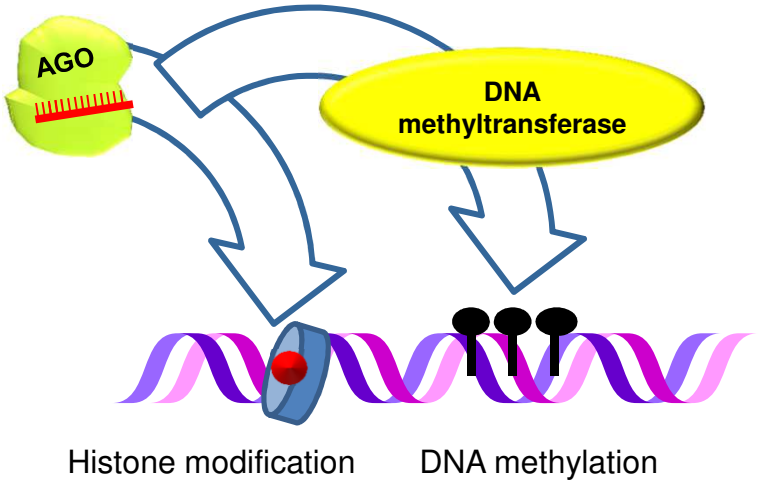
This form of silencing is frequently associated with stably silenced DNA including centromeres and transposons, but also occurs at genes.

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).



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

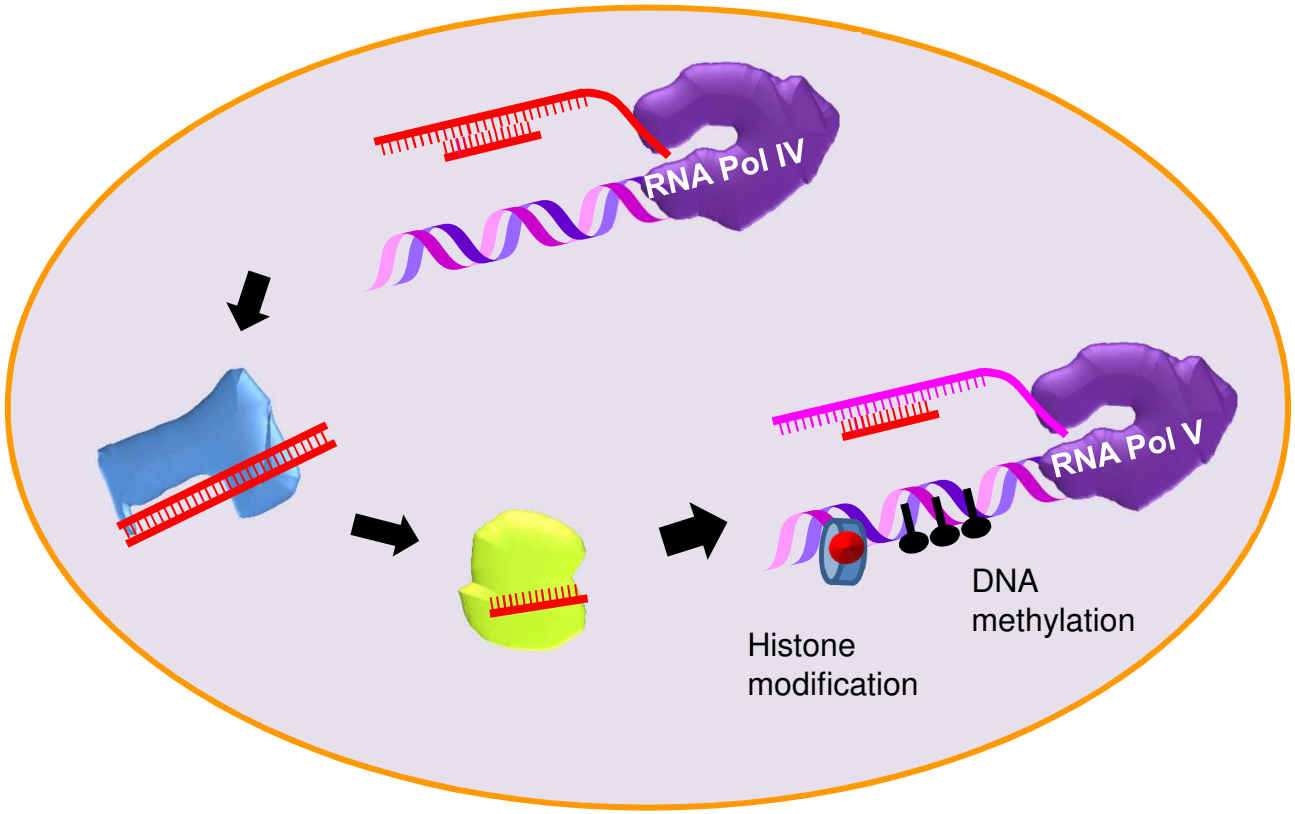
Arabidopsis plant with silenced GFP gene

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.

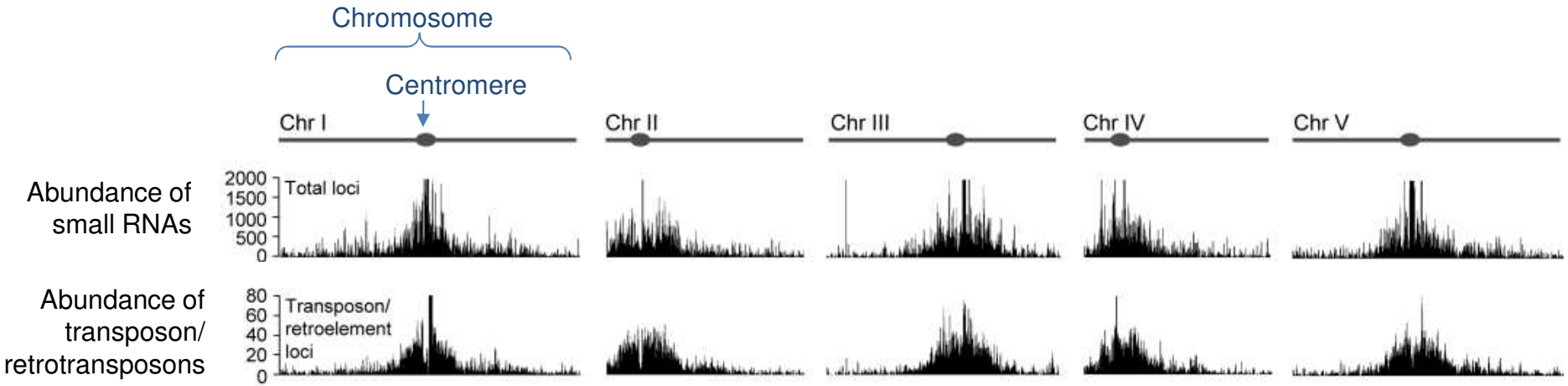
From Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005) RNA polymerase IV directs silencing of endogenous DNA. Science 308: 118–120. Reprinted with permission from AAAS.

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.

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.

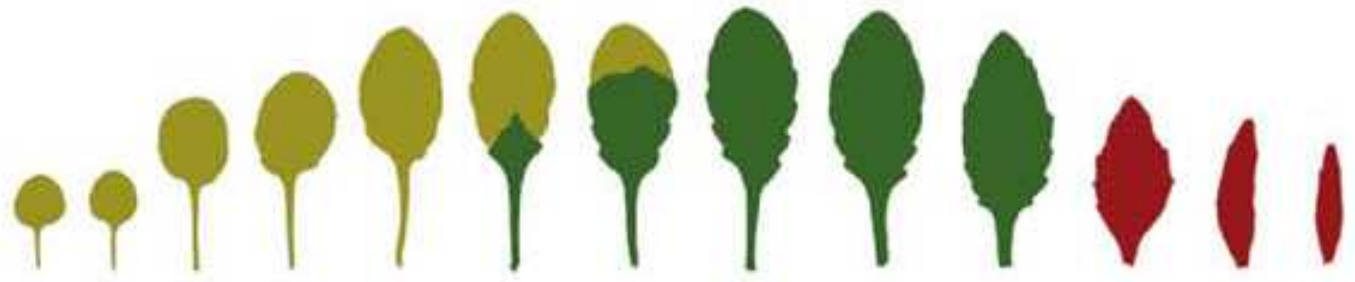
Kasschau, K.D., Fahlgren, N., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., and Carrington, J.C. (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. PLoS Biol 5(3): e57.

siRNAs - summary

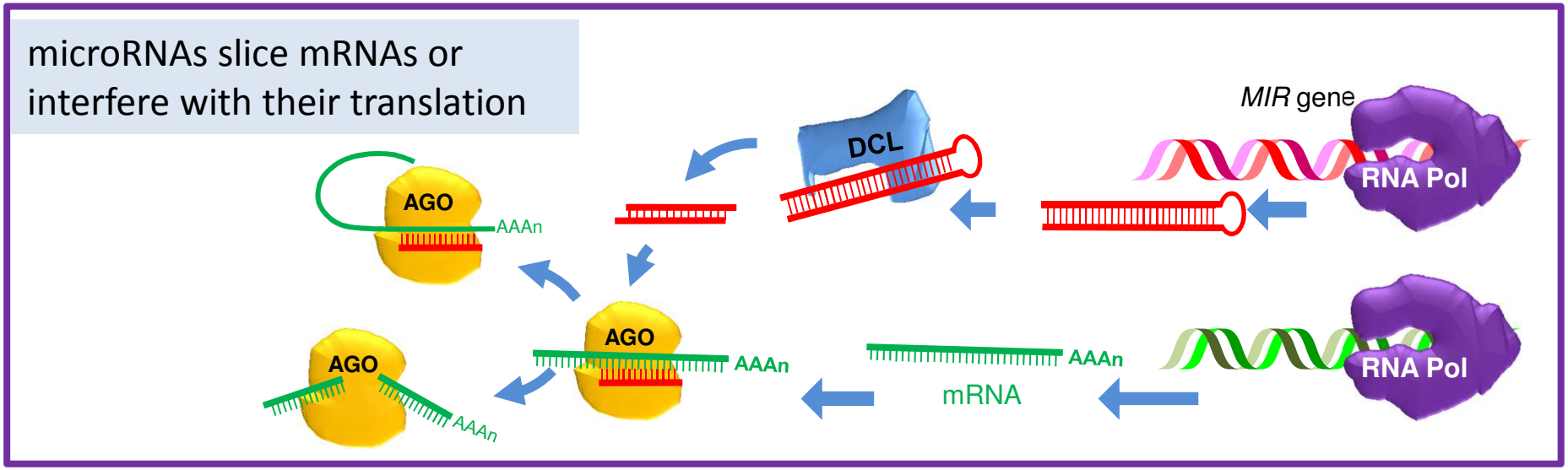
- The siRNA pathway silences foreign DNA, transposons and repetitive elements.
- In plants, siRNAs are produced by the action of Dicer-like proteins dicing dsRNA into 24 nt siRNAs
- The siRNAs associate with AGO proteins and form silencing complexes
- The silencing complexes can act post-transcriptionally 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

microRNAs - miRNAS

- miRNAs are thought to have evolved from siRNAs, and are produced and processed somewhat similarly
- Plants have a small number of highly conserved miRNAs, and a large number of non-conserved miRNAs
- miRNAs are encoded by specific *MIR* genes but act on other genes – they are trans-acting regulatory factors
- miRNAs in plants regulate developmental and physiological events



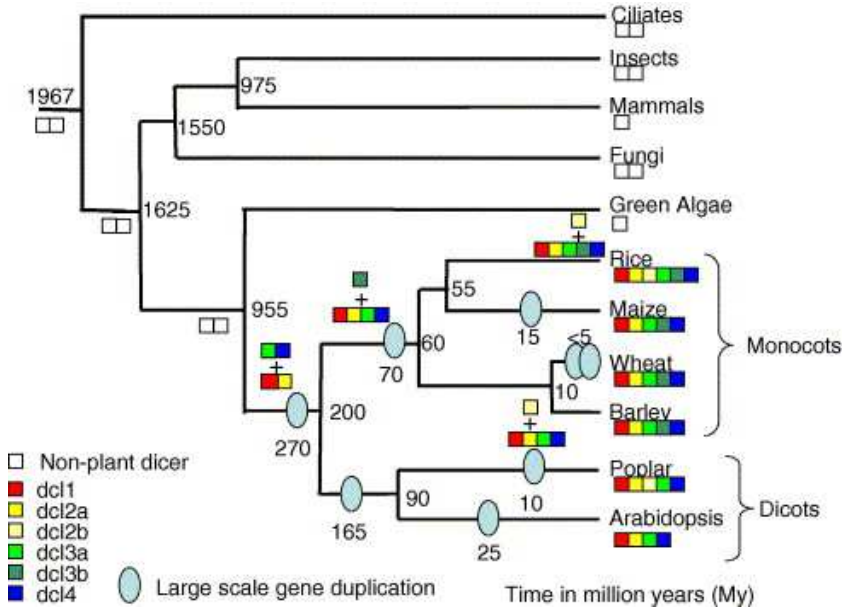
microRNAs - miRNAS



miRNAs and siRNAs are processed by related but different DCL proteins

AtDCL1 produces **miRNA**

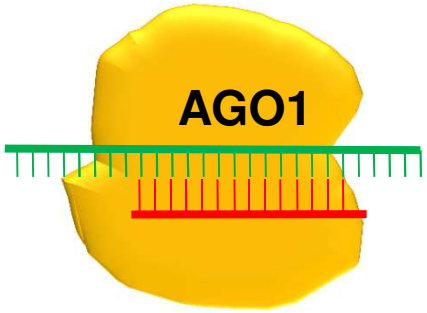
AtDCL2 - 4 produce **siRNA**



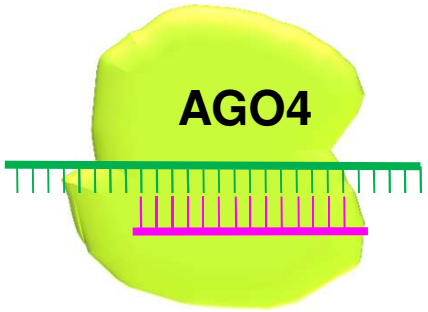
Plants have 4 or more DCL proteins, more than found in other organisms. The amplification of DCL proteins is thought to allow plants great flexibility in pathogen defense responses.

Reprinted from Margis, R., Fusaro, A.F., Smith, N.A., Curtin, S.J., Watson, J.M., Finnegan, E.J., and Waterhouse, P.M. (2006) The evolution and diversification of Dicers in plants FEBS Lett. 580: 2442-2450 with permission from Elsevier.

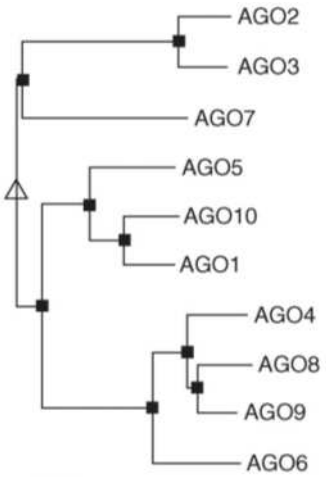
miRNAs and siRNAs associate with several AGO proteins



AGO1 preferentially slices its targets and associates with **miRNAs** but also some **siRNAs**



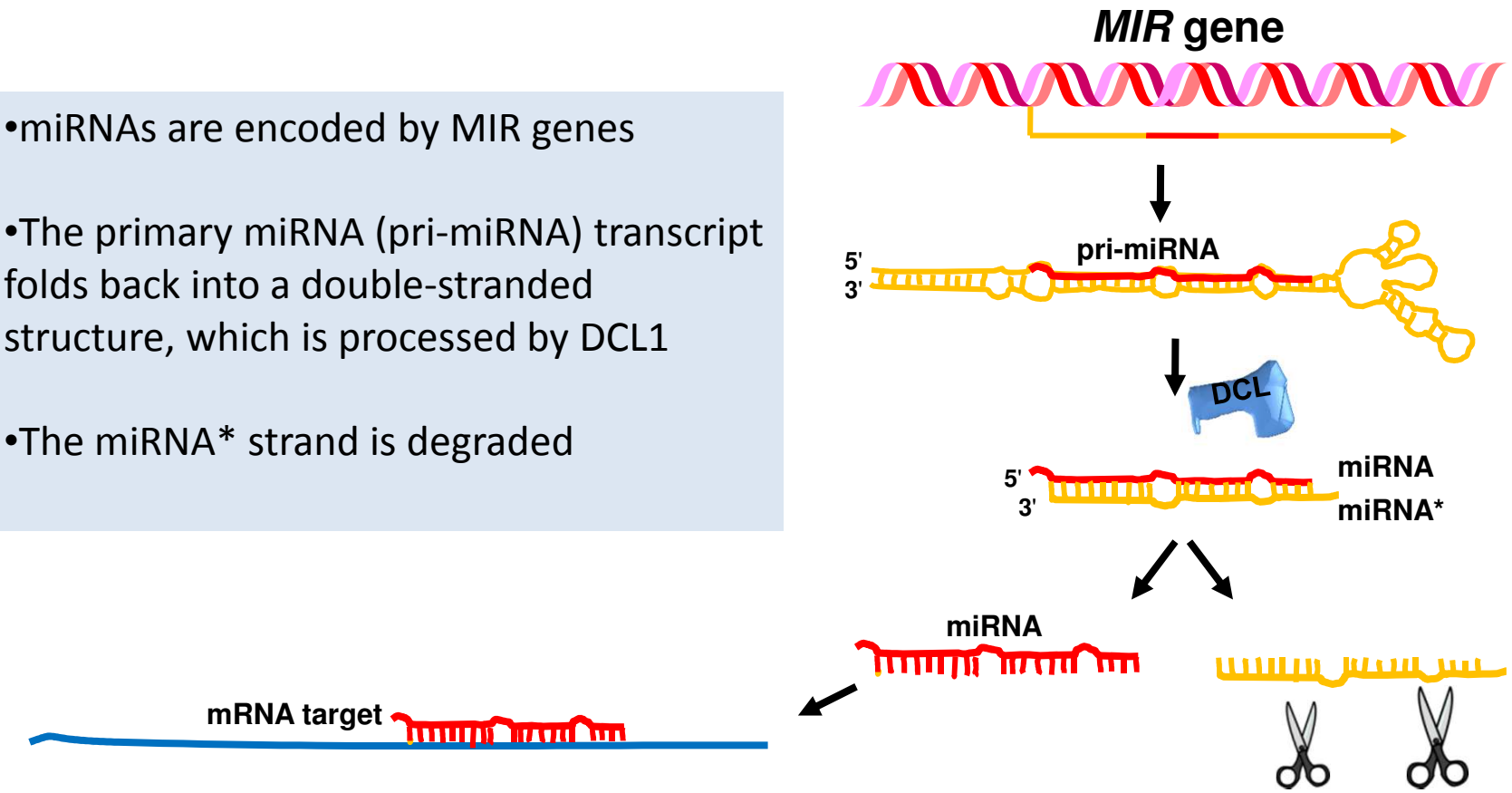
AGO4 preferentially associates with **siRNA** and mediates methylation of source DNA.



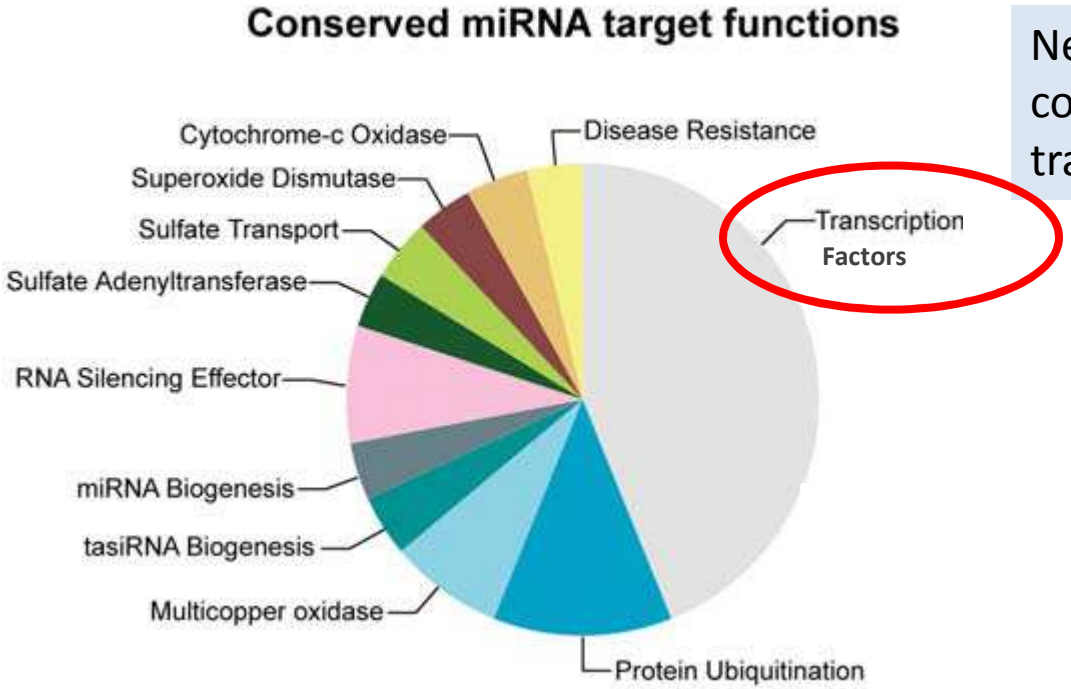
Arabidopsis has 10 AGO proteins. They are not all well characterized and there is some functional overlap.

MIR genes are transcribed into long RNAs that are processed to miRNAs

- miRNAs are encoded by MIR genes
- The primary miRNA (pri-miRNA) transcript folds back into a double-stranded structure, which is processed by DCL1
- The miRNA* strand is degraded



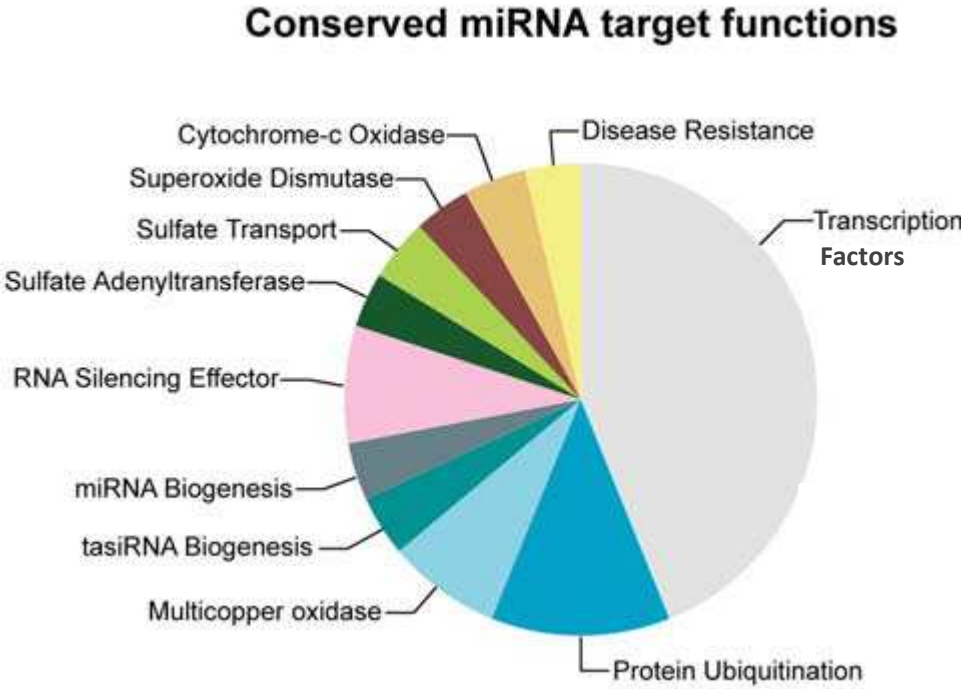
Some miRNAs are highly conserved and important gene regulators



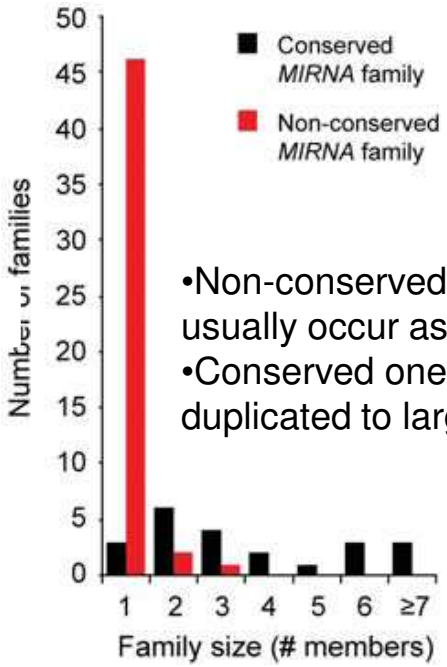
Nearly half of the targets of conserved miRNAs are transcription factors.

Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., Dangl, J.L., and Carrington, J.C. (2007) High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of *MIRNA* genes. PLoS ONE. 2007; 2(2): e219.

Some miRNAs are highly conserved and important gene regulators



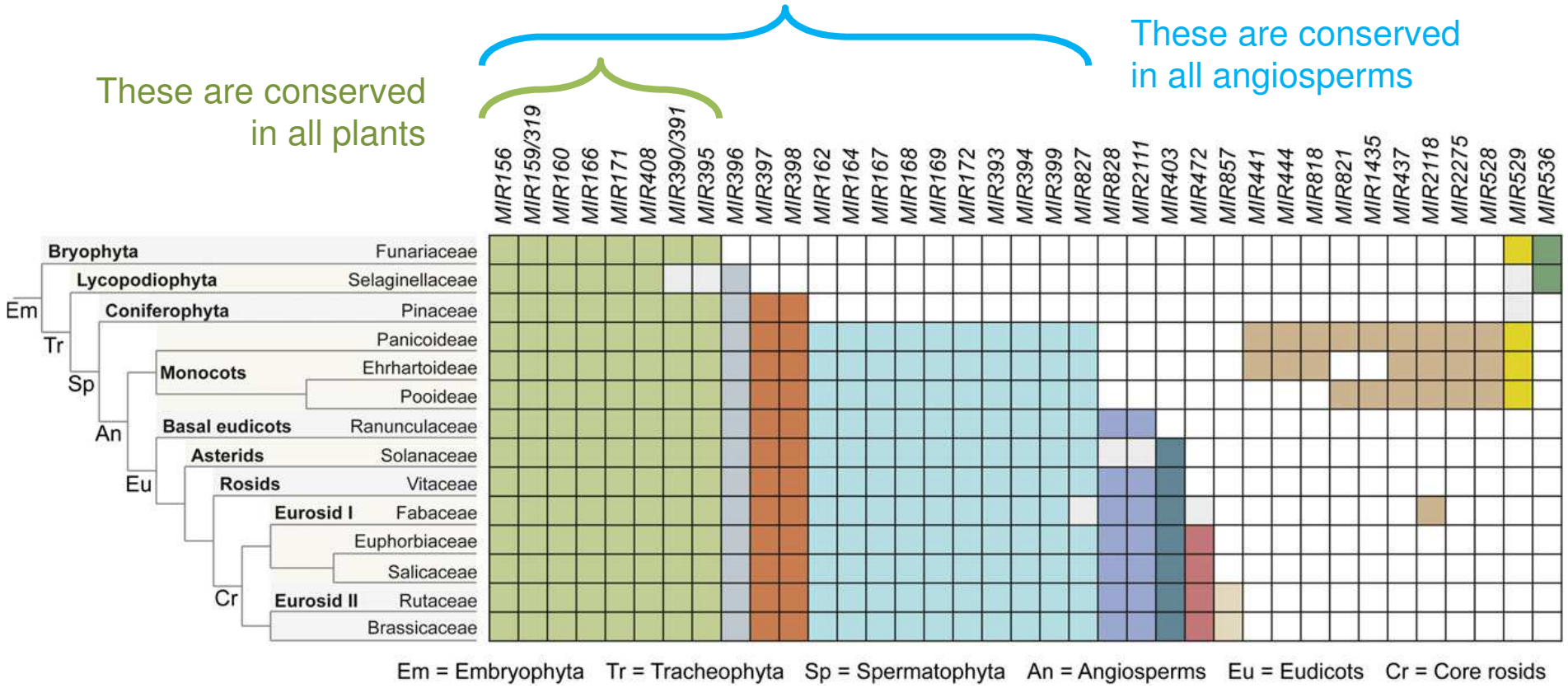
Nearly half of the targets of conserved miRNAs are transcription factors.



- Non-conserved *MIRNA* families usually occur as single genes
- Conserved ones have often duplicated to larger gene families

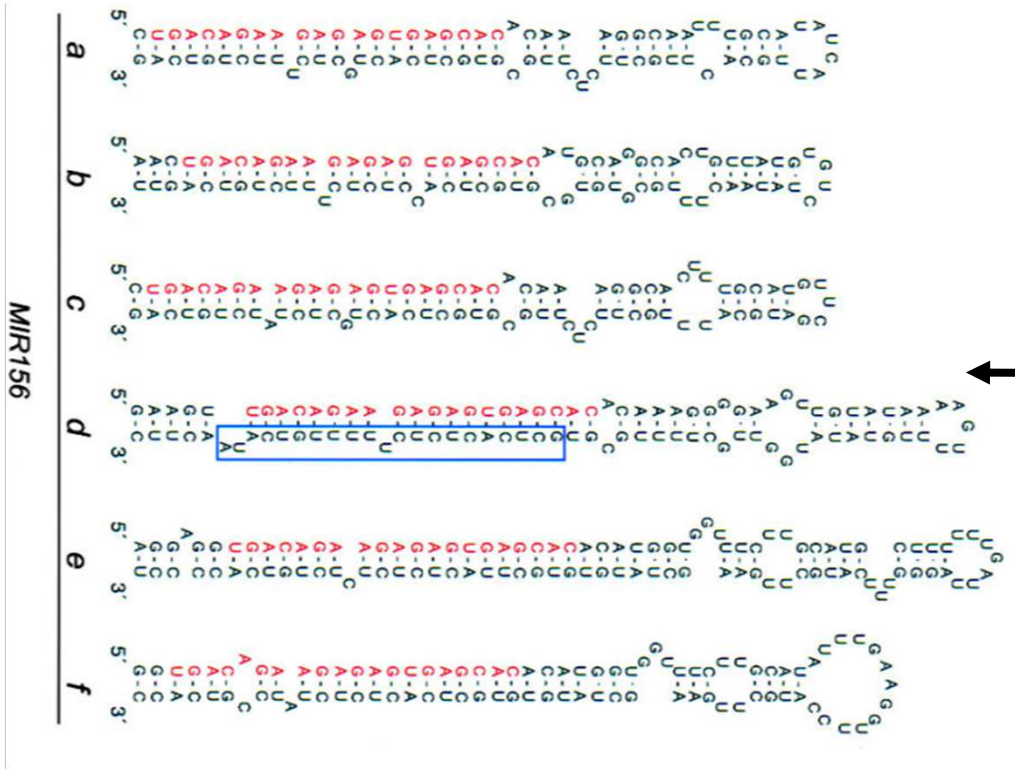
Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., Dangl, J.L., and Carrington, J.C. (2007) High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of *MIRNA* genes. PLoS ONE. 2007; 2(2): e219.

Some *MIR* gene families are present in all plants or all angiosperms



Cuperus, J.T., Fahlgren, N., and Carrington, J.C. (2011). Evolution and Functional Diversification of MIRNA Genes. Plant Cell: tpc.110.082784.

The *MIR156* gene family is highly conserved



- miR156 is highly conserved within the plant kingdom
- miR156 is found in angiosperms as well as mosses
- miR156 is encoded by six or more genes in Arabidopsis
- miR156 targets transcription factors that control developmental phase changes

Reprinted from Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002) MicroRNAs in plants. Genes Dev. 16: 1616–1626.

Targets of some conserved miRNAs

miRNA gene family	Target gene family	Function
156	SPL transcription factors	Developmental timing
160	ARF transcription factors	Auxin response, development
165/6	HD-ZIPIII transcription factors	Development, polarity
172	AP2 transcription factors	Developmental timing, floral organ identity
390	TAS3 (tasiRNA) which acts on ARF transcription factors	Auxin response, development
395	Sulfate transporter	Sulfate uptake
399	Protein ubiquitination	Phosphate uptake

Adapted from Willmann, M.R., and Poethig, R.S. (2007) Conservation and evolution of miRNA regulatory programs in plant development. Curr. Opin. Plant Biol. 10: 503–511..

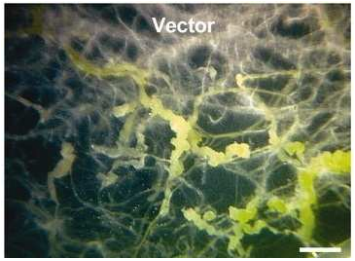
Applications of small RNA technologies

In plants, siRNA or miRNA-forming DNA can be introduced stably into the genome to selectively silence one or more genes.

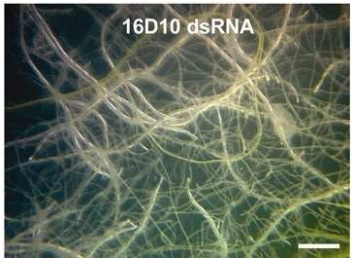
Gene silencing can eliminate allergens from peanuts.

Gene silencing can remove toxic compounds from cotton seed so they can be used as a food source.

Pest Control



Control, infected by parasitic nematode



RNAi-inducing – no infection

Plants expressing dsRNA corresponding to insect or nematode genes are resistant to these pests! When ingested, the dsRNA induces gene silencing.

Huang, G., Allen, R., Davis, E.L., Baum, T.J., and Hussey, R.S. (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proc. Natl. Acad. Sci. USA 103: 14302–14306.

Approaches for RNAi-based Silencing (targeted)

- Antisense-mediated gene silencing
- Hairpin-loop transcript-based RNAi
- Artificial microRNA (amiR)
- VIGS (Virus-induced gene silencing)

Transgene Silencing

Inattivazione epigenetica dovuta a presenza di sequenze/geni espressi a livelli molto elevati (per esempio a causa di copie multiple nel genoma); dipende da interazione tra copie omologhe, i.e. **Homology-Dependent Gene Silencing** (HDGS)

Si distinguono due tipi di HDGS:

- **Transcriptional Gene Silencing (TGS)**
- **Post Transcriptional Gene Silencing (PTGS)**

- Entrambi i tipi di HDGS sono frequentemente associati con "sequence-specific *de novo* methylation"
- Entrambi i tipi di HDGS possono essere associati con la presenza di IRs
- HDGS puo' dare inattivazione sia in *cis* che in *trans*

Transcriptional Inactivation (methylation)

- **CIS Inactivation**

TGS

- Assenza sia dei trascritti maturi che dei precursori

- Lo stato silenziato è mantenuto ad ogni ciclo mitotico, ed è anche trasmesso alla progenie.

- A bassa frequenza, si osserva riattivazione spontanea (reversione) del locus silenziato.

- Geni (endogeni e/o trans) silenziati sono caratterizzati da pattern alterato di metilazione (strategia tipicamente vegetale)

- Struttura cromatinica alterata (come in Drosphila e lievito)

PTGS

PTGS = Post-Transcriptional Gene Silencing

PTGS is a sequence-specific RNA degradation process that targets foreign RNA (details to follow).

- This includes:
  - viral RNA
  - transposon RNA
  - dsRNA, etc.

- **Why is PTGS significant with respect to plant viruses?**
  - PTGS is a mechanism that plants have developed for protection from virus infection (i.e., the plant PTGS system degrades viral RNA)

PTGS

PTGS come soppressione dell'espressione genica x degradazione del (trans)gene RNA

- Avviene nel citoplasma, ed è gene-specifico
- Il livello di trascrizione è inalterato (run-on)
- Rappresenta un meccanismo di difesa dalle infezioni virali

Geni (endogeni e/o trans) silenziati sono caratterizzati da:

- pattern alterato di metilazione (strategia tipicamente vegetale)
- struttura cromatinica alterata (come in Drosphila e lievito)

Post-transcriptional gene silencing across kingdoms.

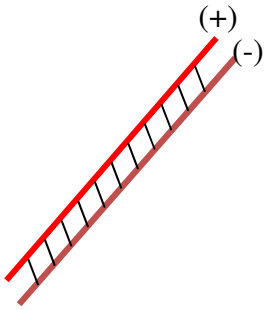
Kingdom	Species	Phenomenon	Trigger
Fungi	*Neurospora*	Quelling	Transgenes
Plants	*Petunia*, *Nicotiana*, *Arabidopsis*, tomato, rice, potato, etc.	PTGS, co-suppression	Transgenes, viruses (dsRNA form)
Animals			
Invertebrates	*C. elegans*	RNAi	dsRNA
	Drosophila	RNAi	dsRNA
	Paramecium	Co-suppression	Transgenes
	Planaria	RNAi	dsRNA
	Hydra	RNAi	dsRNA
	T. brucei	RNAi	dsRNA
Vertebrates	Zebrafish	RNAi	dsRNA
	Mouse	RNAi	dsRNA

RNAi = RNA interference

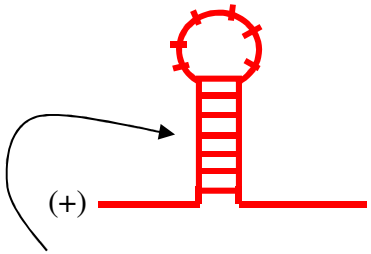
Note different terms used for same phenomenon:
PTGS = quelling = co-suppression = VIGS = RNA interference

What triggers (induces) the PTGS response when a virus infects a plant?

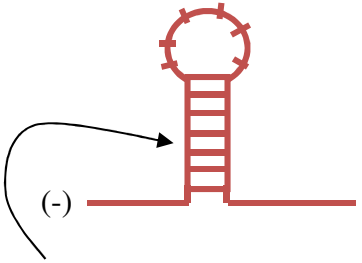
- Inducers of PTGS:
  1. Viral dsRNA (the double-stranded replication intermediate that arises during virus infection)
  2. Viral plus sense RNA (the double-stranded regions)
  3. Viral minus sense RNA (the double-stranded regions)



Viral dsRNA
replication intermediate



ds regions in (+) sense RNA



ds regions in (-) sense RNA

The PTGS pathway has two distinct phases:

1. **Initiation –** the viral RNA triggers the PTGS system to degrade viral RNA into small pieces (called siRNA or small interfering RNA)

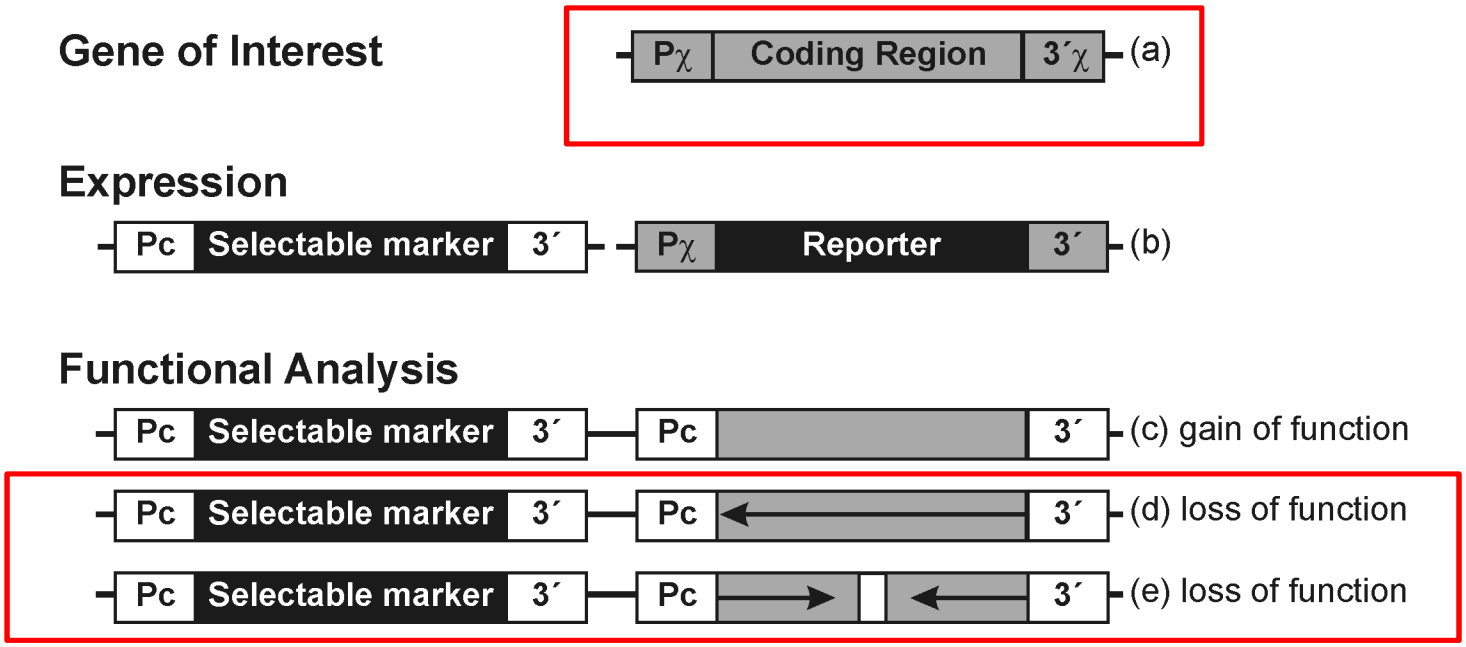
2. **Maintenance-**the siRNA binds to complementary regions in viral RNA and this is either:

   a. degraded by a complex called RISC (RNA-Induced Silencing Complex)
   or

   b. or is used to make more viral RNA via the host RNA dependent RNA polymerase. The resulting dsRNA then feeds back into the system at the point where dsRNA is degraded to siRNA and the cycle continues to repeat.

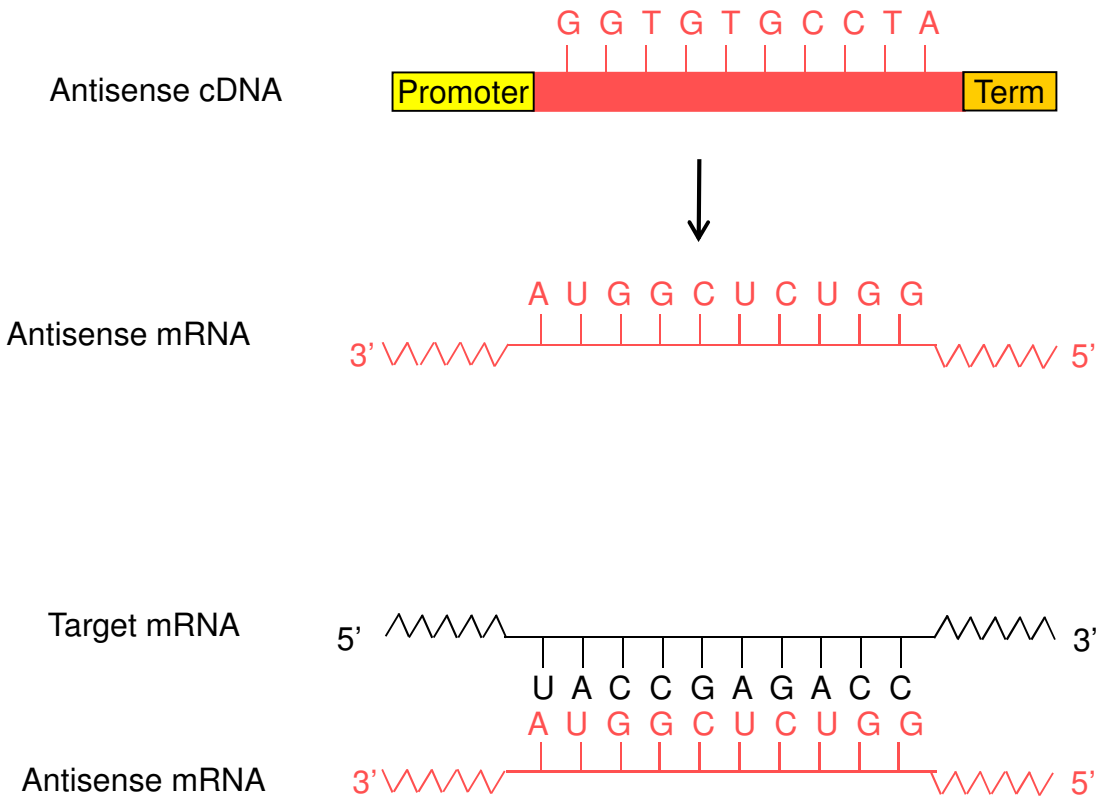
Figure 9.1

Types of cassettes

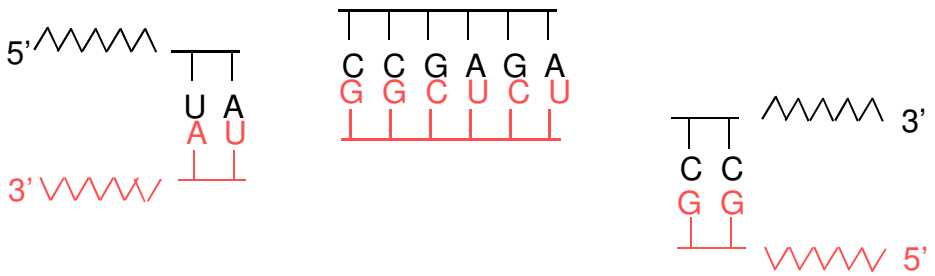


Antisense-mediated gene silencing

To reduce the expression of a target gene by expression of sequences complementary to the target sequence.

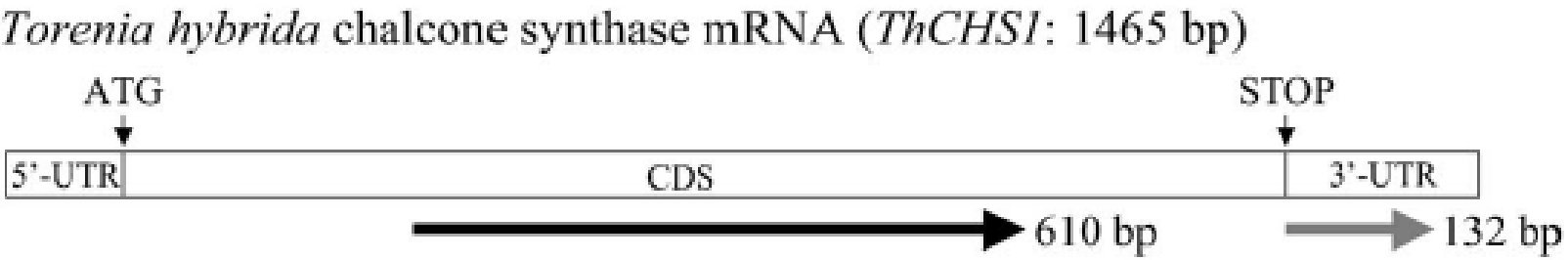


dsRNA Degradation



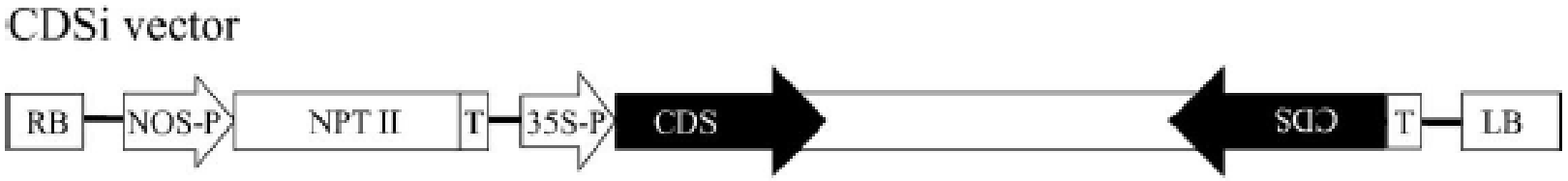
Hairpin loop constructs

Il cDNA della Calcone sintasi

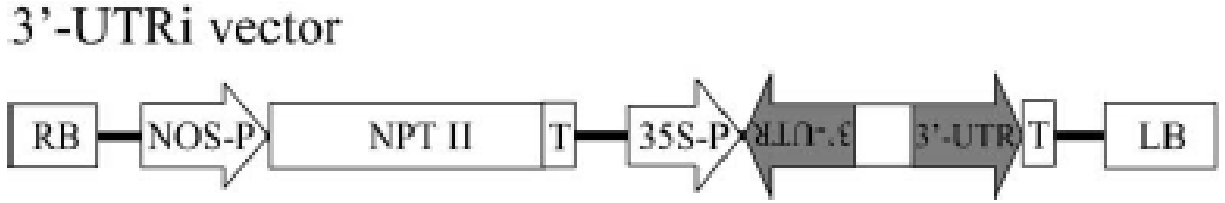


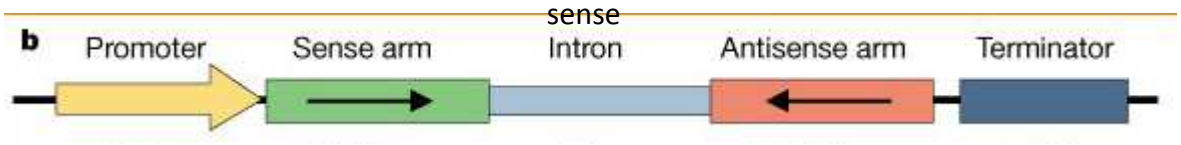
I costrutti per l'RNAi

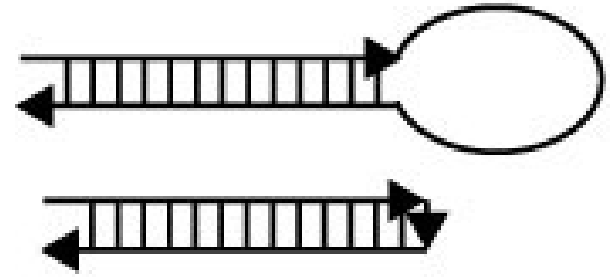
A)



B)




b
Promoter
Sense arm
sense
Intron
Antisense arm
Terminator



VIGS (**V**irus **I**nduced **G**ene **S**ilencing)

- Vettori virali che portano un frammento del gene target per generare un dsRNA che ne induce il silenziamento

- Possibilità di silenziare un gene specifico senza trasformare geneticamente la pianta studiata

- Permette di vincere il problema della ridondanza funzionale legato al knock-down di un gene target

Constructs for RNAi

- Hairpin Loop
- miRNA artificiali

- **Virus a DNA :** derivato del *Cabbage leaf curl geminivirus* (CbLCV)

Svantaggi:

-difficoltà nell'inserire il virus nella pianta tramite bombardamento di particelle

-Dimensioni limitate dell'inserto

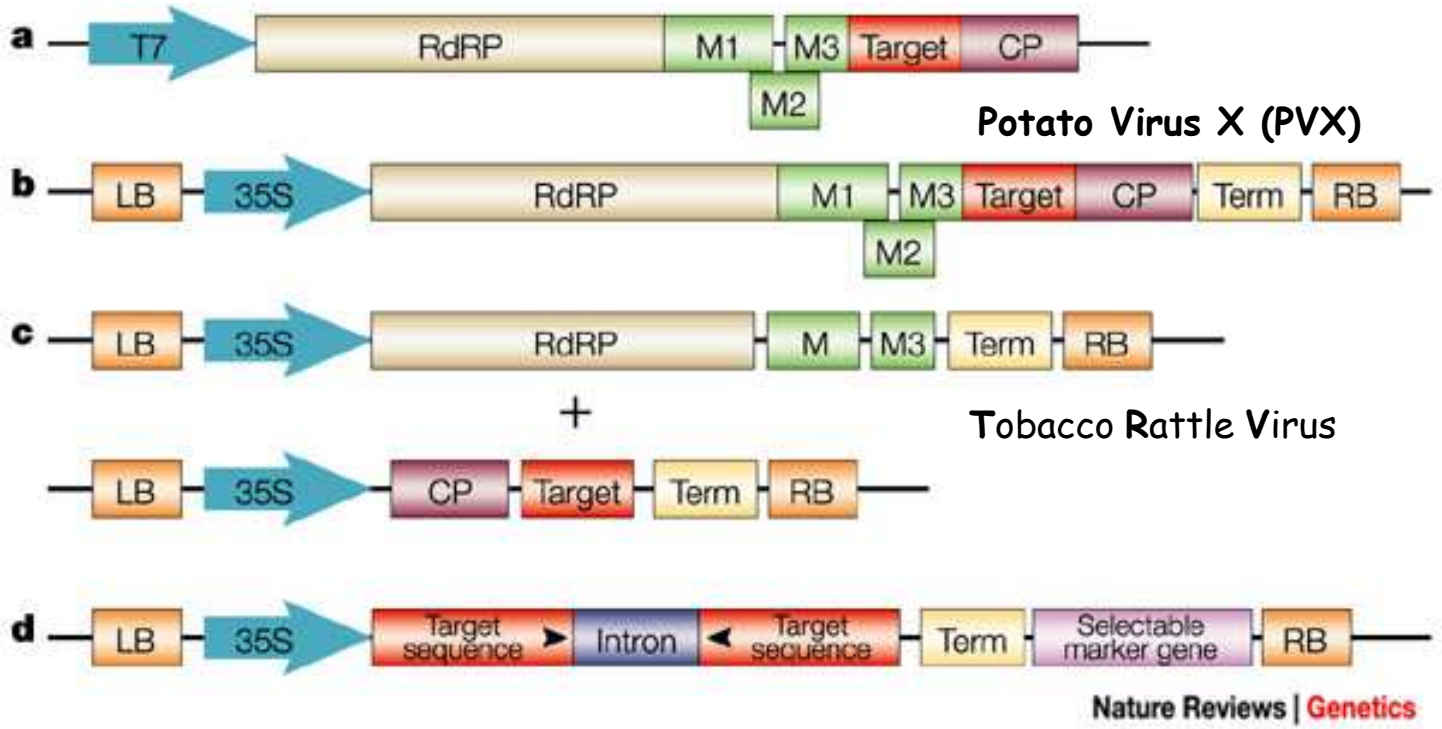
2. Virus a RNA : ***T****obacco* ***R****attle* ***V****irus* (TRV), RNA1 e RNA2

Vantaggi:

-In natura infetta più di 100 specie, in laboratorio più di 400

-Capacità di mediare VIGS in assenza di sintomi indotti dal virus

-Capacità di veicolare gli inserti nei punti di crescita della pianta (meristemi)



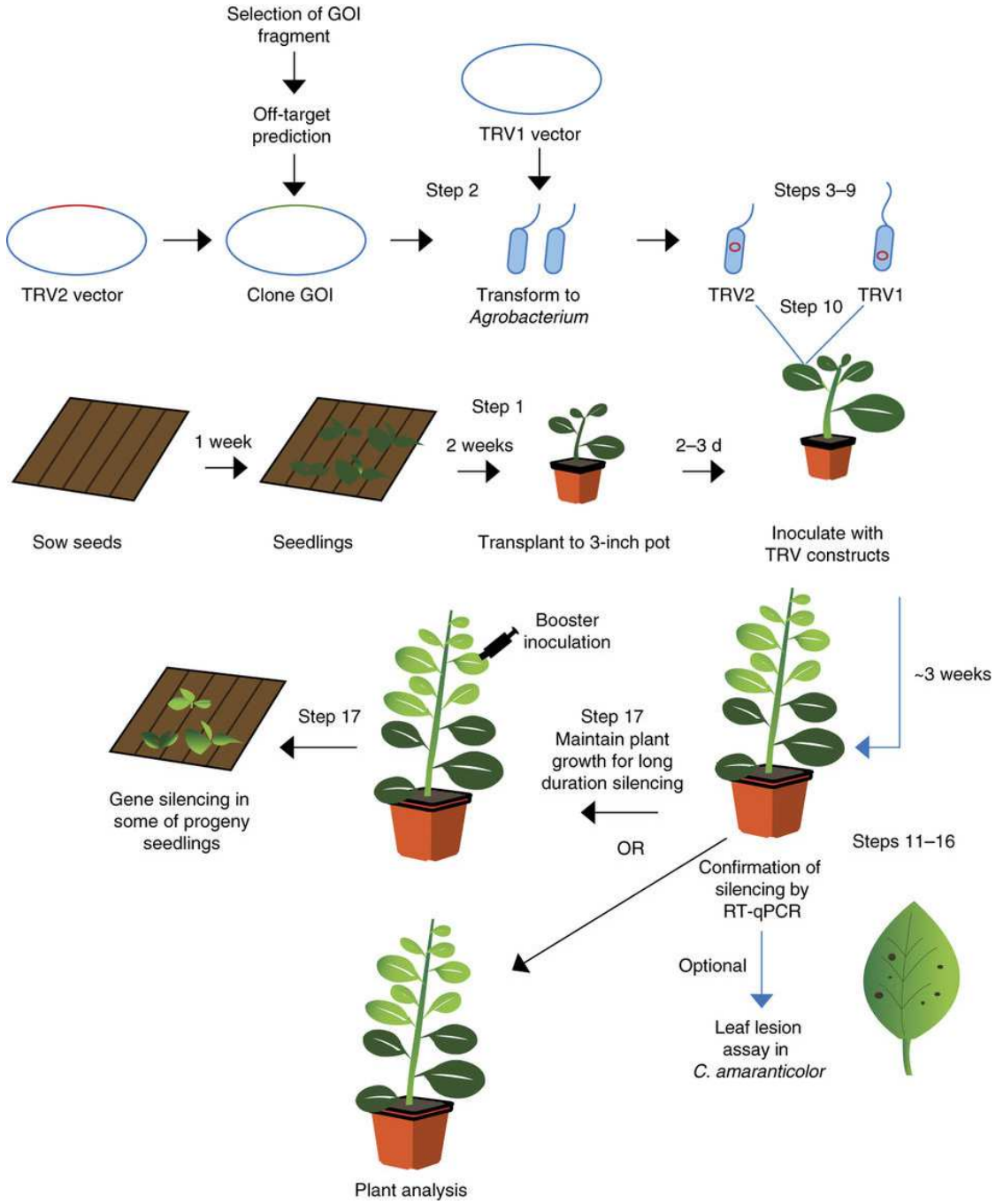
DNA constructs for RNA-mediated gene silencing. **a** | A DNA plasmid that can be propagated in *Escherichia coli* from which infectious potato virus X (PVX) RNA can be transcribed *in vitro*, using T7 polymerase. The PVX cassette contains sequence derived from the gene to be targeted. **b** | A transferred (T)-DNA plasmid that is propagated in *Agrobacterium*. When this plasmid-carrying *Agrobacterium* is inoculated onto a plant, it transfers the DNA between its left (LB) and right (RB) borders into the plant's cells. The region between the borders contains the viral sequences shown in part **a**, but in this vector, the T7 promoter has been replaced with the cauliflower mosaic virus promoter. This enables the transferred DNA to be transcribed by the plant's endogenous transcription machinery to generate infectious PVX (plus insert sequence) RNA. In amplicon transgene vectors, a selectable maker gene is also present between the left and right borders of this plasmid, enabling plants to be stably transformed with the transferred DNA. **c** | The tobacco rattle virus (TRV) virus-induced gene-silencing (VIGS) system. Two T-DNA plasmids that encode the TRV genome (one encoding TRV RNA1 and the other encoding TRV RNA2, which carries the inserted target sequence) are propagated separately in *Agrobacterium* and used to co-infect plant tissue. **d** | A typical T-DNA plasmid for the expression of hairpin RNAs (hpRNAs). This plasmid can be transiently introduced into plants by bombardment or stably introduced by agroinfiltration. A generic silencing precursor construct (pHANNIBAL) that enables hpRNA vectors to be easily constructed has different multiple cloning sites either side of the intron to enable the rapid insertion of target sequences in forward and reverse orientations. 35S, CaMV 35S promoter; CP, coat protein; M1,2,3, movement proteins 1, 2, 3; RdRP, RNA-dependent RNA polymerase; T7, T7 promoter; Term, transcription termination sequence.

V I G S

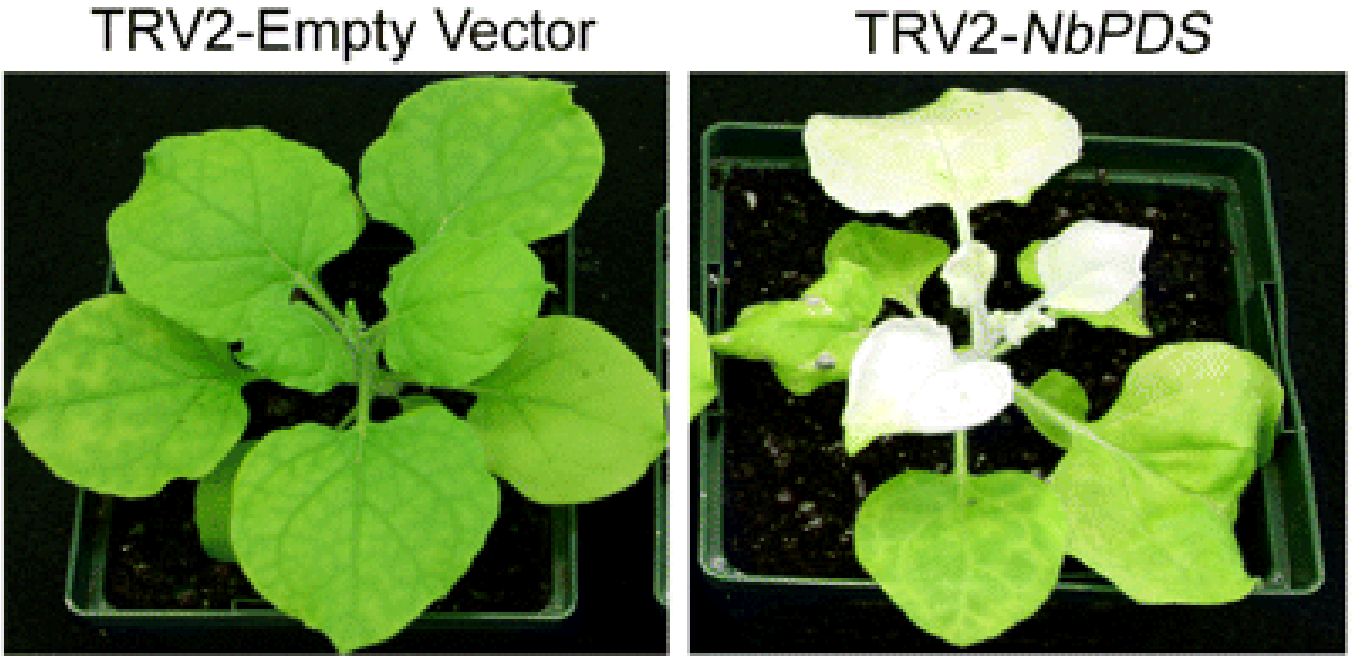
• Non necessita di trasformazione genetica delle piante
• Consente lo studio del silenziamento di *geni letali*
• Supera il problema della ridondanza genica attraverso al silenziamento di famiglie geniche

Svantaggi

• Distorsione fogliare dovuta all'infezione
• Necrosi tissutale dovuta all'infezione
• Bassa efficienza d'infezione degli *apici meristematici*

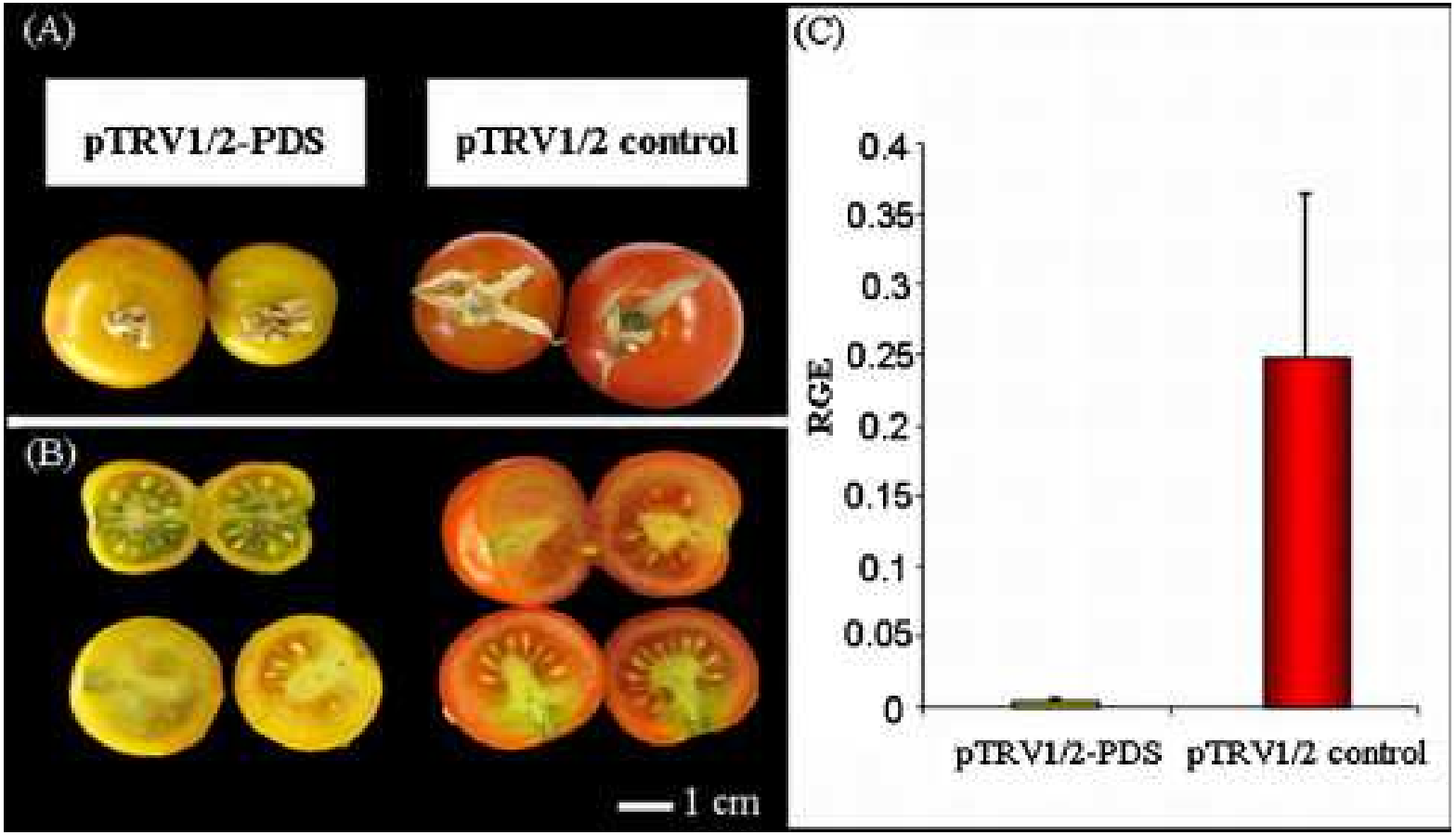

Selection of GOI fragment
Off-target prediction
TRV1 vector
Step 2
Steps 3–9
TRV2 vector
Clone GOI
Transform to Agrobacterium
TRV2
TRV1
Step 10
Step 1
1 week
2 weeks
2–3 d
Sow seeds
Seedlings
Transplant to 3-inch pot
Inoculate with TRV constructs
Booster inoculation
~3 weeks
Step 17
Step 17
Maintain plant growth for long duration silencing
Gene silencing in some of progeny seedlings
OR
Steps 11–16
Confirmation of silencing by RT-qPCR
Optional
Leaf lesion assay in C. amaranticolor
Plant analysis

VIGS del gene FITOENE DESATURASI (PDS) in *Nicotiana benthamiana*



N.B.: PDS serve alla sintesi dei carotenoidi -> l'assenza di carotenoidi porta a photobleaching (sbiancamento per stress ossidativo)

VIGS del gene FITOENE DESATURASI (PDS) in frutti di pomodoro



Some Uses of PTGS in Agriculture

- **Functional genomics/identification of gene function**
  - Expression of specific genes with unknown function can be silenced via *Agrobacterium* or a viral vector (i.e., VIGS) and the effect on the plant can be analyzed.
- **Overexpression of genes (use of viral suppressor)**
  - Sometimes difficult to express high levels of proteins due to PTGS. Viral suppressors of gene silencing can be used to inhibit PTGS and therefore achieve high level expression
- **Inhibiting virus expression**
  - Transform plant with a virus gene. The plant then becomes resistant to virus infection via PTGS pathway

Table 2. Use of RNAi for virus resistance in plants

Name of virus	Family	Region targeted	Results	System used	Genome	Refs
Potato virus Y	*Potyviridae*	HC-Pro	Immunity	Potato	RNA	[5]
Mungbean yellow mosaic India virus (MYMIV)	*Geminiviridae*	Bidirectional promoter	Recovery from infection	*Vigna mungo* (black gram)	DNA	[23]
African cassava mosaic virus (ACMV)	*Geminiviridae*	Replication-associated protein gene	Reduced virus accumulation	Tobacco protoplast	DNA	[26]
Tomato yellow leaf curl Sardinia virus	*Geminiviridae*	Replication-associated protein gene	Poor resistance	Tomato	DNA	[27]
Pepper mild mottle virus (PMMoV)	*Tobamoviridae*	Arbitrary sequence	Block in viral infectivity	Tobacco	RNA	[11]
Tobacco etch virus (TEV)	*Potyviridae*	Arbitrary sequence	No viral-specific symptoms appeared	Tobacco	RNA	[11]
Alfalfa mosaic virus (AMV)	*Bromoviridae*	Arbitrary sequence	Recovery from infection	Tobacco	RNA	[11]
Beet necrotic yellow vein virus (BNYVV)	*Benyviridae*	Coat protein	Tolerance	Tobacco	RNA	[28]
Tobacco mosaic virus (TMV)	*Tobamoviridae*	Replication-associated protein	Inhibition of TMV replication	Tobacco	RNA	[52]

Abbreviation: HC-PRO, helper-component proteinase gene.

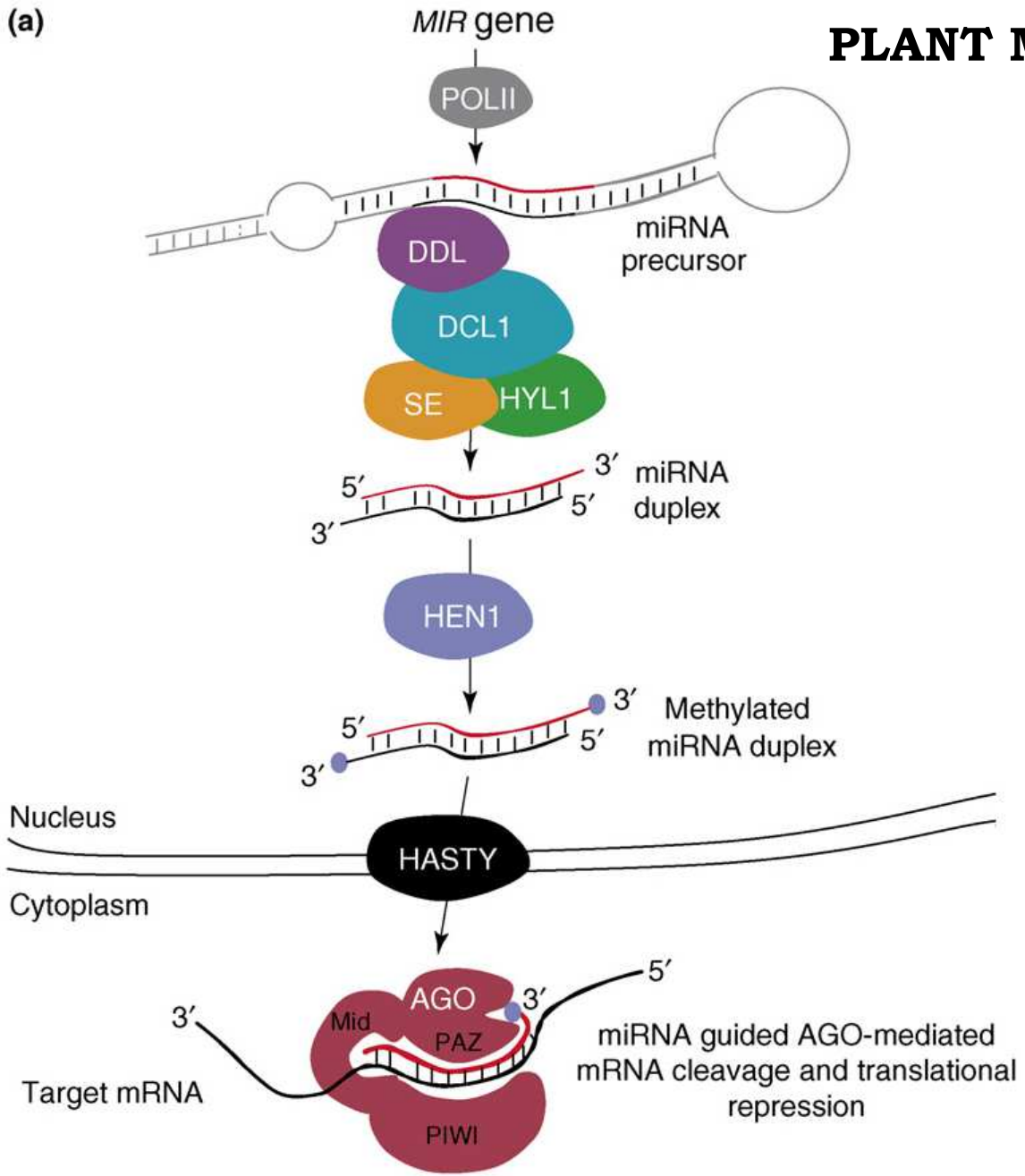
Table 1. Use of RNAi in metabolic engineering of plants

Trait	Target gene	Host plant	Potential benefit	Refs
Enzymatic browning	Polyphenyl oxidase gene	Potato	Extended storage life	[7]
Increased stearic acid and oleic acid content of seed oil	ghSAD-1 and ghFAD2-1 genes	Cotton	Useful for cooking applications without the need for hydrogenation	[13]
Reduced caffeine production	CaMxMt 1 gene	Coffee bean plant	Decaffeinated coffee	[15]
Reduced or absent petals	BP1 gene	Oilseed rape	Improved photosynthesis	[16]
Non-narcotic alkaloid production	Codeine reductase (COR) gene	Opium poppy	–[a]	[17]
Increased carotenoid and flavonoid content	DET1 gene	Tomato	Consumer health benefits	[18]
Flower colour	CHI gene	Tobacco	–[a]	[49]
Maize quality	Starch branching enzyme	Maize	Up to 50% increase in amylose content	[50]
Allergy	Lol p1 and Lol p2	Ryegrass (*Lolium* spp.)	Hypo-allergic ryegrass	[14]
Reduced ethylene sensitivity	1-Aminocyclo propane-1-carboxylate oxidase	Tomato	Longer shelf life (slower ripening)	[51]
Increased arsenic uptake	ACR2 gene	*Arabidopsis*	Phytoremediation of soils	[19]

[a]No direct benefit because these experiments were designed to demonstrate 'proof of concept'.

SILENZIAMENTO INDOTTO DA microRNA ARTIFICIALI

PLANT MICRO-RNA BIOGENESIS



Allison C Mallory, Taline Elmayan and Herve´ Vaucheret, Current Opinion in Plant Biology 2008

The Plant Cell, Vol. 18, 1121–1133, May 2006, www.plantcell.org © 2006 American Society of Plant Biologists

RESEARCH ARTICLES

Highly Specific Gene Silencing by Artificial MicroRNAs in *Arabidopsis*

Rebecca Schwab, Stephan Ossowski, Markus Riester, Norman Warthmann, and Detlef Weigel[1]
Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

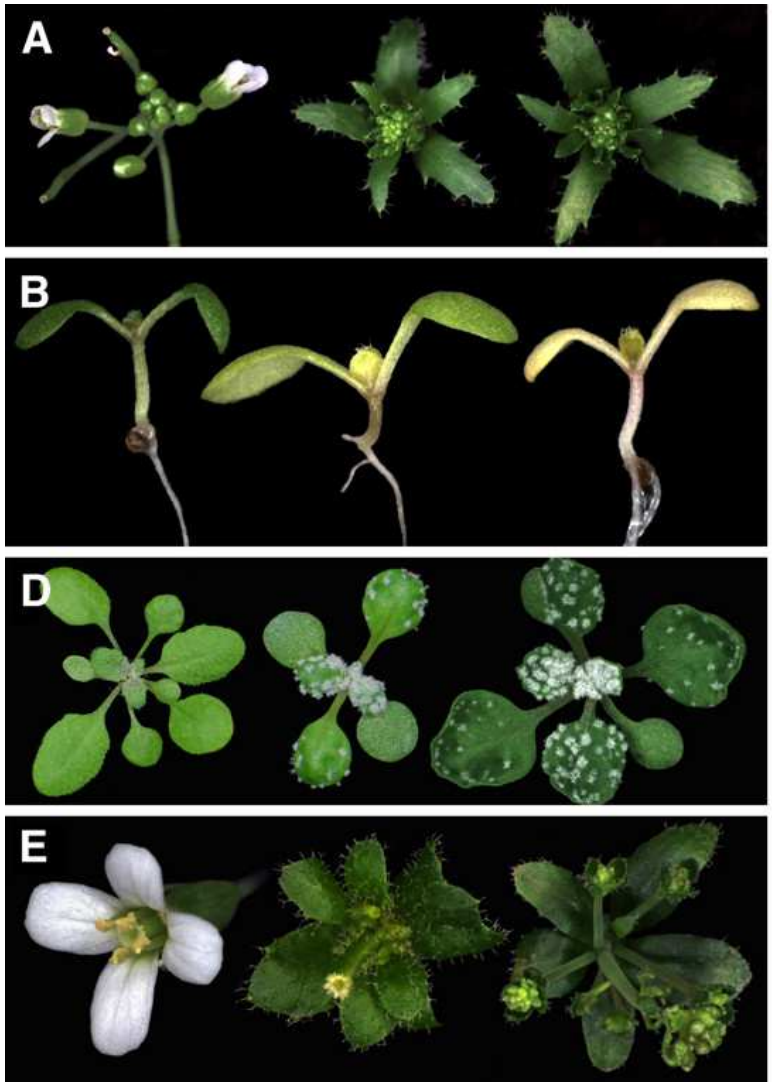
Compared with conventional RNAi, amiRNAs offer several advantages:

1) miRNA precursors generally generate only a single effective small RNA of known sequence. By contrast, several siRNAs with undefined 5’ and 3’ ends are produced as a silencing trigger from hairpin constructs. Therefore, potential off-targets of amiRNAs can be more accurately predicted than those of longer hairpin constructs.

2) because of their exquisite specificity, amiRNAs can possibly be adapted for allele-specific knockouts.

3) as with natural miRNAs, amiRNAs are likely to be particularly useful for targeting groups of closely related genes, including tandemly arrayed genes. Approximately 4000 genes in Arabidopsis are found in tandem arrays (Arabidopsis Genome Initiative, 2000), and no convenient tool exists for their knockout.

PHENOTYPES OF amiRNA OVEREXPRESSERS



(A) Inflorescences. From left to right: the wild type, lfy-12, and amiR-lfy-1 (MIR172a backbone) overexpresser.

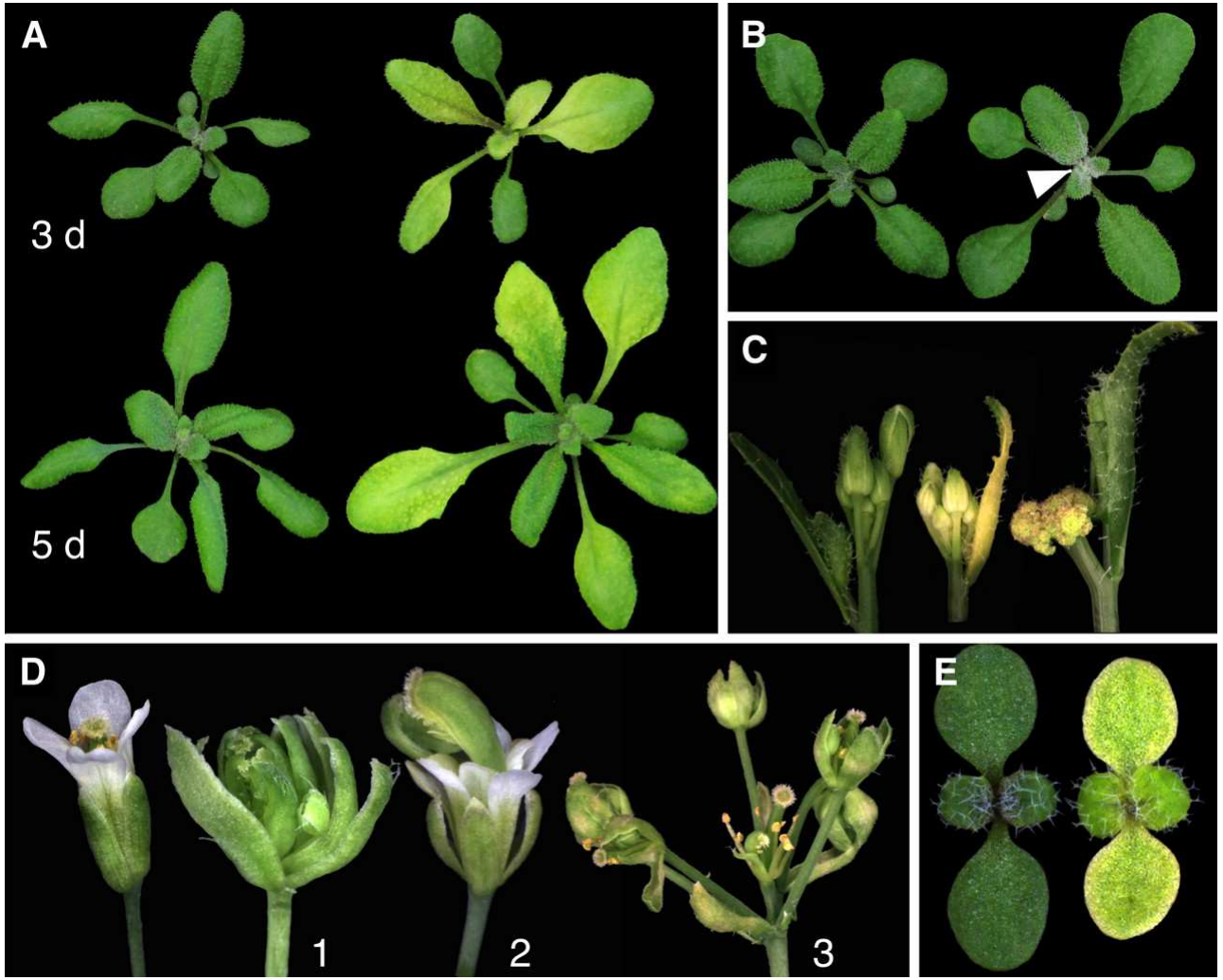
(B) Seedlings. From left to right: the wild type, gun4-1, and amiR-white-1 (MIR172a backbone) overexpresser. Bleaching of cotyledons is more pronounced in the amiR-white plants than in gun4-1, consistent with the more severe molecular profile of the amiR-white overexpressers.

(D) Leaf rosettes. From left to right: the wild type, try cpc double mutants, and amiR-trichome (MIR319a backbone) overexpresser. Clustered trichomes are evident even at low magnification.

(E) Flowers. From left to right: the wild type, weak amiR-mads-2 (MIR319a backbone) overexpresser, and strong amiR-mads-2 (MIR319a backbone) overexpresser. In the strong line, secondary inflorescences replace the central gynoecium.

Schwab R. et.al. Plant Cell 2006:18:1121-1133

INDUCIBLE AND TISSUE-SPECIFIC EXPRESSION OF AMIRNAs



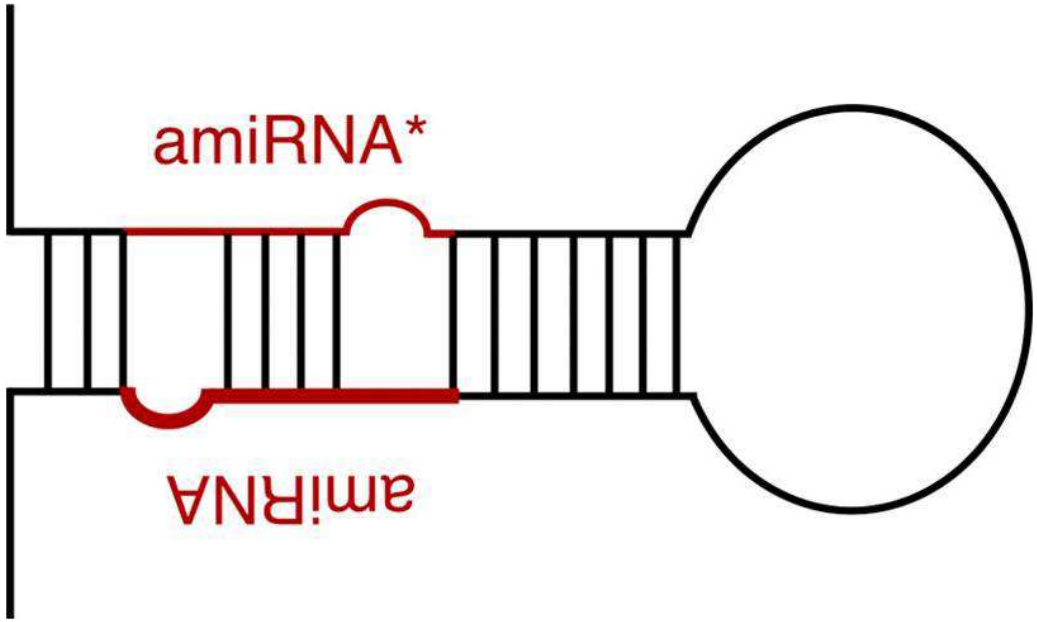
Schwab R. et.al. Plant Cell 2006:18:1121-1133

ENGINEERING OF AMIRNAs

miR319 (BACKBONE):

caaacacacgctcggacgcatattacacatgttcatacacttaatactcgctgttttgaatt
gatgttttaggaatatatatgt**agagagagcttccttgagtccattcacaggtcgtgatatgattaatta**
gcttccgactcattcatccaaataccgagtcgccaaaattcaaactagactcgttaaatgaatgaatgatgcg
gtagacaaattggatcattgattctctttgattggactgaagggagctccctctctcttttgtatccaatt
ttcttgattaatctttcctgcacaaaaacatgcttgatccactaagtgacatatatgctgcc
ttcgtatatatagttctggtaaaattaacattttgggtttatctttatttaaggcatcgcca
tg

miRNA319
miRNA319*



Schwab R. et.al. Plant Cell 2006:18:1121-1133

ENGINEERING OF AMIRNAS

http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd

ARTIFICIAL MICRORNA SELECTION CRITERIA

There are still some criteria, which have to be considered when choosing the final amiRNA. Most of them have been implemented into the ranking process, and they should be considered here again, especially when multiple genes are targeted simultaneously.
We prefer (not require):

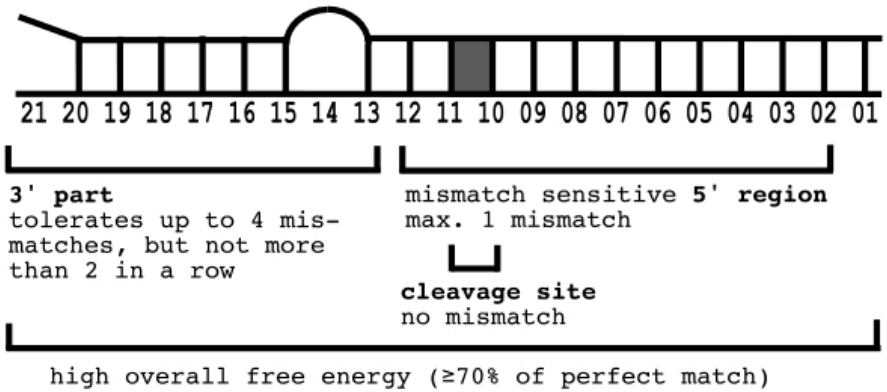
1. No mismatch between positions 2 and 12 of the amiRNA for all targets.
   Mismatches are not allowed for the target gene that is used as a template, but they might come up for additional intended targets since the target determinants allow for one mismatch.

2. One (or two) mismatches at the amiRNA 3? end (pos.18-21).
   There is no evidence for transitive formation of secondary siRNAs from amiRNA targets, but if there was, this mismatch should reduce the process.

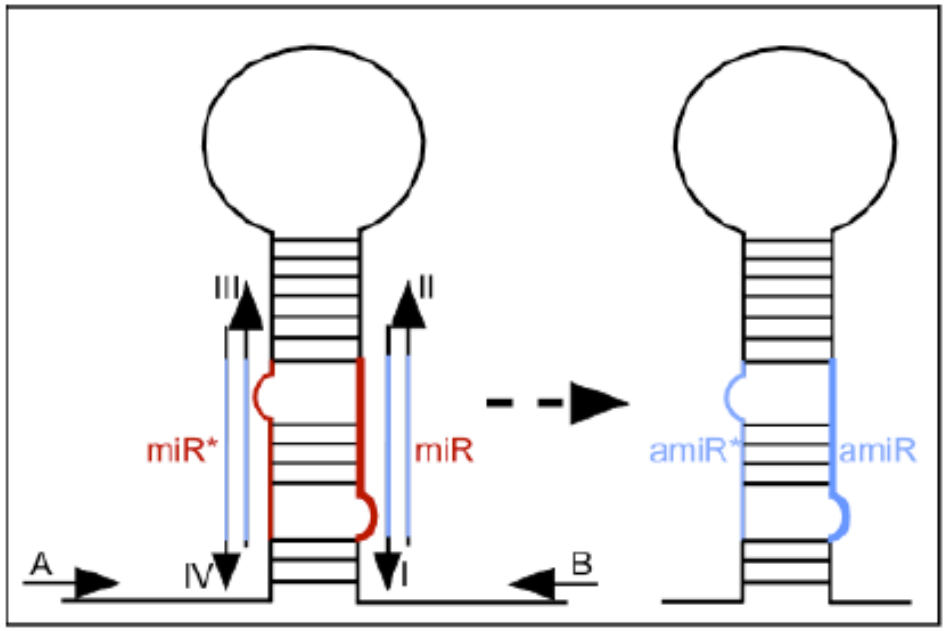
3. Similar mismatch pattern for all intended targets.
   There is no evidence that the pattern of mismatches matters, but similar patterns definitely don't hurt.

4. Absolute Hybridization energy between -35 and -38 kcal/mole.
   These are the values observed for most endogenous miRNA targets. We don?t consider amiRNAs which pair to intended targets with energies higher than -30 kcal/mole.

5. Target site position.
   There is no evidence that the position of the target site in the target transcript has an effect on effectiveness, but target sites in most endogenous miRNA targets are found towards the 3' end of the coding regions. Examples in the 3'UTR are also not uncommon.



CLONING STRATEGY



I: microRNA forward
II: microRNA reverse
III: microRNA* forward
IV: microRNA* reverse

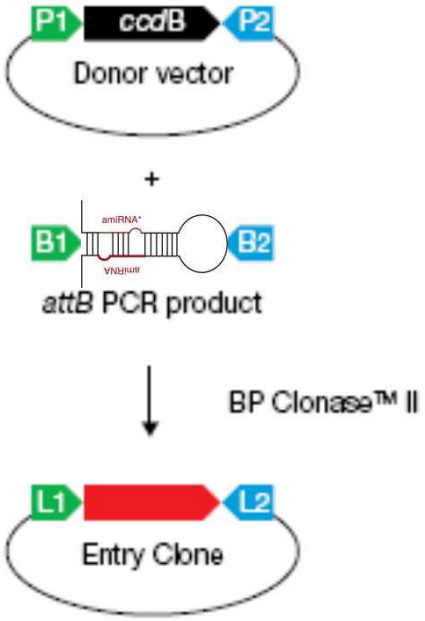
	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

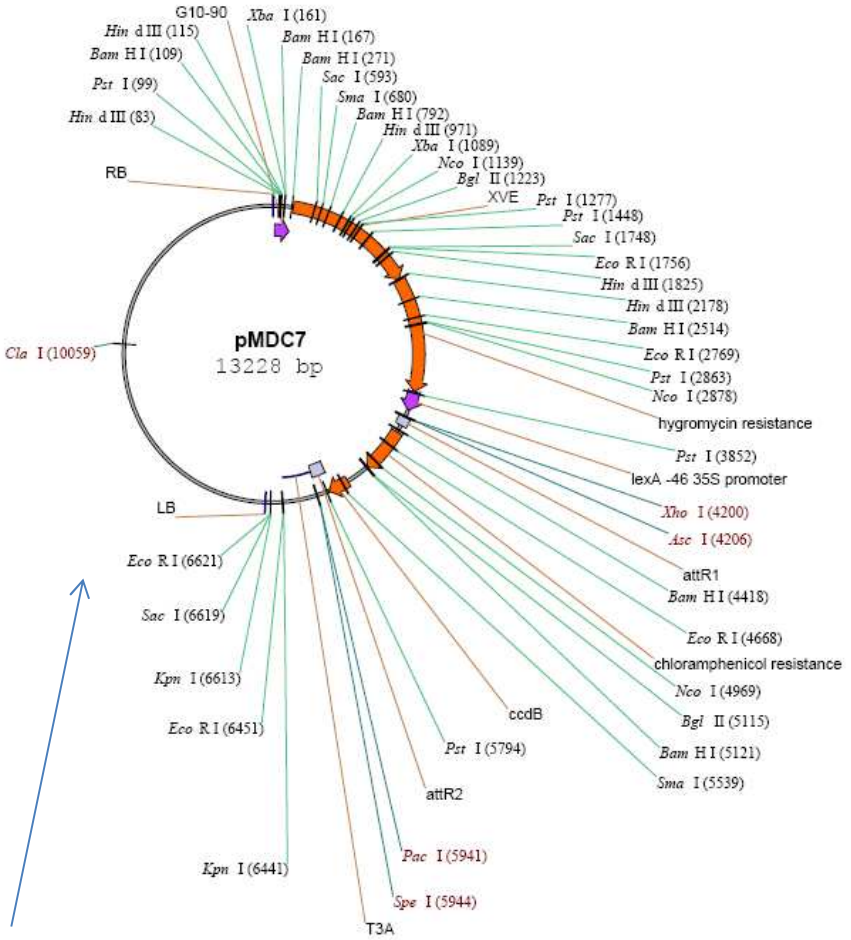
Schwab R. et.al. Plant Cell 2006:18:1121-1133

DNA CLONING USING IN VITRO SITE-SPECIFIC RECOMBINATION

The Gateway reactions:

1)





2)

The Plant Cell, Vol. 14, 1109–1120, May 2002, www.plantcell.org © 2002 American Society of Plant Biologists

An Arabidopsis Mitogen-Activated Protein Kinase Kinase Kinase Gene Family Encodes Essential Positive Regulators of Cytokinesis

Patrick J. Krysan,[1,2] Peter J. Jester, Jennifer R. Gottwald, and Michael R. Sussman

Biotechnology Center, University of Wisconsin–Madison, 425 Henry Mall, Madison, Wisconsin 53706

Hormone Sensitivity

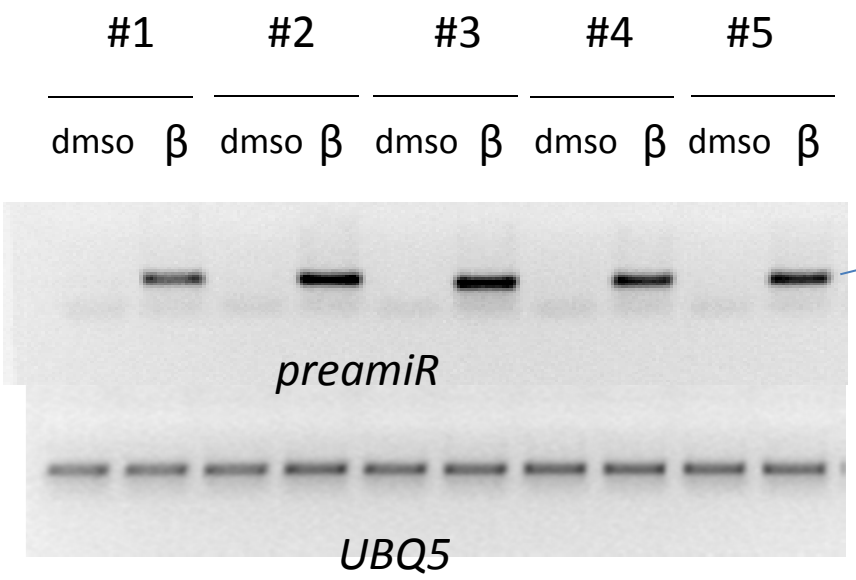
The results described above demonstrate that the *ANP* genes are involved in the control of cellular growth and division. Therefore, we tested the sensitivity of the *anp2 anp3* plants to the phytohormones abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, and gibberellin using agar plate assays. Vertically oriented plates were grown either in the dark for 3 days or in constant light for 1 week. During growth in the light, the plants were observed daily. None of these exogenous hormone treatments "rescued" the mutant phenotype of the double mutants. In addition, all of the mutants displayed a level of sensitivity to each hormone treatment that was equivalent to that of the wild type (i.e., the degree of growth inhibition or stimulation was the same; data not shown). Furthermore, we observed no qualitative changes in growth that were unique to the mutant genotypes under the various hormone treatments.

Genome-Wide Gene Expression Analysis

To gain additional insight into the signaling pathways affected by the *ANP* mutations, we used the Affymetrix Arabidopsis Gene Chip to compare the RNA levels for 8200 genes in *anp2 anp3* double-mutant plants and wild-type plants. Plants were grown in soil for 11 days under constant light, and the aerial tissue then was harvested and used for RNA analysis. Overall, RNA levels increased threefold or greater for 211 genes, whereas levels decreased threefold or greater for 30 genes (see supplemental material at www.biotech.wisc.edu/krysan/). The most striking result from the gene chip experiment was that a number of pathogen- and stress-related genes were upregulated in the *anp2 anp3* plants. These genes include numerous disease-resistance genes, chitinases, glucanases, peroxidases, glutathione *S*-transferases, and several heat shock–related genes

TRANSGENIC PLANTS SELECTON AND ANALYSIS

Hygromycin resistance



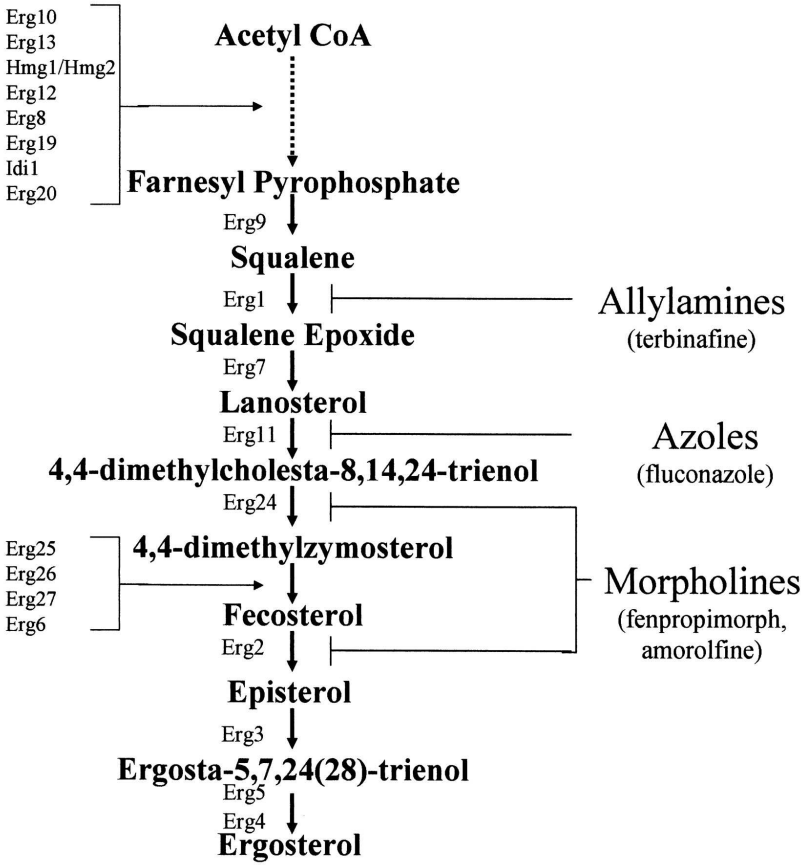
miR PRECURSOR (miR319 BACKBONE):

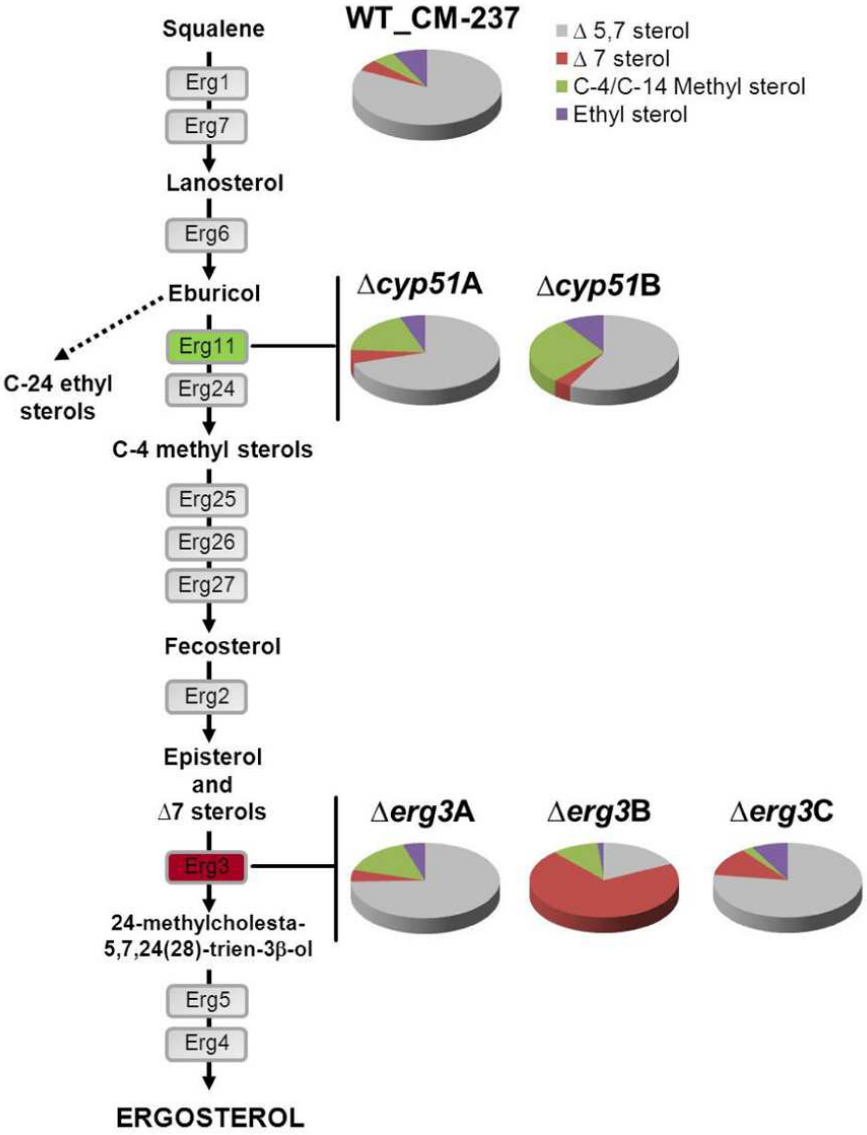
CAAACACACGCTCGGACGCATATTACACATGTTC
ATACACTTAATACTCGCTGTTTTGAATTGATGTTTT
AGGAATATATATGT**AGCAAGTAGTCGTGATTTGA**
ATTTCACAGGTCGTGATATGATTCAATTAGCTT
CCGACTCATTCATCCAAATACCGAGTCGCCAAA
ATTCAAACTAGACTCGTTAAATGAATGAATGAT
GCGGTAGACAAATTGGATCATTGATTCTCTTTG
ATATTCAATTCACGACTACCTGCTCTCTTTTGTA
TTCCAATTTTCTTGATTAATCTTTCCTGCACAAAAA
CATGCTTGATCCACTAAGTGACATATATGCTGCC
TTCGTATATATAGTTCTGGTAAAATTAACATTTTG
GGTTTATCTTTATTTAAGGCATCGCCATG

amiR * - CAAGTAGTCGTGATTTGAATT
amiR – TATTCAATTCACGACTACCTG

INDUCIBILE PTGS

SILENZIAMENTO DI GENI DI PATOGENI INDOTTO DA RNAi IN PIANTA


Erg10
Erg13
Hmg1/Hmg2
Erg12
Erg8
Erg19
Idi1
Erg20
Acetyl CoA
Farnesyl Pyrophosphate
Erg9
Squalene
Erg1
Squalene Epoxide
Erg7
Lanosterol
Erg11
4,4-dimethylcholesta-8,14,24-trienol
Erg24
Erg25
Erg26
Erg27
Erg6
4,4-dimethylzymosterol
Fecosterol
Erg2
Episterol
Erg3
Ergosta-5,7,24(28)-trienol
Erg5
Erg4
Ergosterol
Allylamines
(terbinafine)
Azoles
(fluconazole)
Morpholines
(fenpropimorph,
amorolfine)


Squalene
WT_CM-237
Δ 5,7 sterol
Δ 7 sterol
C-4/C-14 Methyl sterol
Ethyl sterol
Erg1
Erg7
Lanosterol
Erg6
Eburicol
Δcyp51A
Δcyp51B
C-24 ethyl
sterols
Erg11
Erg24
C-4 methyl sterols
Erg25
Erg26
Erg27
Fecosterol
Erg2
Episterol
and
Δ7 sterols
Δerg3A
Δerg3B
Δerg3C
Erg3
24-methylcholesta-
5,7,24(28)-trien-3β-ol
Erg5
Erg4
ERGOSTEROL

A)

Clone sequences of CYP51A (294nt)

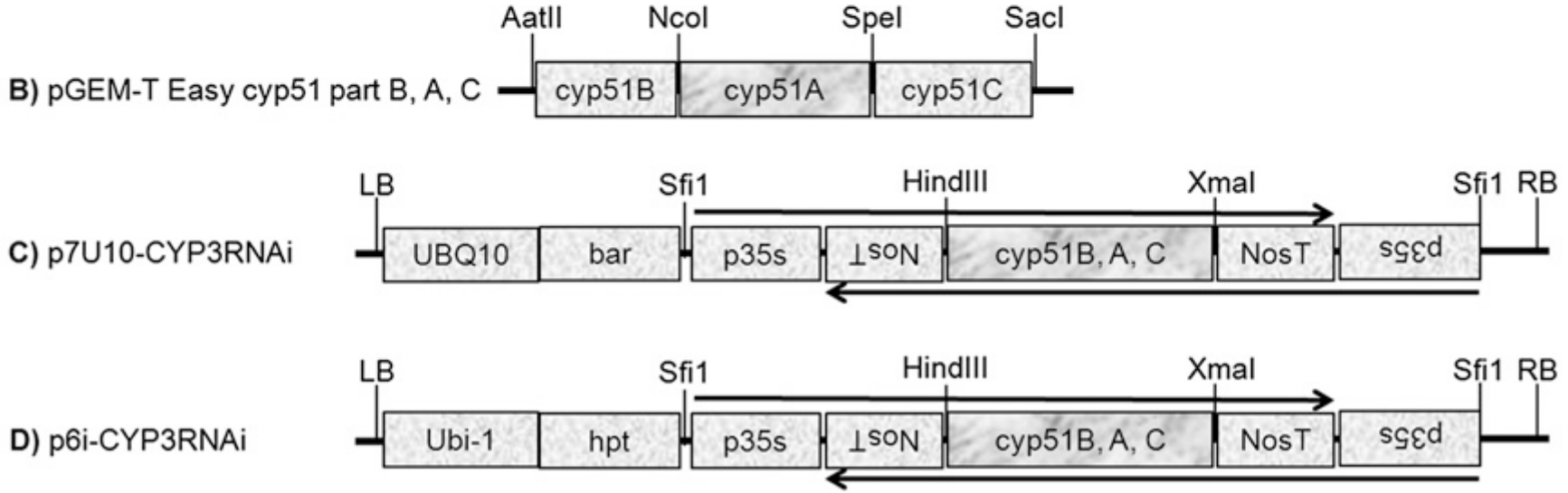
CGGTCCATTGACAATCCCCGTCTTTGGTAGCGATGTCGTATACGATTGTCCCAACTCGAAGCTCATGGAACAAAAGAAGT
TTGTCAAGTTTGGCCTTACGCAAAAAGCACTCGAGTCACACGTCCAGTTAATCGAGCGAGAGGTTCTTGACTACGTCGA
AACTGATCCATCCTTTTCTGGCAGAACTAGCACCATCGATGTCCCCAAGGCAATGGCTGAGATAACAATCTTTACTGCCT
CACGTTCTTTGCAGGGTGAGGAAGTTCGGAGAAAACTCACTGCCGAGTTTGCTGC

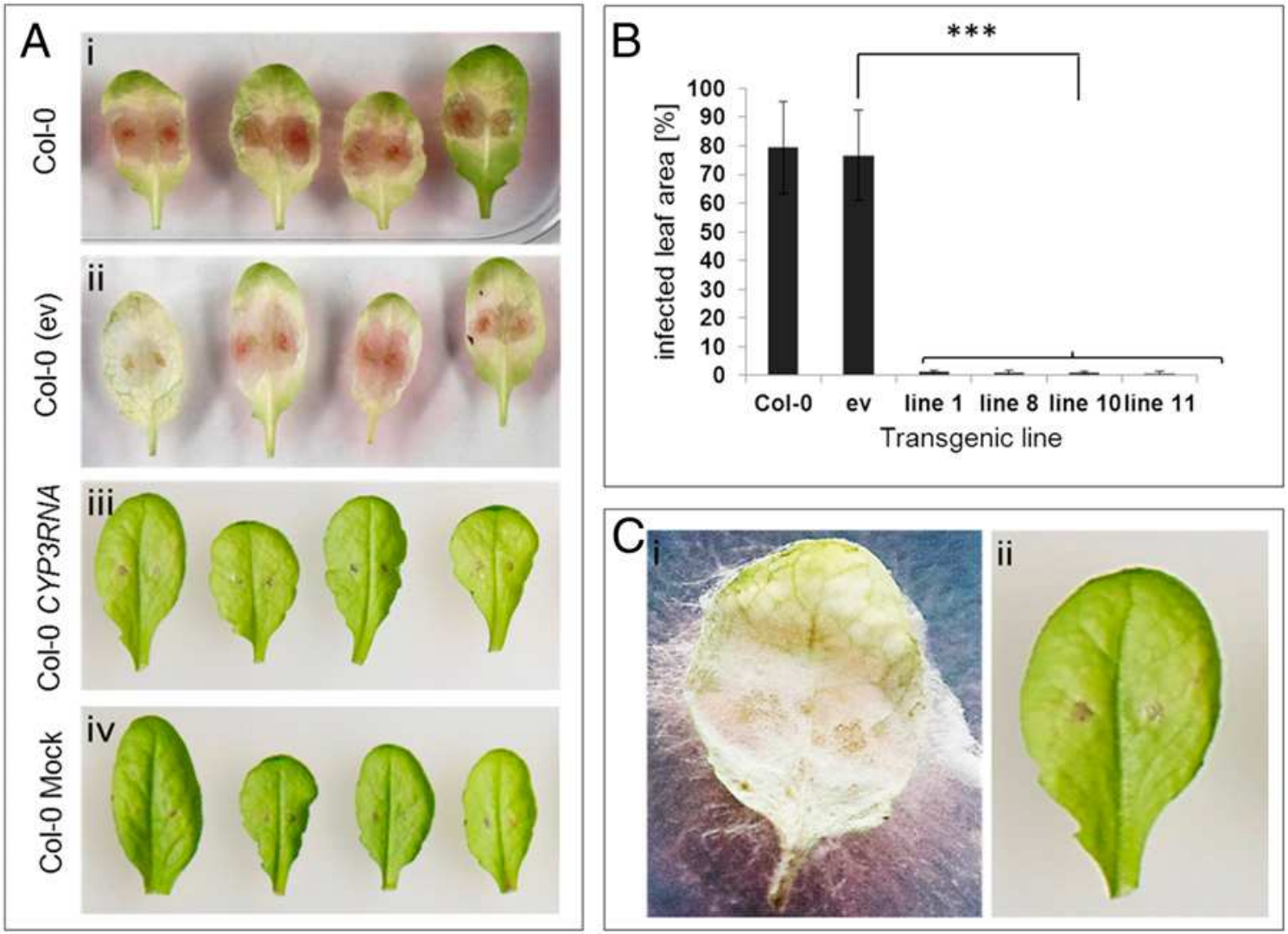
Clone sequences of CYP51B (220nt)

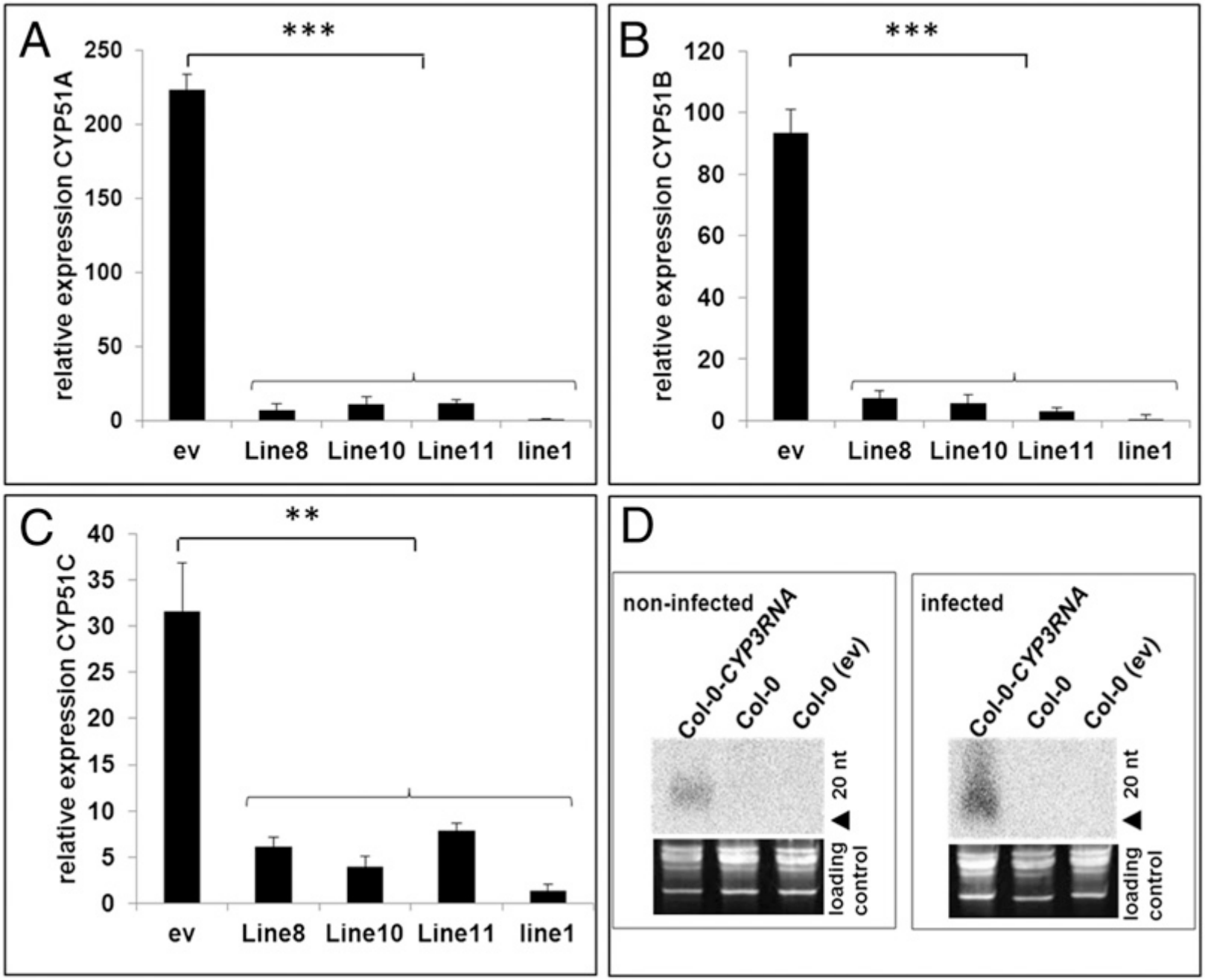
CAGCAAGTTTGACGAGTCCCTGGCCGCTCTCTACCACGACCTCGATATGGGCTTCACCCCCATCAACTTCATGCTTCAC
TGGGCCCCTCTCCCCTGGAACCGTAAGCGCGACCACGCCCAGCGCACTGTTGCCAAGATCTACATGGACACTATCAAG
GAGCGCCGCGCCAAGGGCAACAACGAATCCGAGCATGACATGATGAAGCACCTTATGAACTCT

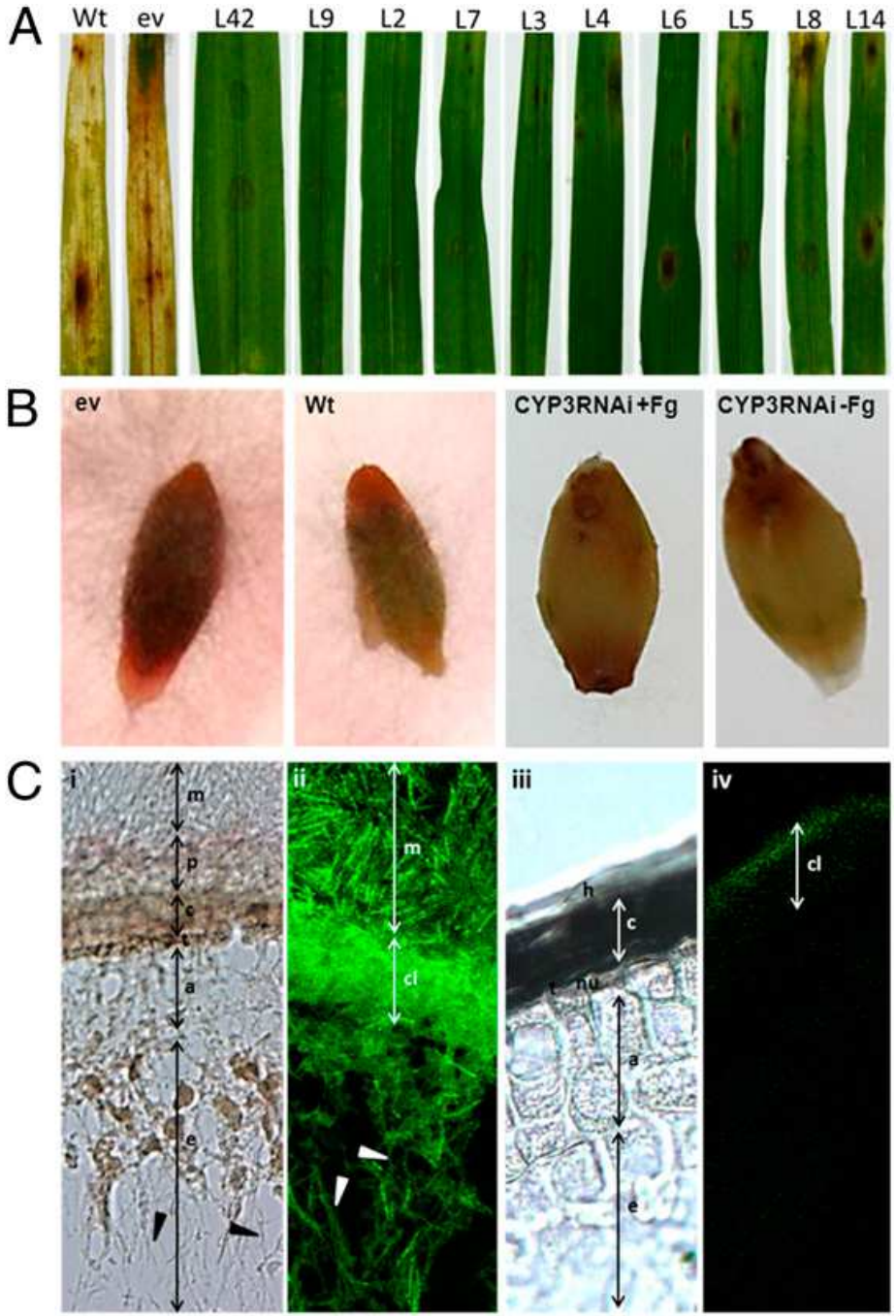
Clone sequences of CYP51C (238nt)

ATTGGAAGCACCGTACAATATGGCATCGACCCGTACGCTTTTTTCTTCGACTGCAGAGATAAATACGGCGACTGCTTTAC
CTTTATTCTCCTTGGCAAATCAACGACTGTCTTTCTTGGTCCCAAGGGCAATGACTTTATCCTCAACGGCAAACACGCCG
ATCTCAACGCCGAGGACGTTTATGGGAAACTTACCACGCCCGTGTTTGGTGAGGAGGTTGTTTATGACTGCTCCAATG




A
i
Col-0
ii
Col-0 (ev)
iii
Col-0 CYP3RNA
iv
Col-0 Mock
B
***
infected leaf area [%]
100
90
80
70
60
50
40
30
20
10
0
Col-0
ev
line 1
line 8
line 10
line 11
Transgenic line
C
i
ii


A
***
relative expression CYP51A
250
200
150
100
50
0
ev
Line8
Line10
Line11
line1
B
***
relative expression CYP51B
120
100
80
60
40
20
0
ev
Line8
Line10
Line11
line1
C
**
relative expression CYP51C
40
35
30
25
20
15
10
5
0
ev
Line8
Line10
Line11
line1
D
non-infected
Col-0-CYP3RNA
Col-0
Col-0 (ev)
20 nt
loading control
infected
Col-0-CYP3RNA
Col-0
Col-0 (ev)
20 nt
loading control


A
Wt
ev
L42
L9
L2
L7
L3
L4
L6
L5
L8
L14
B
ev
Wt
CYP3RNAi+Fg
CYP3RNAi-Fg
C
i
m
p
c
t
a
e
ii
m
cl
iii
h
c
t
hu
a
e
iv
cl

RESEARCH ARTICLE

An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery

Aline Koch[1], Dagmar Biedenkopf[1], Alexandra Furch[2], Lennart Weber[3], Oliver Rossbach[4], Eltayb Abdellatef[1], Lukas Linicus[1], Jan Johannsmeier[1], Lukas Jelonek[5], Alexander Goesmann[5], Vinitha Cardoza[6], John McMillan[6], Tobias Mentzel[7], Karl-Heinz Kogel[1]*

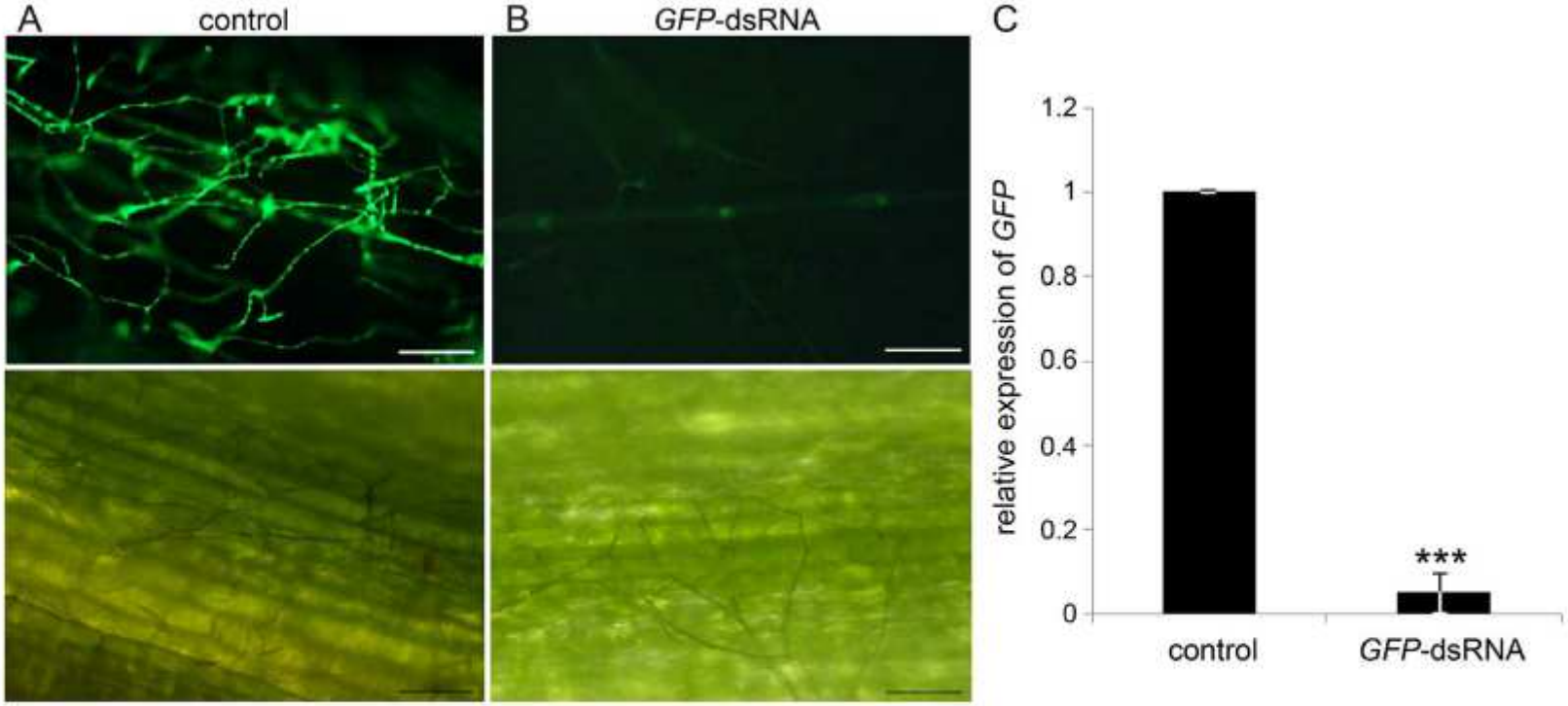


Fig 1. (A-C) Spray-induced gene silencing (SIGS) of *GFP* expression in *Fusarium graminearum* strain Fg-IFA65$_{GFP}$. Detached second leaves of three-week-old barley plants were locally sprayed with Tris-EDTA (TE, **A**, control) or *GFP*-dsRNA **(B)**. Forty-eight hours after spraying, distal, non-sprayed leaf segments were drop-inoculated with Fg-IFA65$_{GFP}$ (20 μL of a solution containing 2 x 10^4 conidia mL^{-1}). *GFP* silencing efficiency was visualized 6 dpi using confocal microscopy. **(C)** *GFP* transcripts were quantified by qPCR at 6 dpi. The reduction in fungal *GFP* expression on leaves sprayed with *GFP*-dsRNA and infected with Fg-IFA65$_{GFP}$ compared with TE-sprayed controls was statistically significant (***$P < 0.001$; Student´s *t* test). Bars represent mean values ± SDs of three independent experiments. Scale bars represent 100 μm.

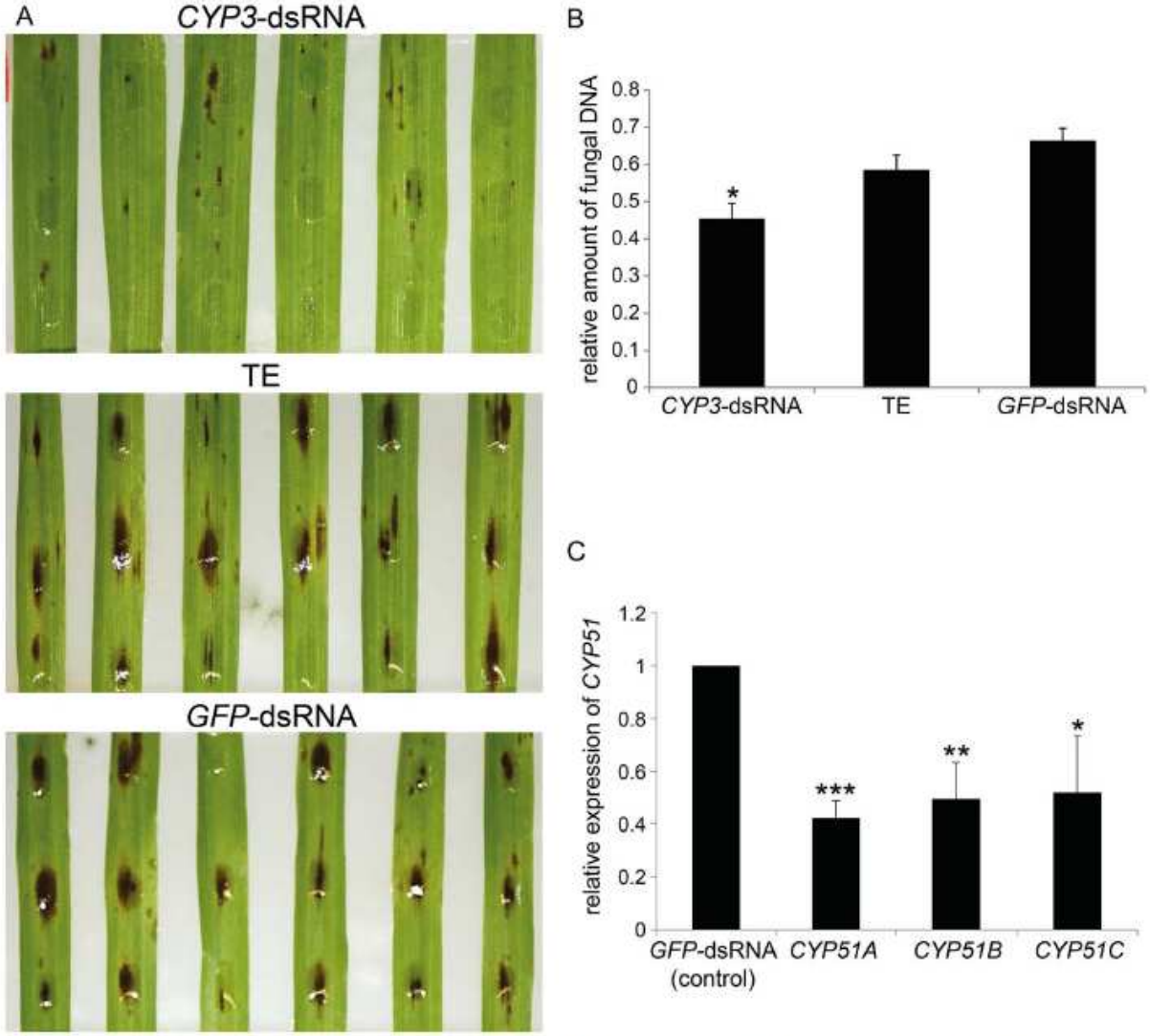


Fig 2. (A-C) SIGS-mediated control of *F. graminearum* on leaves sprayed with *CYP3*-dsRNA. (A) Detached second leaves of three-week-old barley were sprayed evenly with *CYP3*-dsRNA, TE (mock control), and *GFP*-dsRNA (negative control), respectively. After 48 hours, leaves were drop-inoculated with 2×10^4 conidia mL^{-1} of Fg-IFA65 onto the sprayed area and evaluated for necrotic lesions at 6 dpi. **(B)** The relative amount of fungal DNA at 6 dpi as measured by qPCR was reduced in *CYP3*-dsRNA-treated leaves compared to control leaves. Bars represent mean values ± SDs of three independent experiments. The reduction of fungal growth on *CYP3*-dsRNA vs. TE- or *GFP*-dsRNA-sprayed leaves was statistically significant (*$P < 0.05$; Student´s t test). **(C)** Gene-specific qPCR analysis of fungal *CYP51A*, *CYP51B*, and *CYP51C* transcripts at 6 dpi (corresponding to 8 d after spraying). The reduction in fungal *CYP51* gene expression on *CYP3*-dsRNA-sprayed leaves as compared with *GFP*-dsRNA-sprayed controls was statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student´s t test).

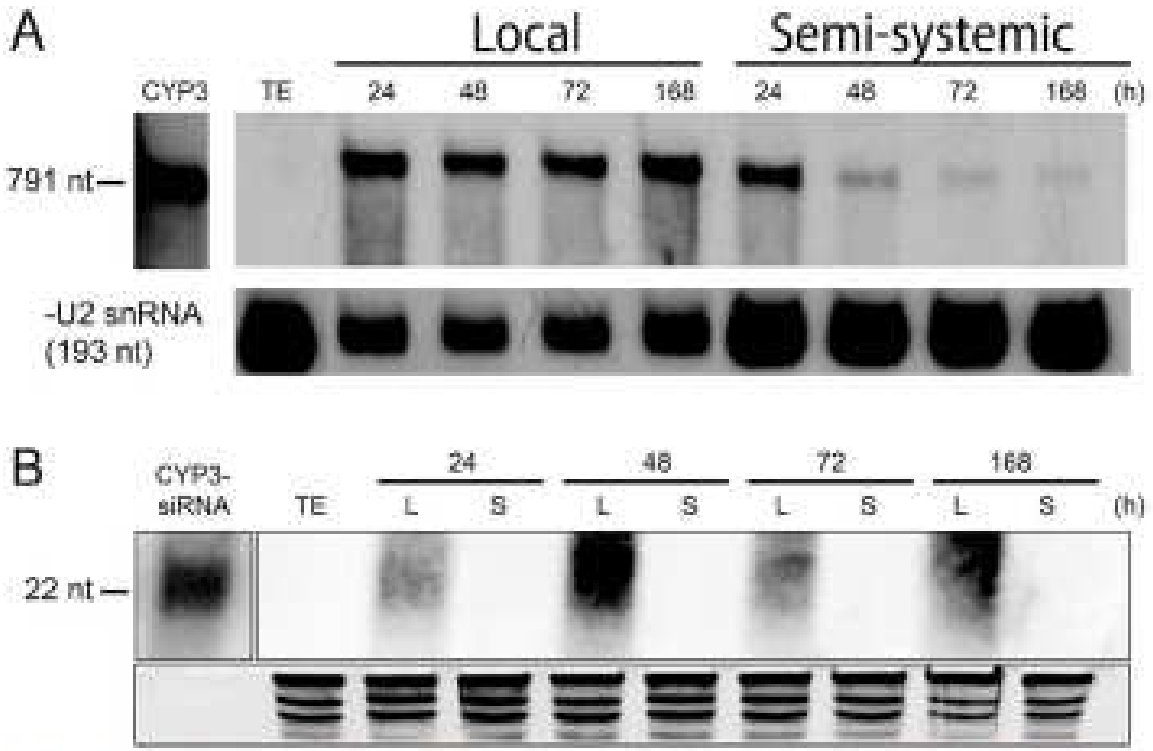


Fig 4. (A,B) Northern gel blot analysis of *CYP3*-dsRNA and *CYP3*-dsRNA-derived siRNA accumulation in local and distal (semi-systemic) barley leaf areas. **(A)** Detection of 791 nt long *CYP3*-dsRNA precursor in pooled leaf tissue from non-infected leaves using [α-32P]-dCTP labeled *CYP3*-dsRNA as probe. Local (L) and distal (semi-systemic [S]) leaf segments were sampled separately at the indicated times after spraying with *CYP3*-dsRNA. No signal was detected in samples from TE-sprayed plants. **(B)** Recording *CYP3*-dsRNA-derived small RNAs in local and distal (semi-systemic) leaf areas using [α-32P]-dCTP labeled *CYP3*-dsRNA as probe. In this experiment, small RNAs could not be detected in distal (non-sprayed) tissues. siRNA generated *in vitro* by a commercial Dicer preparation from *CYP3*-dsRNA was used as positive control. No signal was detected in samples from TE-sprayed plants. Ethidium bromide-stained rRNA served as the loading control. Signals originate from the same membrane but different exposure times.