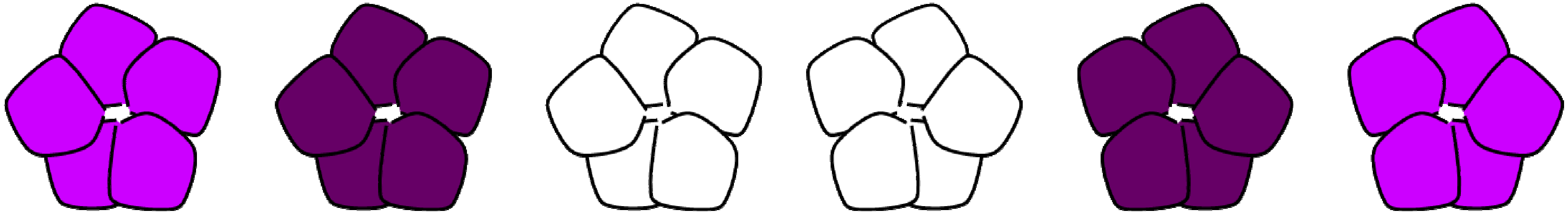
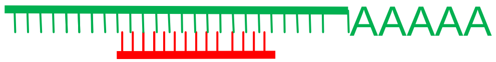
Small RNAs



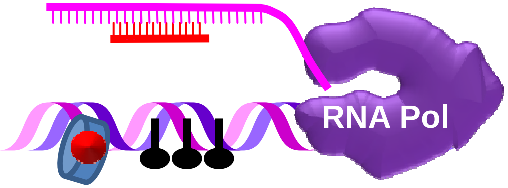
What are small RNAs?

- 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

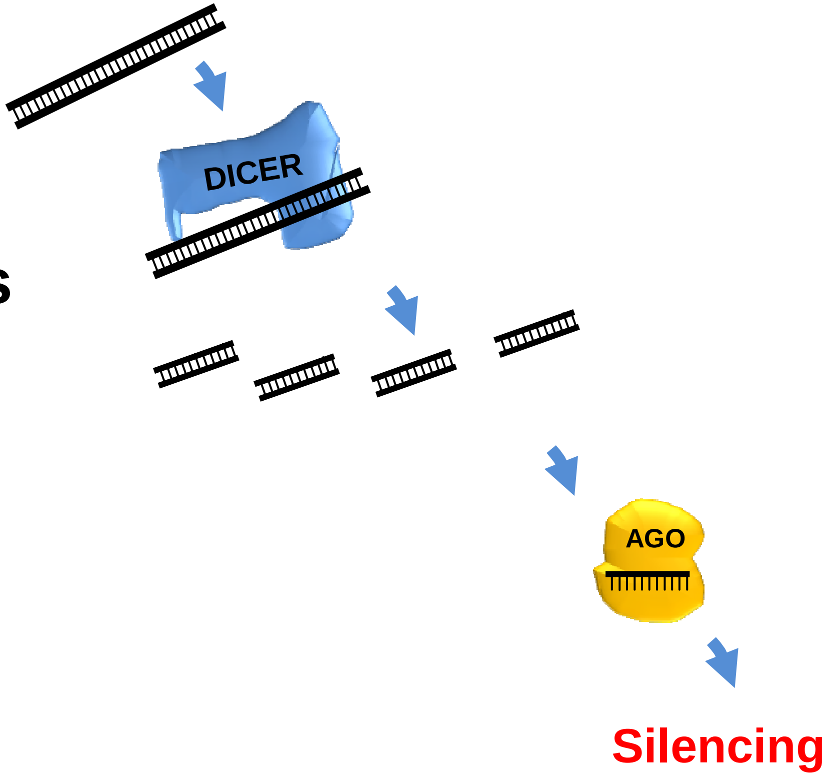


Histone modification, DNA methylation

The core of RNA silencing: Dicers and Argonautes

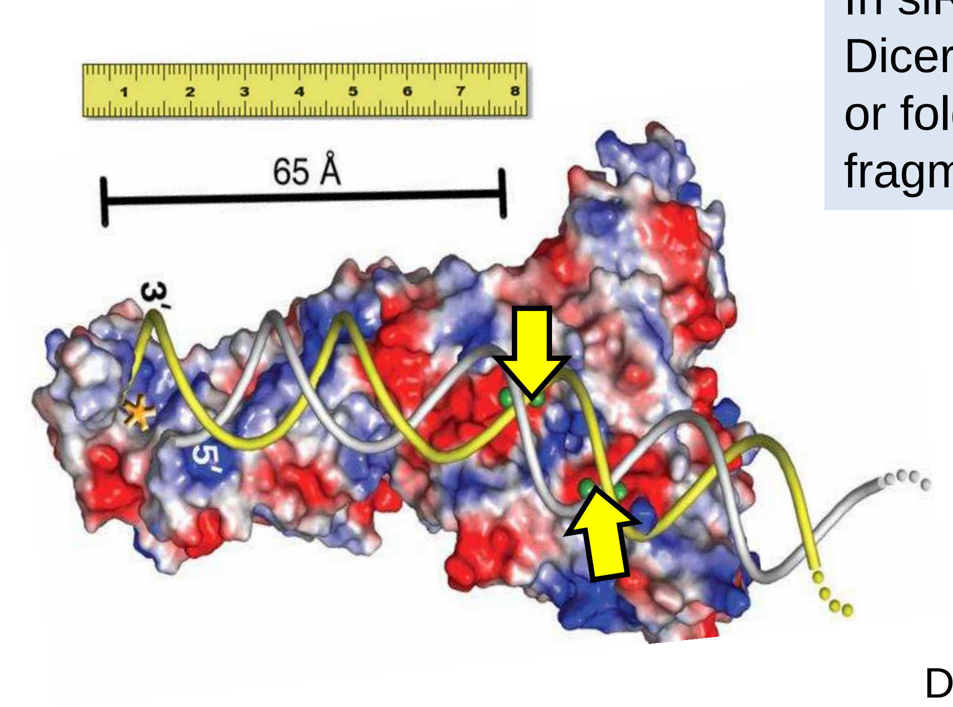
RNA silencing uses a set of core reactions in which **double-stranded RNA (dsRNA)** is processed by **Dicer** or **Dicer-like proteins** into **short RNA duplexes**.

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.

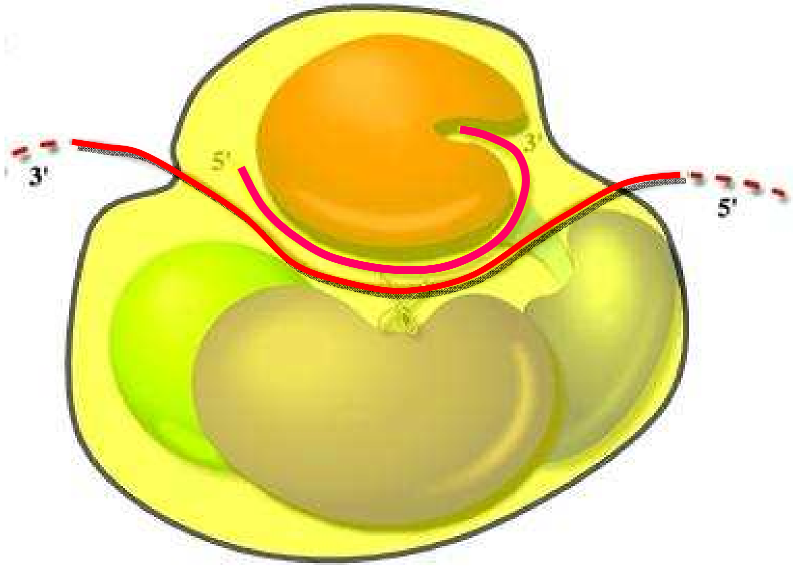


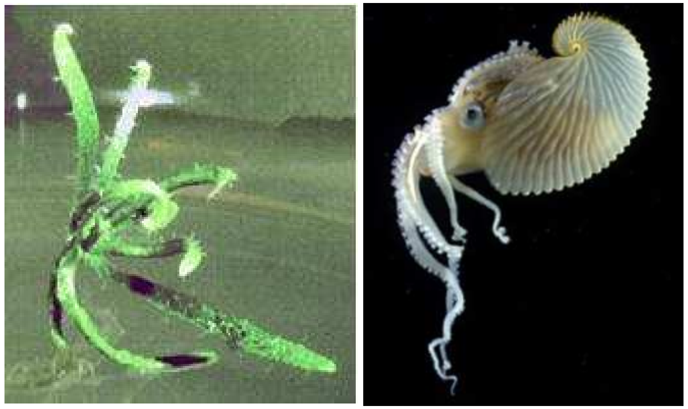
Dicer’s structure allows it to measure the RNA it is cleaving. Like a cook who “dices” a carrot, Dicer chops RNA into uniformly-sized pieces.

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.





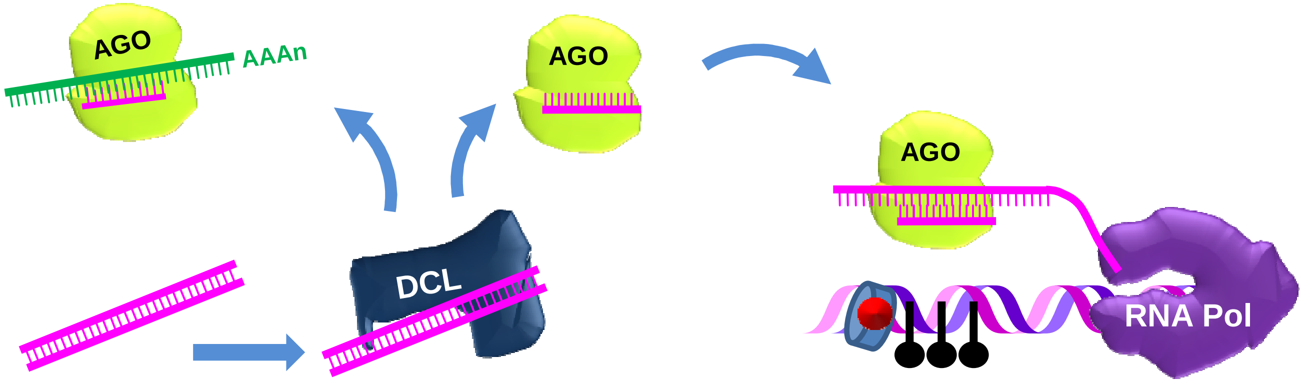
The Arabidopsis *ago1* mutant and the octopus *Argonauta argo*

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.

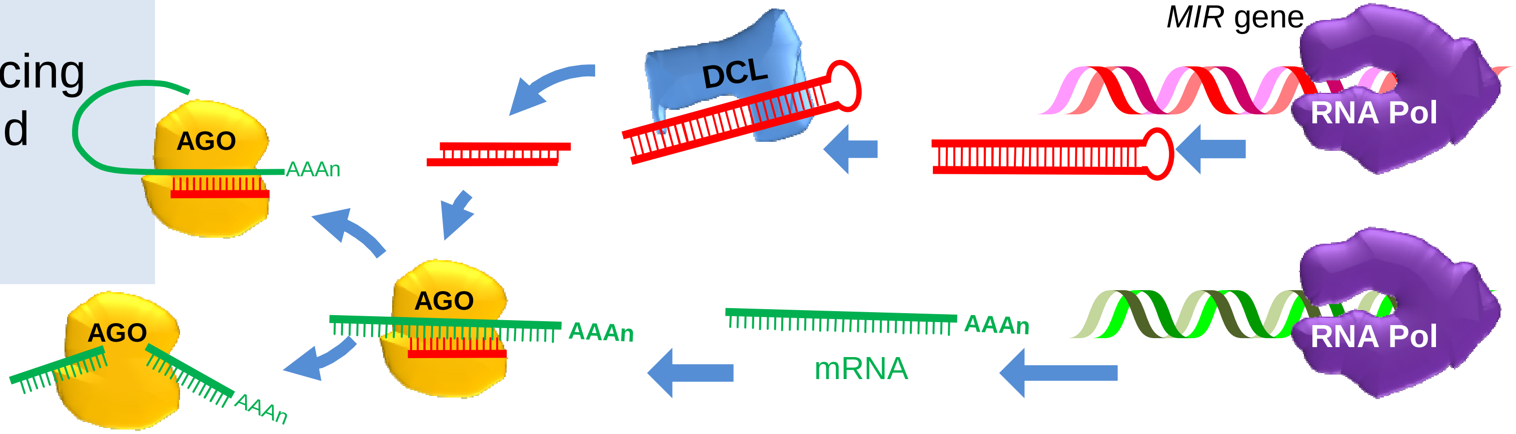
Reprinted by permission from Macmillan Publishers Ltd: EMBO J. Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J. 17: 170–180. Copyright 1998; Reprinted from Song, J.-J., Smith, S.K., 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

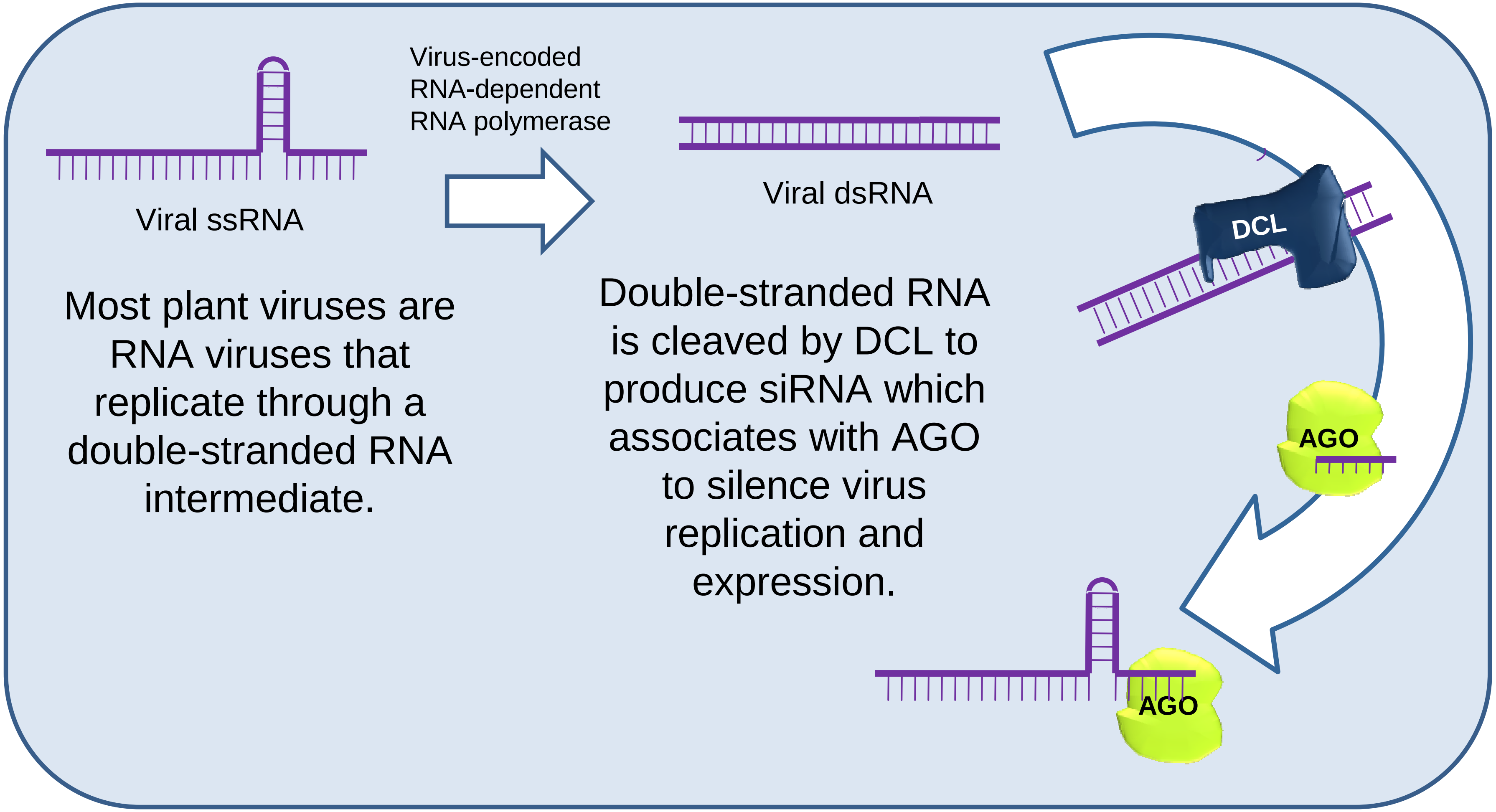
siRNAs protect the genome by

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

siRNAs also maintain some genes in an epigenetically silent state

Reprinted by permission from Macmillan Publishers, Ltd: Nature. Lam, E., Kato, N., and Lawton, M. (2001) Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411: 848-853.
Copyright 2001.

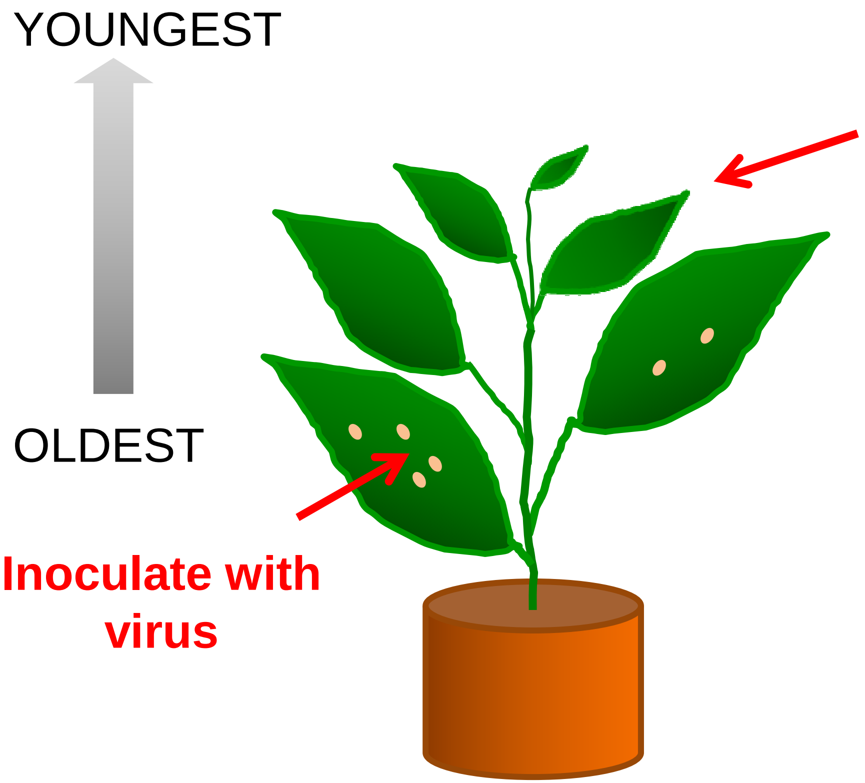
Viral induced gene silencing - overview



Most plant viruses are RNA viruses that replicate through a double-stranded RNA intermediate.

Double-stranded RNA is cleaved by DCL to produce siRNA which associates with AGO to silence virus replication and expression.

Plants can recover from viral infection and become resistant



Inoculate with virus

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

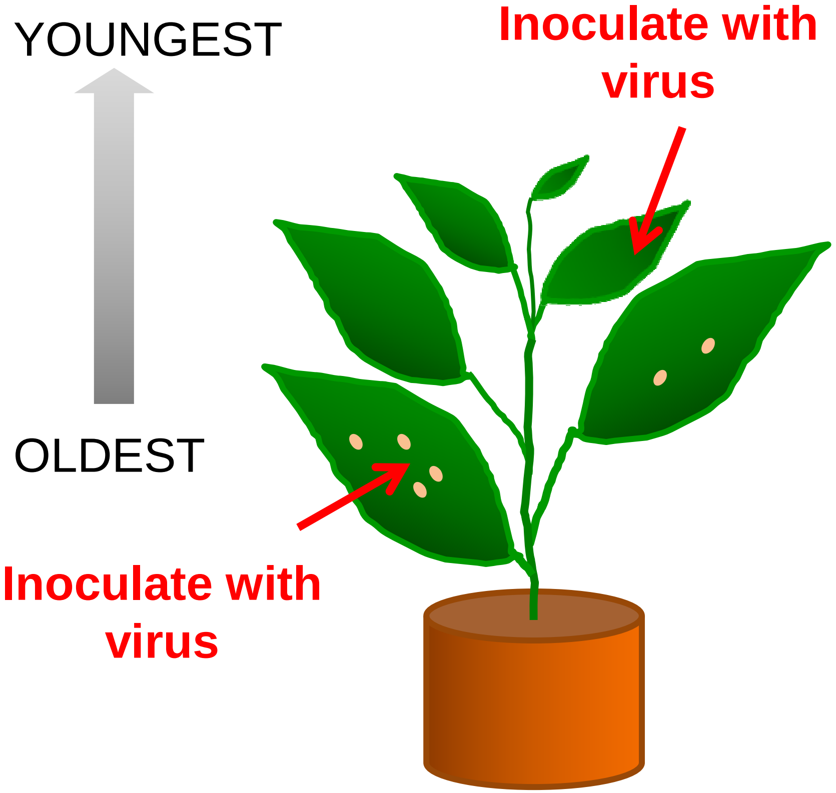
Virus-induced cell death

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


YOUNGEST

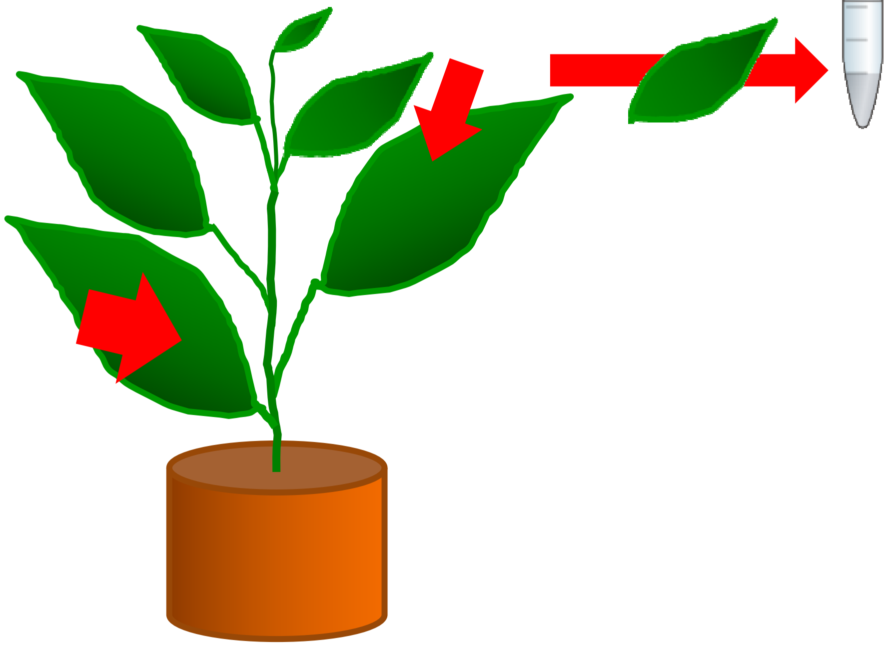
OLDEST

Inoculate with virus

Inoculate with virus

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

Viral resistance involves siRNA-mediated silencing

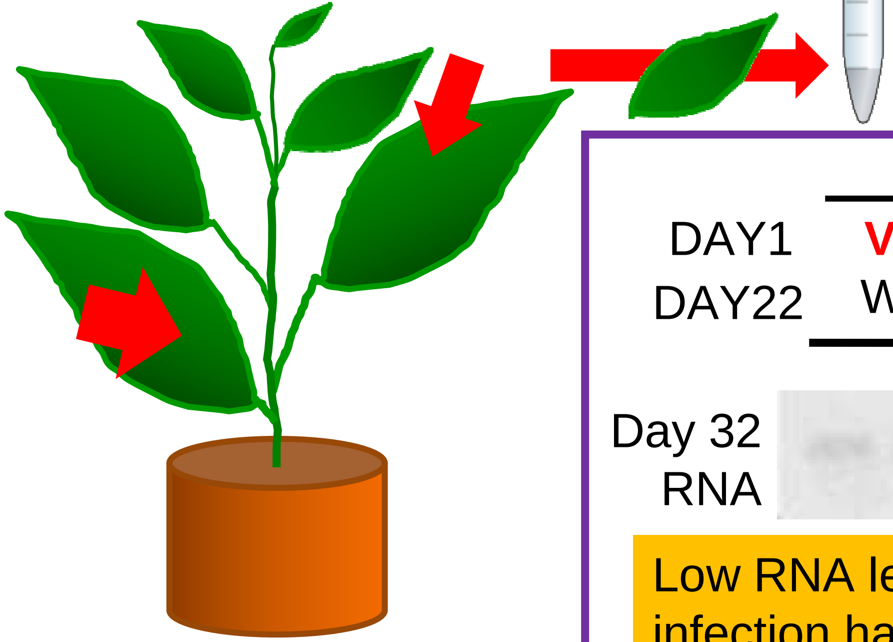


DAY 22 – Inoculate younger leaf with virus or water

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

DAY 1 – Inoculate leaf with virus or water

Viral resistance involves siRNA-mediated silencing



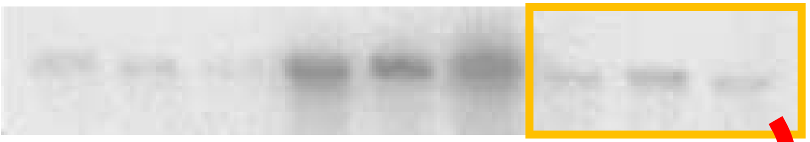
DAY 1 – Inoculate leaf with virus or water

DAY 22 – Inoculate younger leaf with virus or water

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

	Inoculum		
DAY1	**Virus**	Water	**Virus**
DAY22	Water	**Virus**	**Virus**

Day 32 RNA

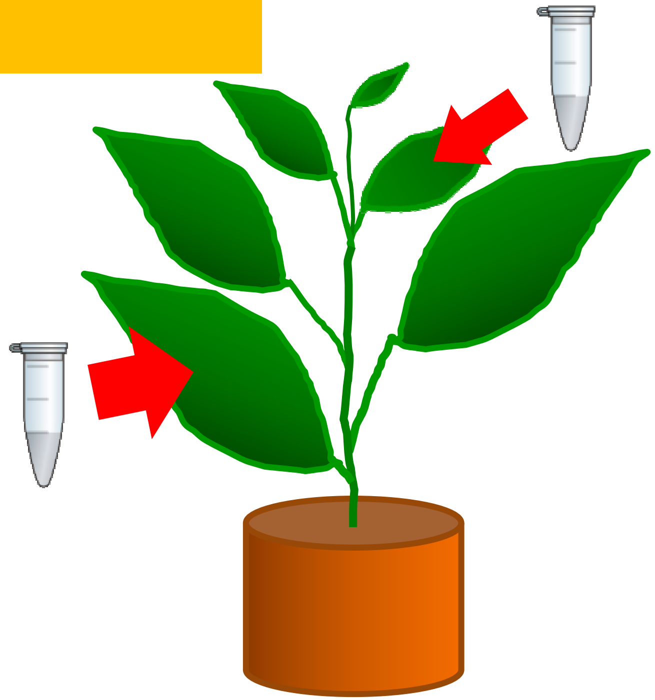


Low RNA levels show that the first viral infection has induced **RNA silencing**, preventing subsequent viral replication.

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.

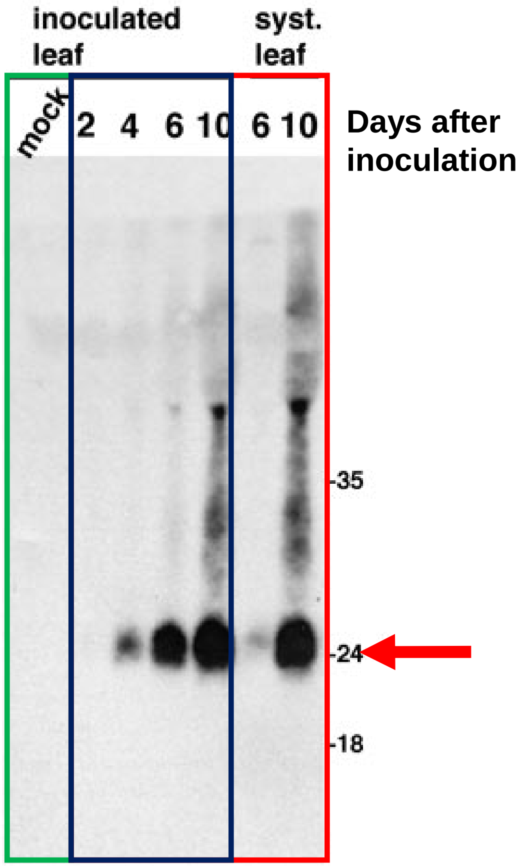
Small RNAs are correlated with viral-induced gene silencing

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



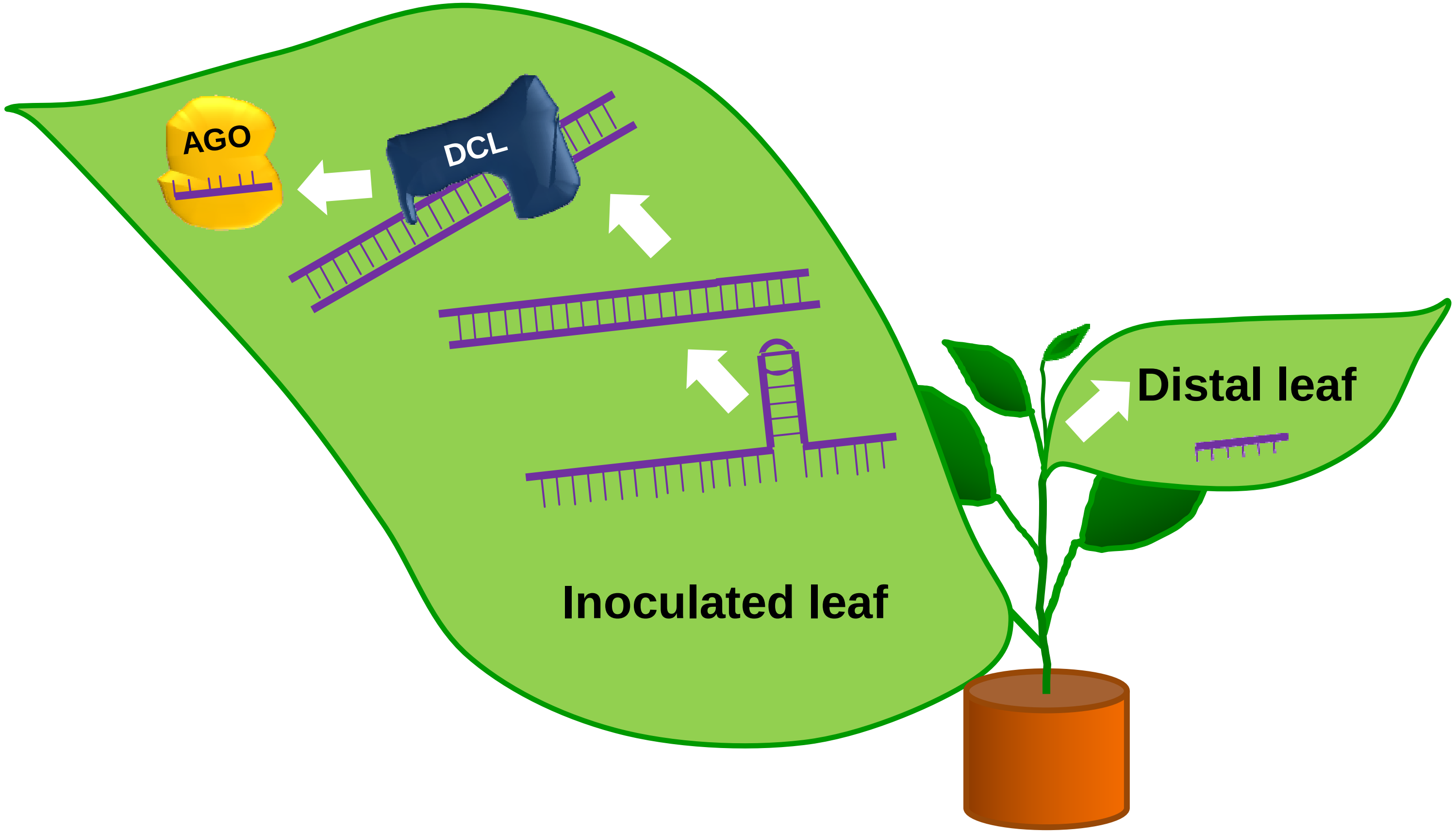
Systemic leaf

Inoculated leaf

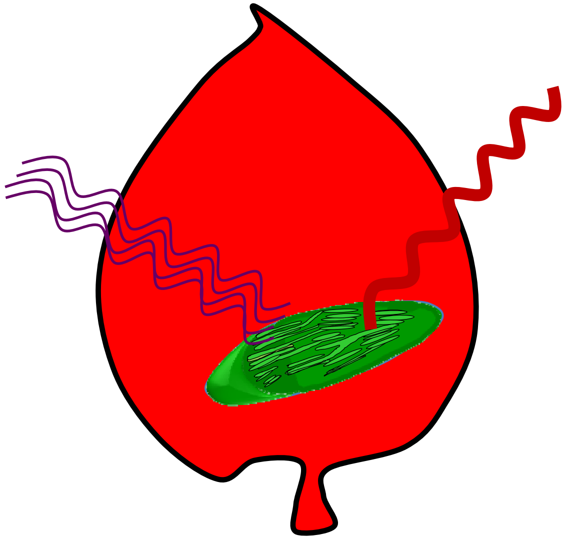


From Ratcliff, F., Henderson, B.D., and Baulcombe, D.C. (1997) A similarity between viral defense and gene silencing in plants. Science 276: 1558–1560. Reprinted with permission from AAAS.

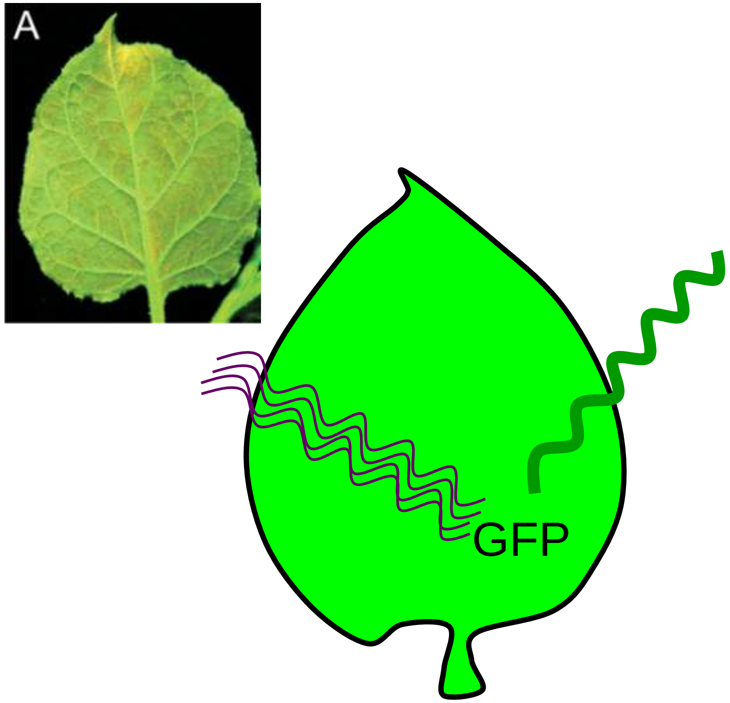
Virus infection causes systemic siRNA accumulation



How does RNA silencing spread systemically???



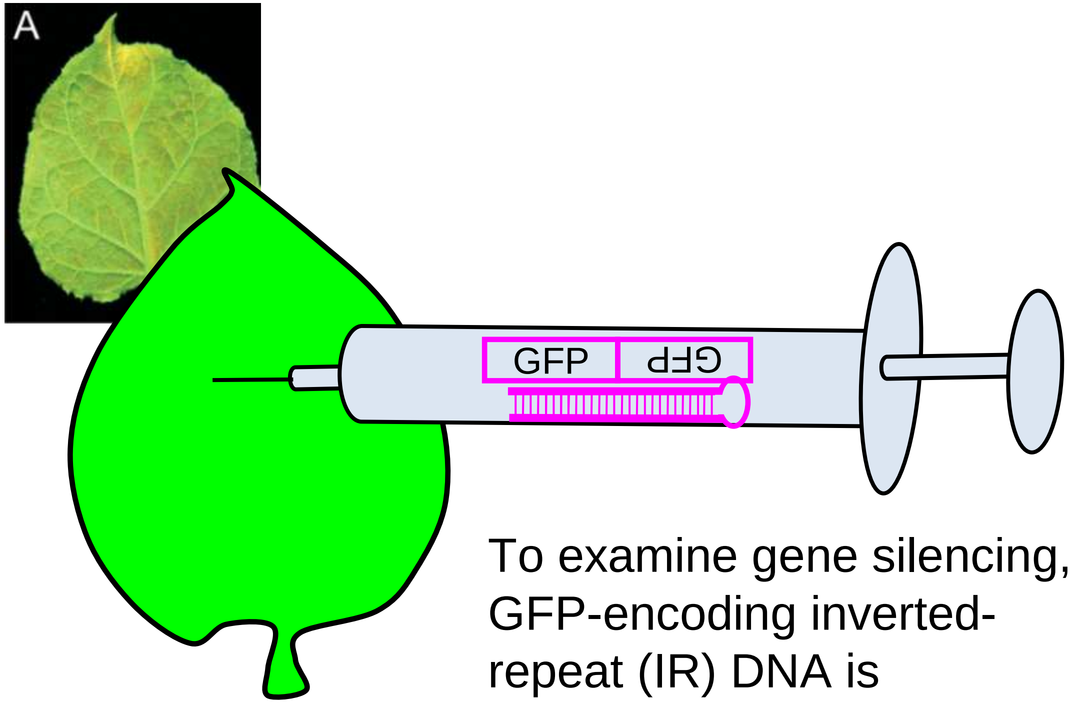
Under UV light, wild-type leaves fluoresce **red,** from chlorophyll in the chloroplasts.



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

Reprinted with permission from Kalantidis, K., Schumacher, H.T., Alexiadis, T., and Helm, J.M. (2008) RNA silencing movement in plants. Biol. Cell 100: 13–26; (c) the Biochemical Society.

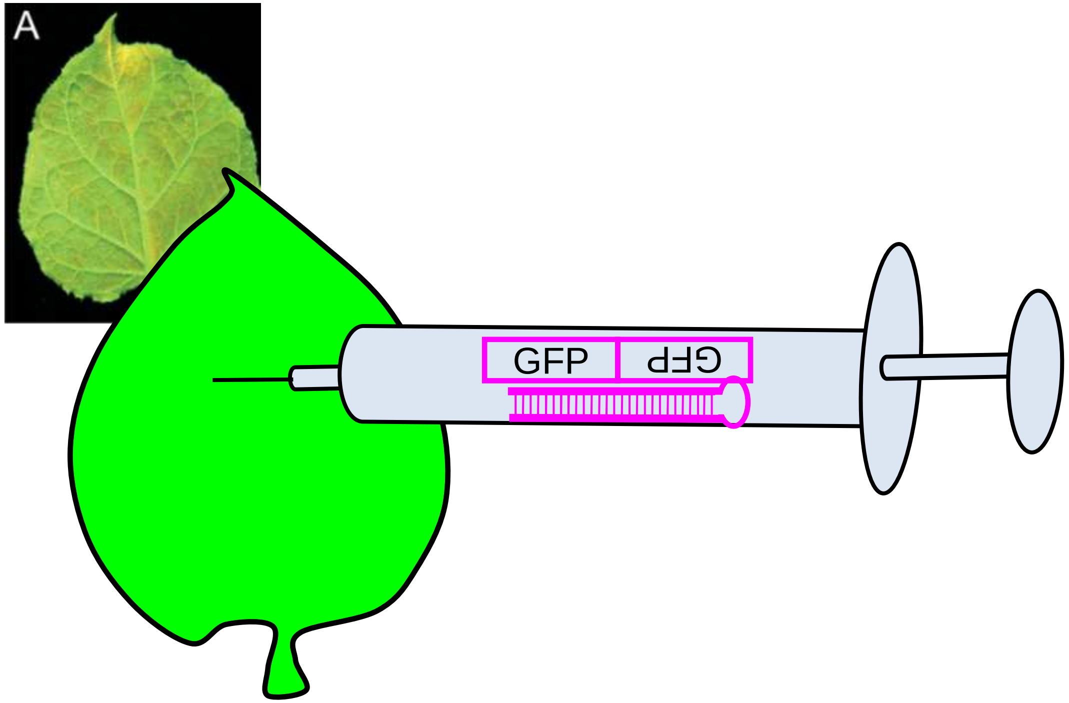
Spreading of RNA silencing

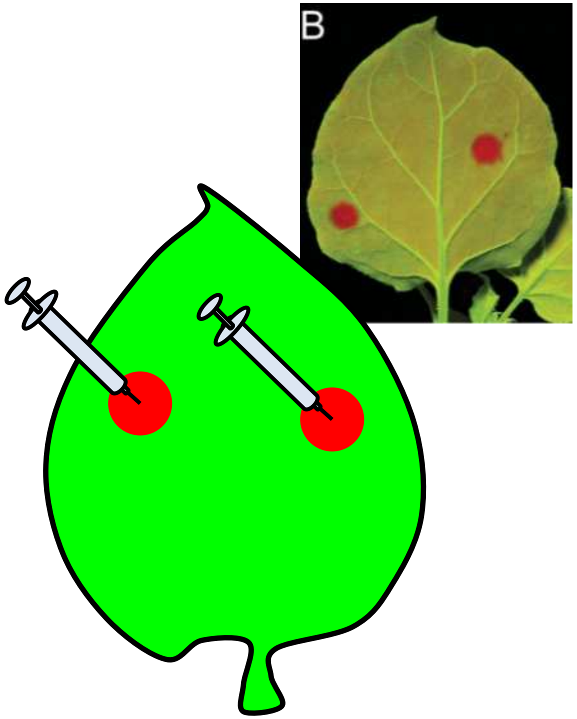


To examine gene silencing, GFP-encoding inverted-repeat (IR) DNA is introduced into the GFP-expressing cells.

Reprinted with permission from Kalantidis, K., Schumacher, H.T., Alexiadis, T., and Helm, J.M. (2008) RNA silencing movement in plants. Biol. Cell 100: 13–26; (c) the Biochemical Society.

Spreading of RNA silencing

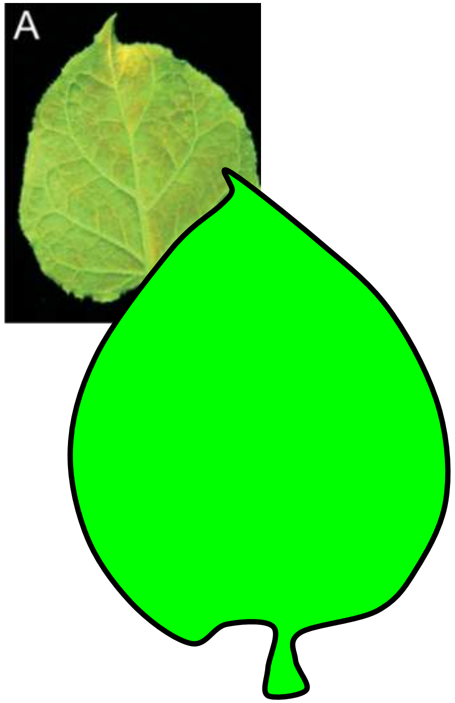




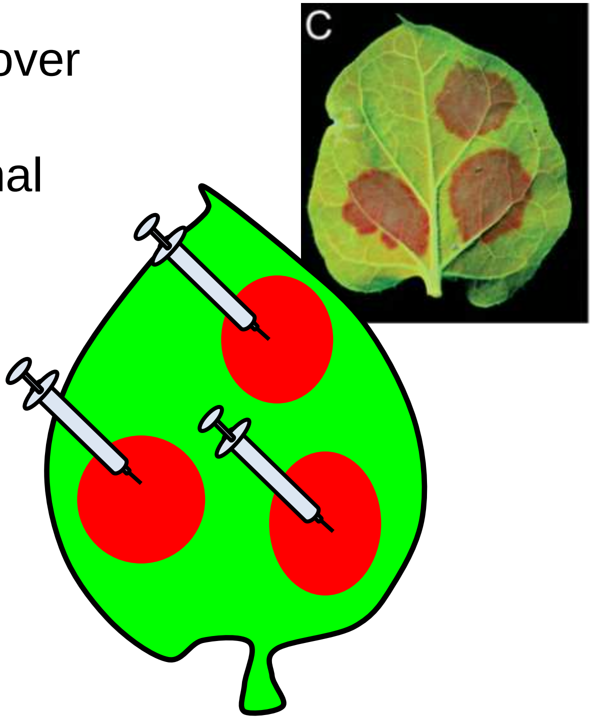
When GFP is silenced, the red chlorophyll fluorescence becomes visible.

Reprinted with permission from Kalantidis, K., Schumacher, H.T., Alexiadis, T., and Helm, J.M. (2008) RNA silencing movement in plants. Biol. Cell 100: 13–26; (c) the Biochemical Society.

Silencing can spread locally

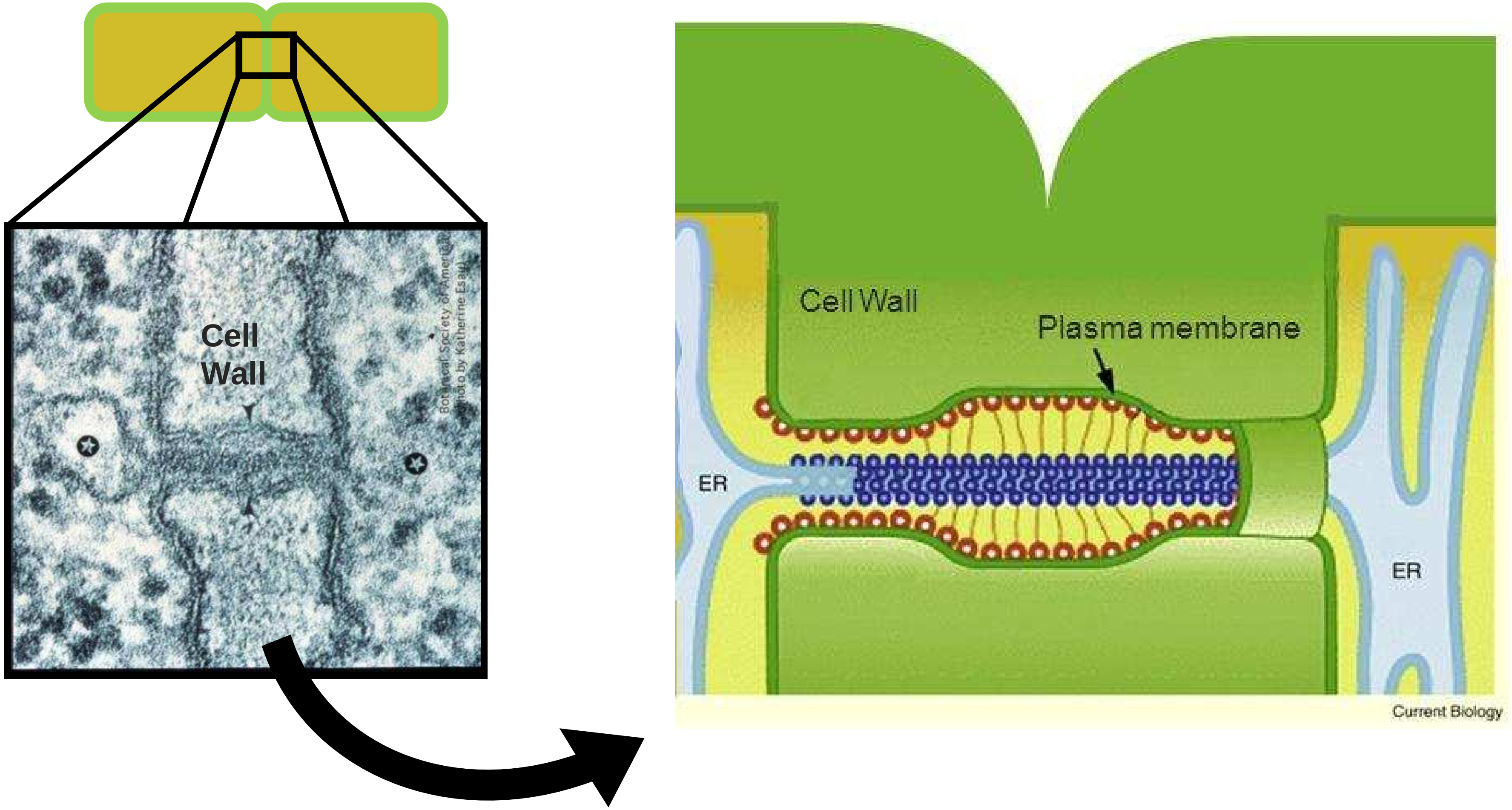


Often the silencing spreads over up to 15 cells, probably by diffusion of the silencing signal through the plasmodesmata.



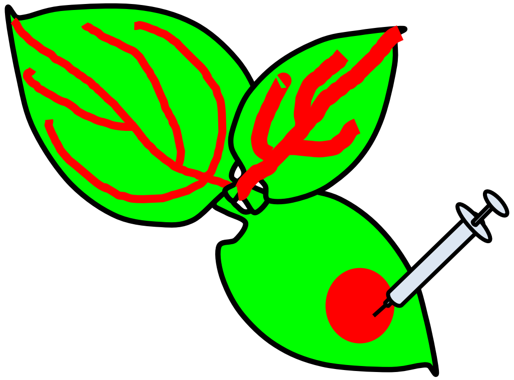
Reprinted with permission from Kalantidis, K., Schumacher, H.T., Alexiadis, T., and Helm, J.M. (2008) RNA silencing movement in plants. Biol. Cell 100: 13–26; (c) the Biochemical Society.

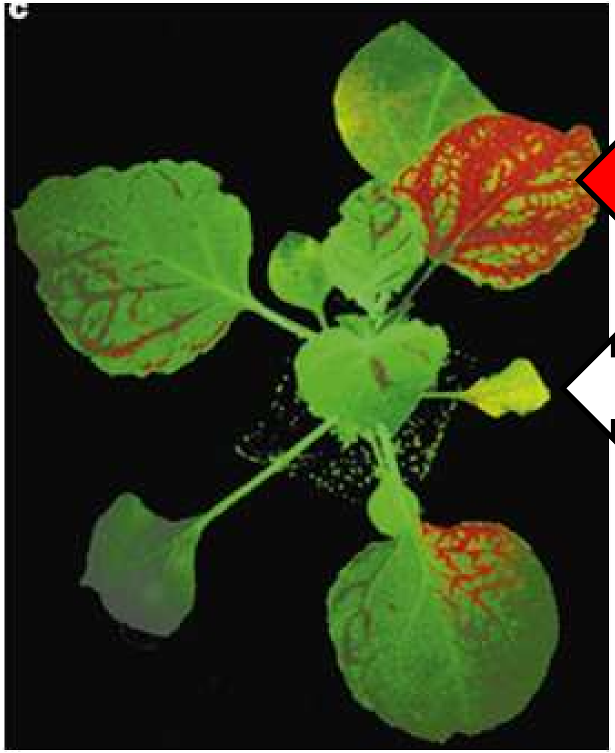
Plasmodesmata are regulated connections between plant cells



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





Systemic silencing

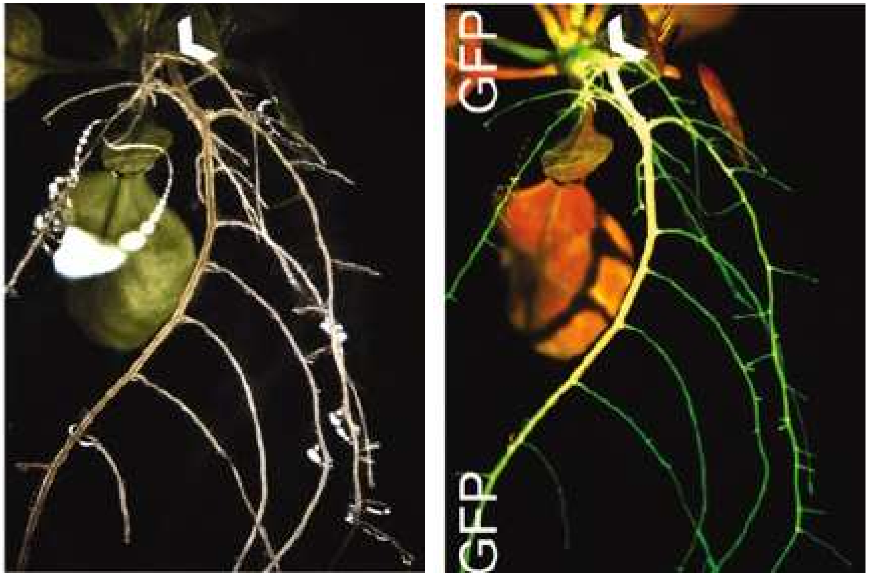
Injected leaf

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

Reprinted by permission from Macmillan Publishers, Ltd: Nature Copyright 1997.
Voinnet, O., and Baulcombe, D. (1997) Systemic silencing in gene silencing. Nature 389: 553.

Small RNAs can move from shoot to root in Arabidopsis

Control GFP expressing plant showing GFP in shoot and root



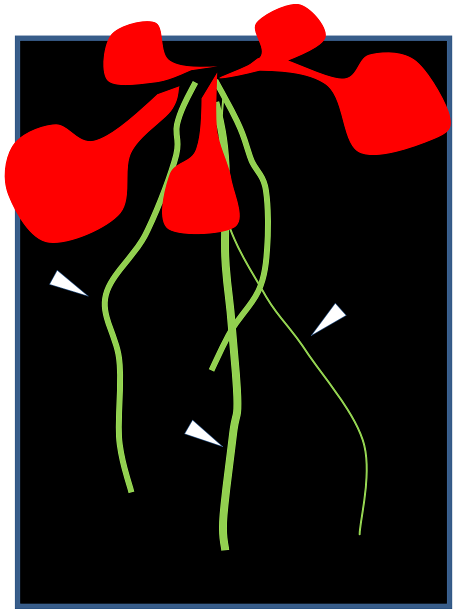
White light   Fluorescence

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

White light   Fluorescence

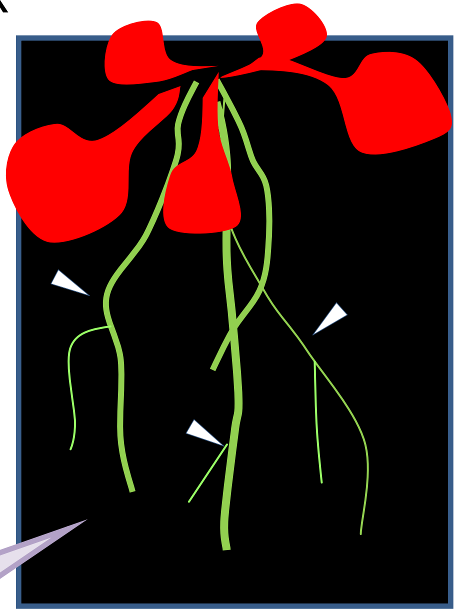
From Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science **328:** 872-875; reprinted with permission from AAAS.

Dicer activity for sRNA production in the shoot is sufficient



GFP-IR
GFP

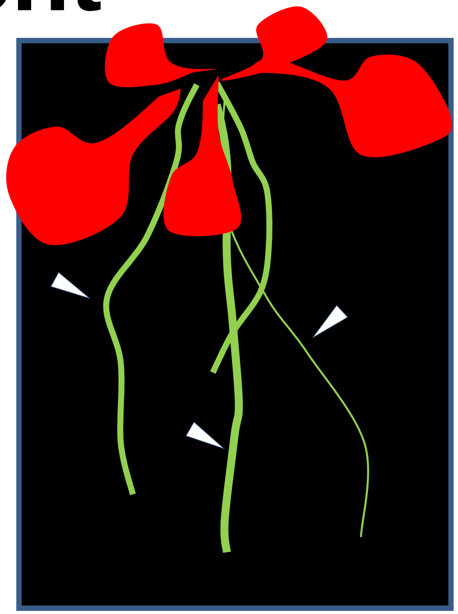
GFP silenced



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

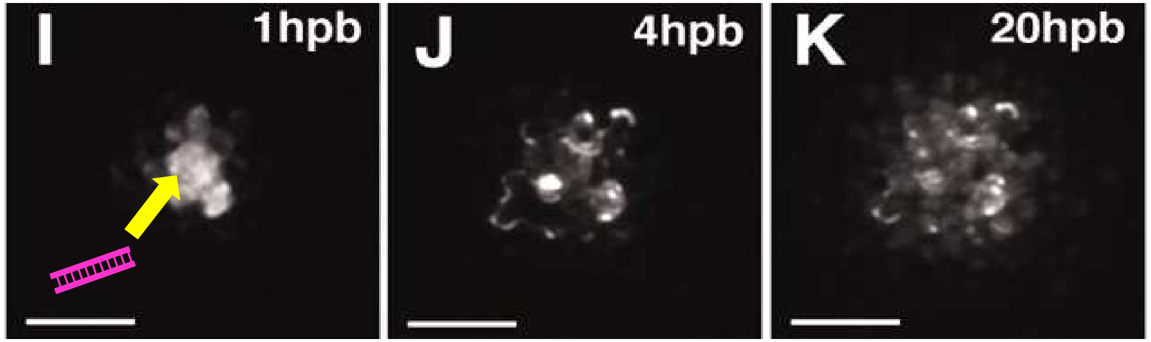


GFP-IR
GFP
dcl2,3,4

GFP silenced; sRNAs in root are derived from shoot

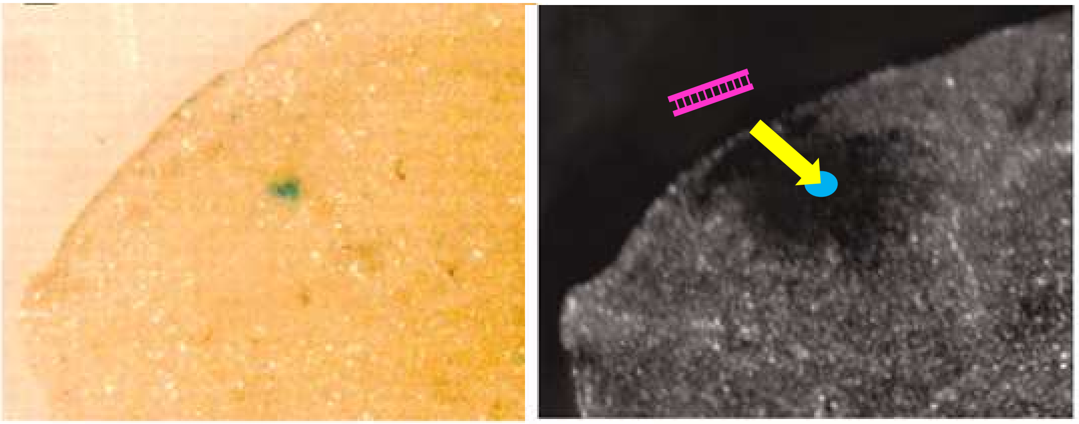
From Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science **328:** 872-875; reprinted with permission from AAAS.

siRNA duplexes move between cells and are sufficient to confer silencing



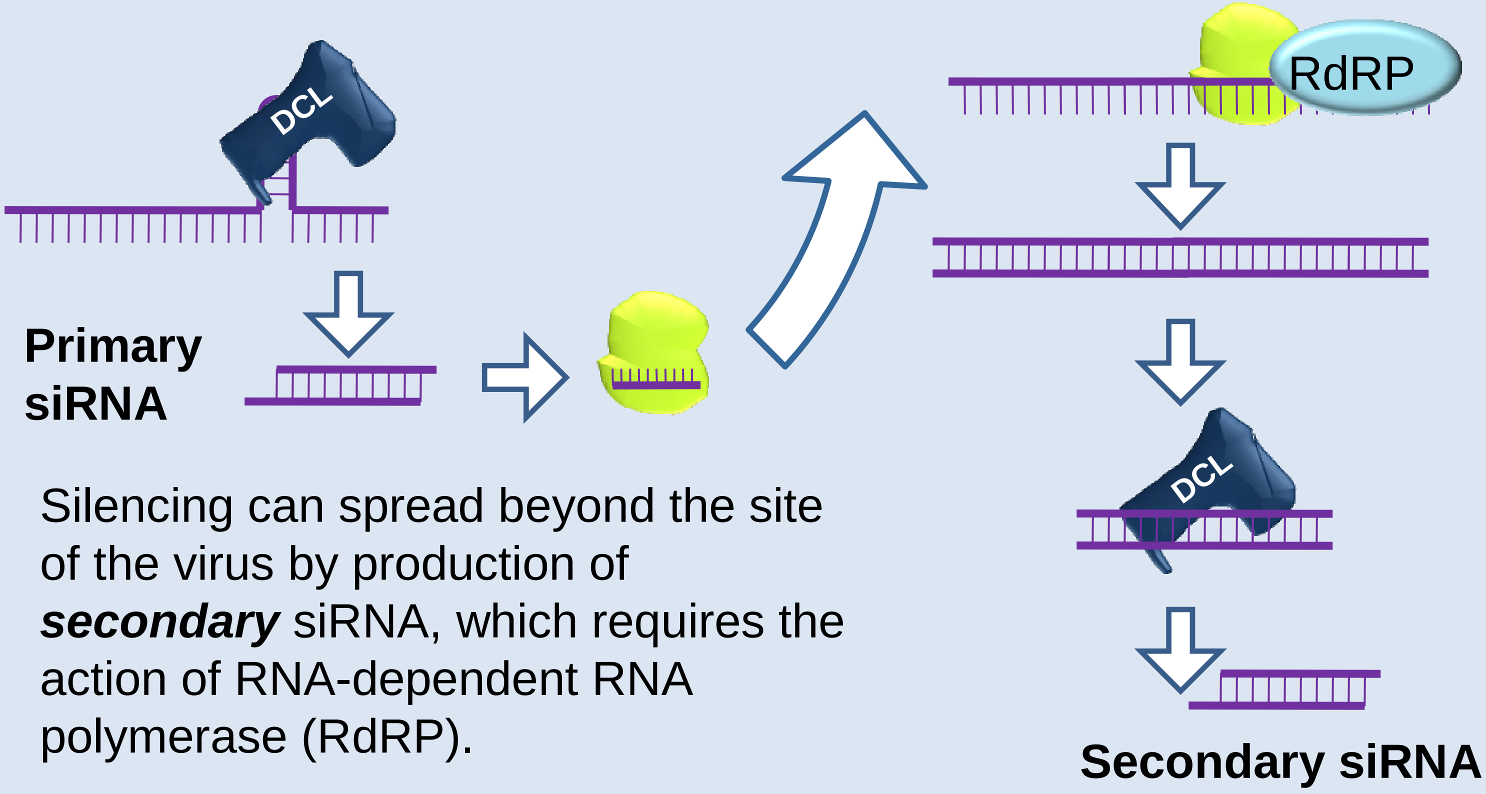
Fluorescently labeled duplex siRNA was bombarded into a single cell. Spreading to adjacent cells is visible at 20 hours post-bombardment.

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



Primary siRNA

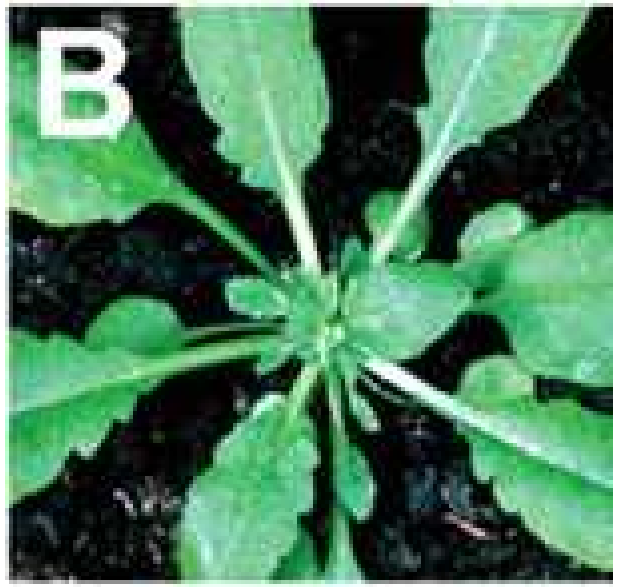
Secondary siRNA

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.

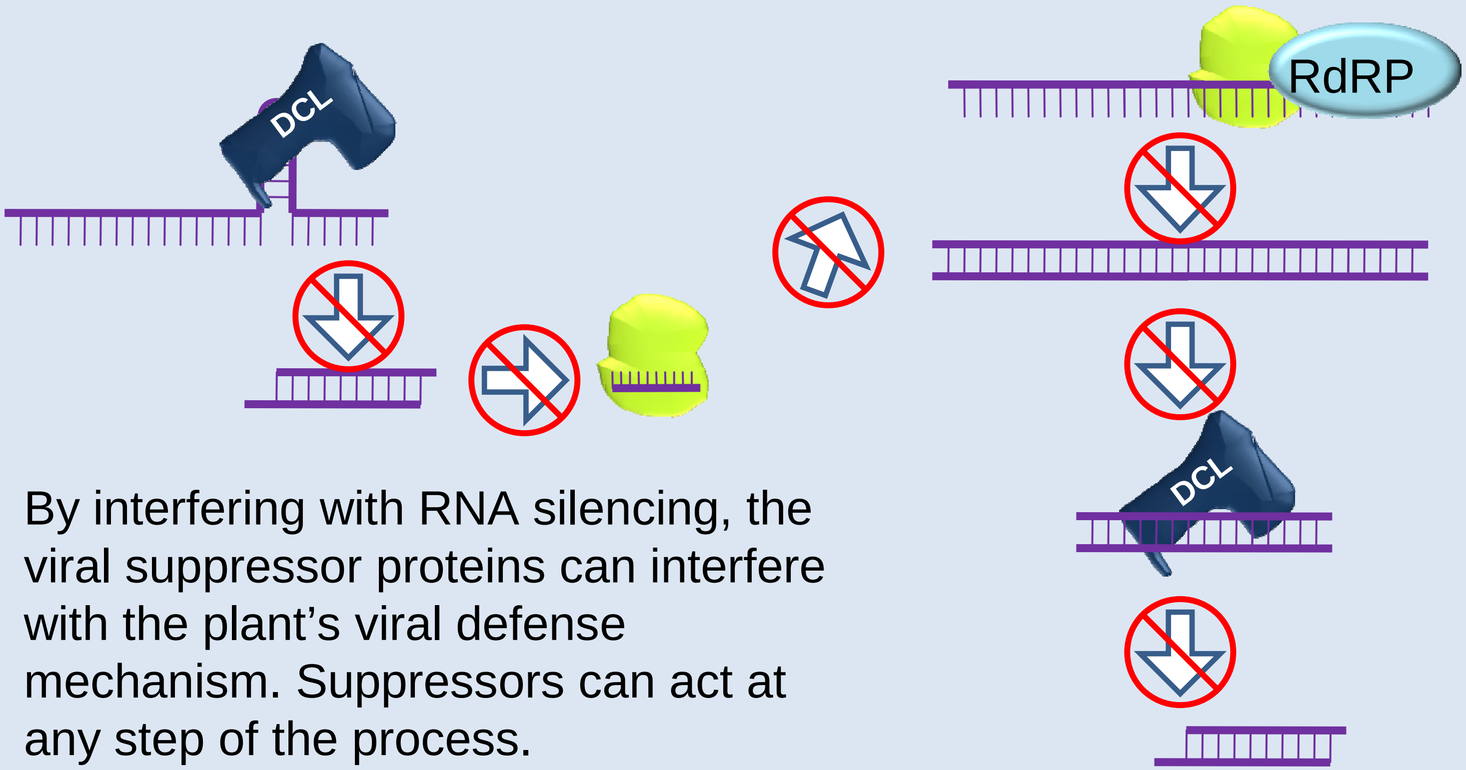
WT Arabidopsis inoculated with TRV



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

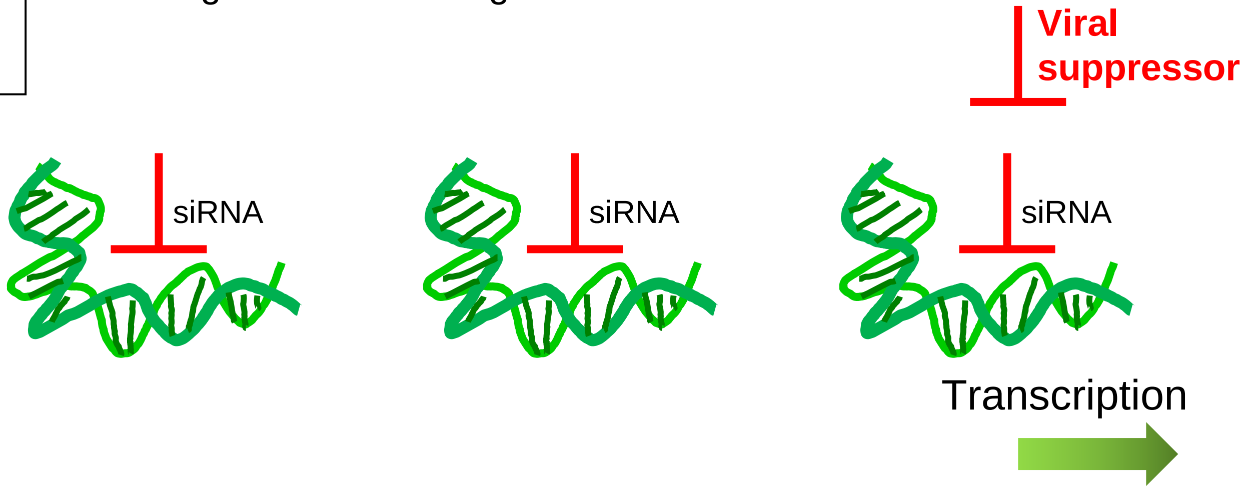
Genes encoding functional, mutant, or no viral suppressor proteins were introduced into plants carrying a silenced GUS gene. The plants were inoculated with a virus expressing GUS. Blue spots indicate GUS expression.

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.

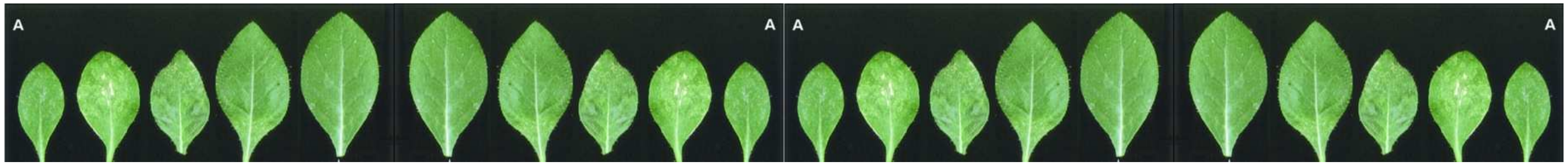
Small RNAs also protect plants against bacterial pathogens

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.

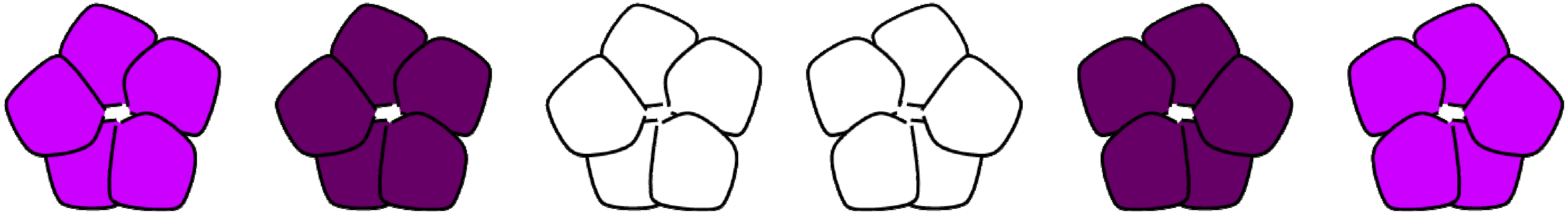
Viral-induced gene silencing summary

- 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



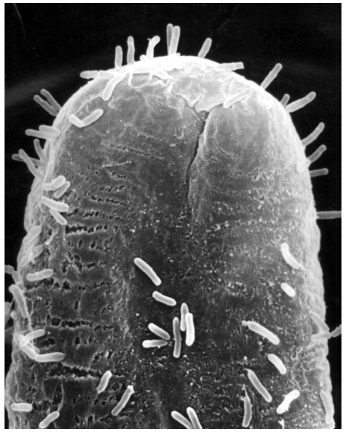
Silencing of transgenes

- 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.

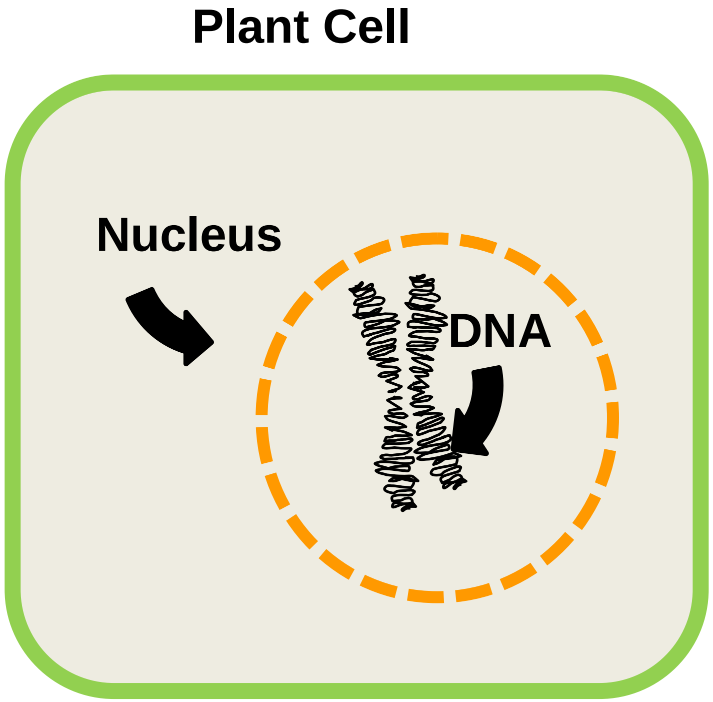
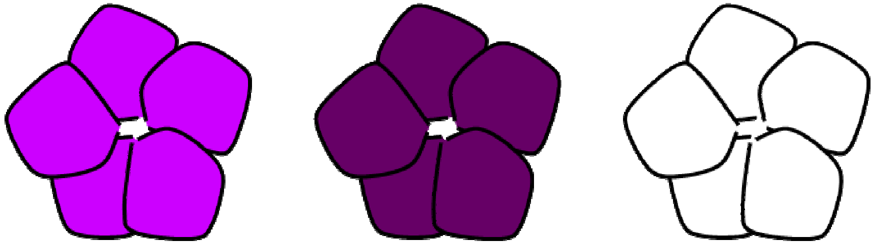


Photo credits: Martha Hawes, University of Arizona

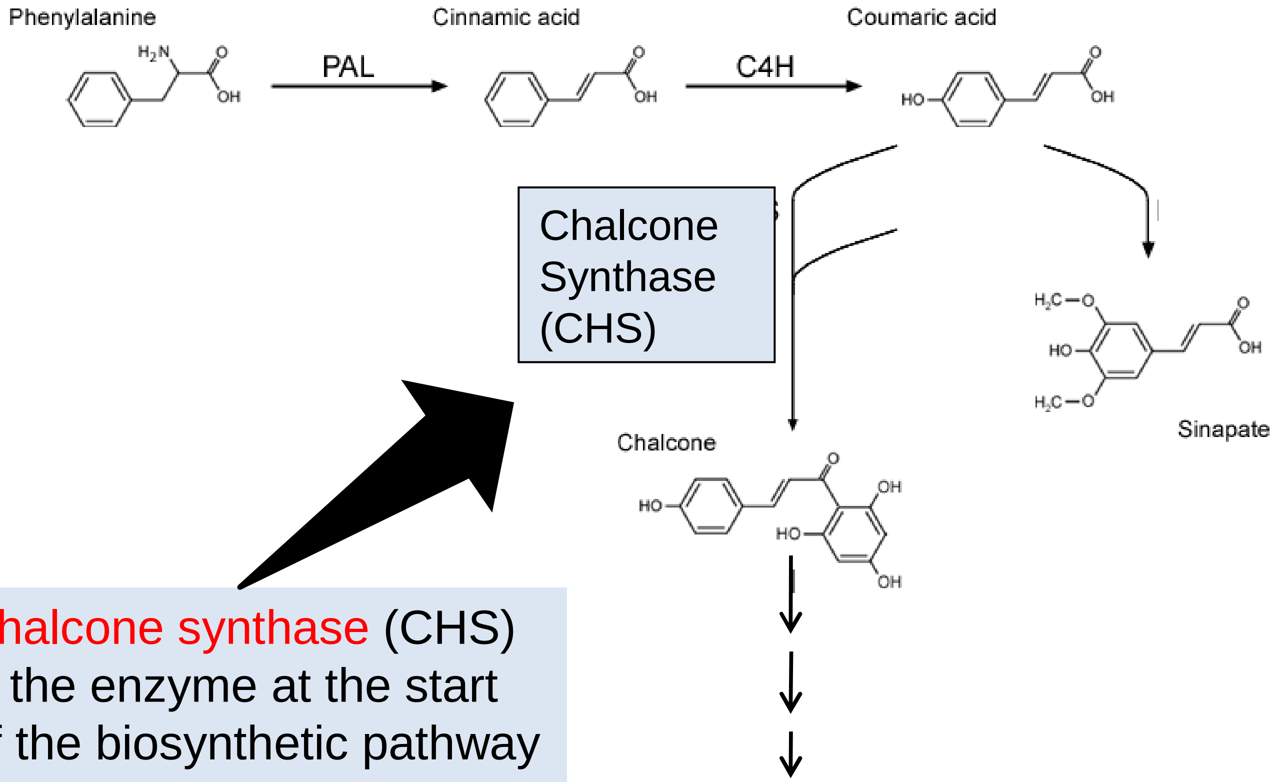
Transgene-induced post-transcriptional silencing



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)

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

Anthocyanins

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

Expectation – sense RNA production would enhance pigmentation...

Endogenous gene

PRO ORF

mRNA

Protein translated

mRNA

Transgene

Sense construct:

PRO ORF

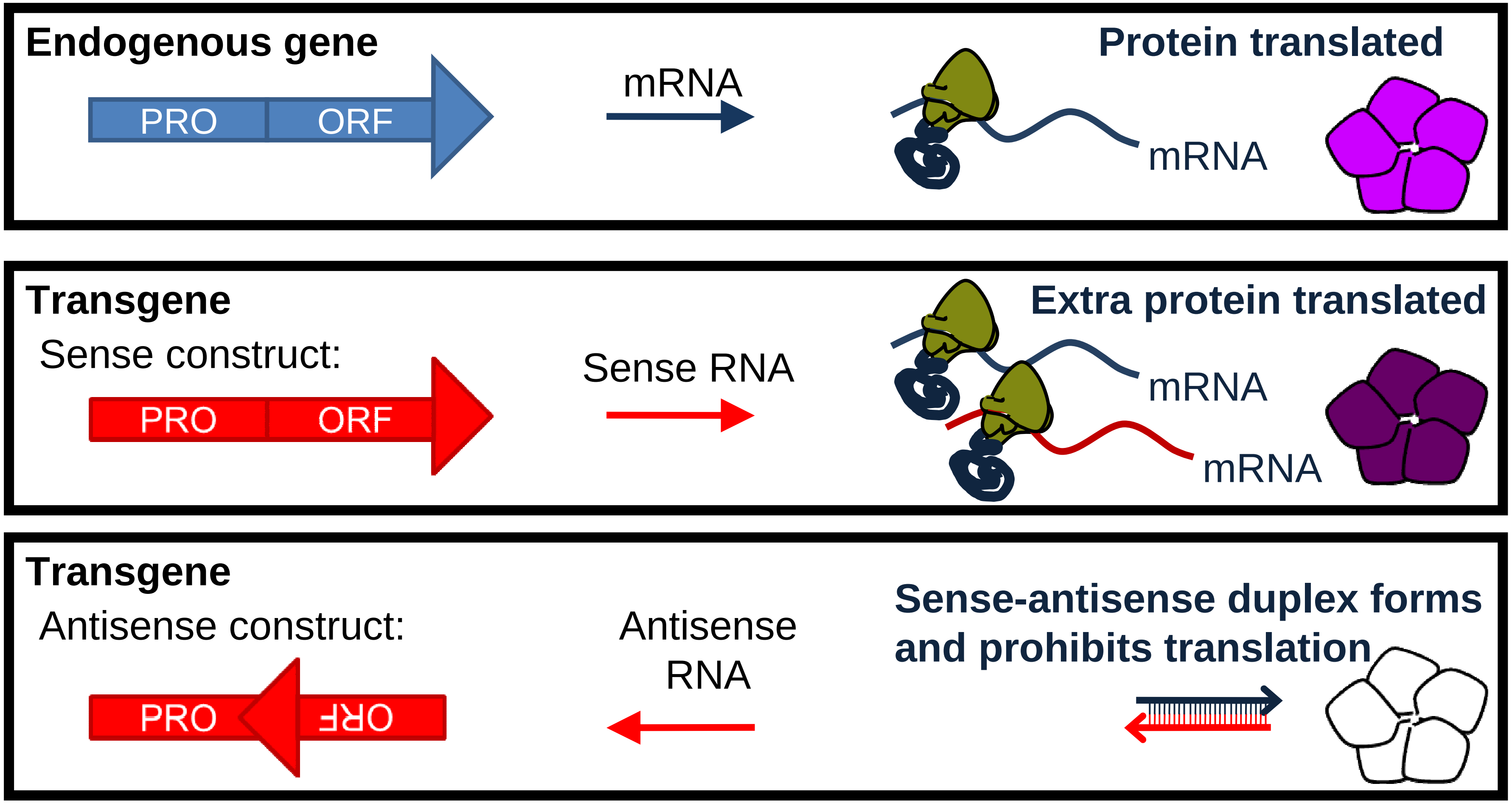
Sense RNA

Extra protein translated

mRNA

mRNA

..and antisense RNA production would block pigmentation



Endogenous gene

Protein translated

Transgene

Sense construct:

Sense RNA

Extra protein translated

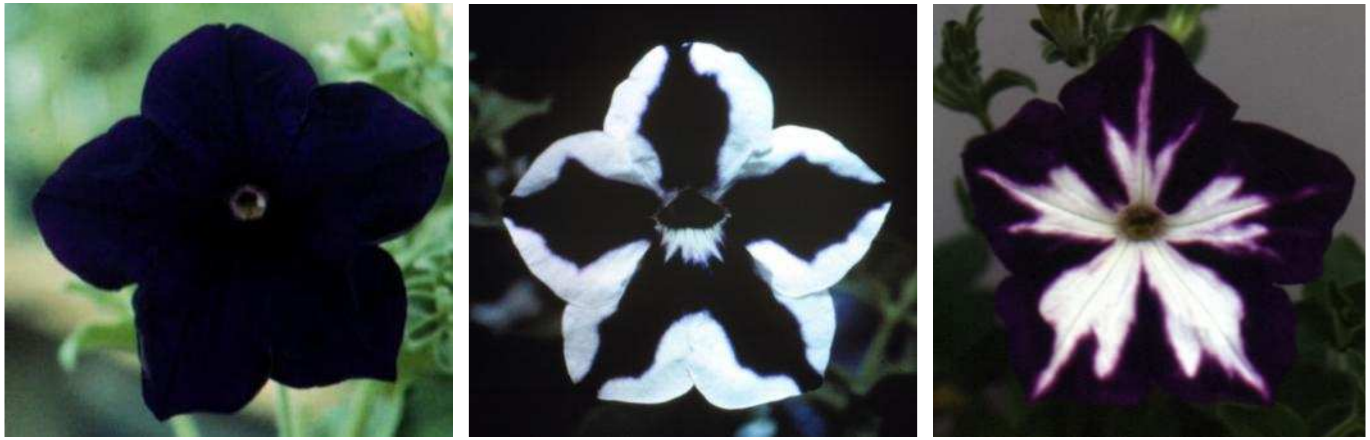
Transgene

Antisense construct:

Antisense RNA

Sense-antisense duplex forms and prohibits translation

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



Plants carrying CHS transgene

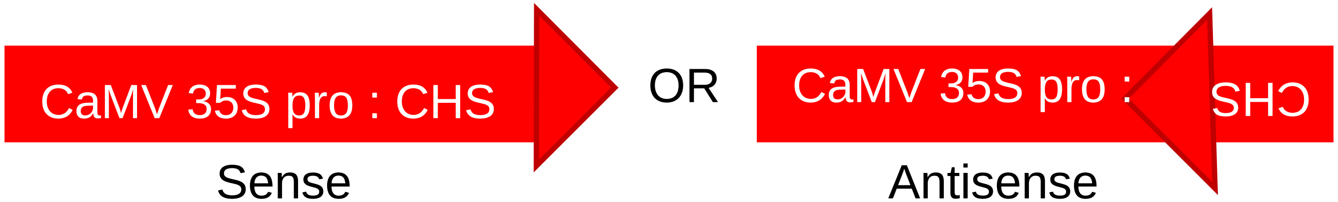
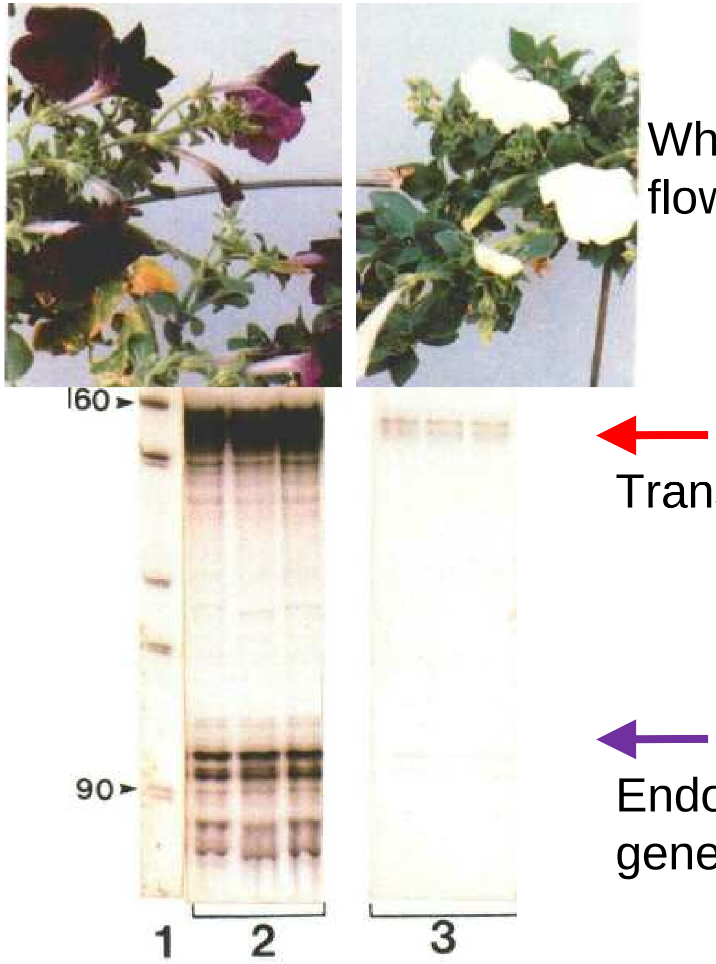


Photo credit Richard Jorgense

Silenced tissues do not express endogenous or introduced CHS



Purple flowers

White flowers

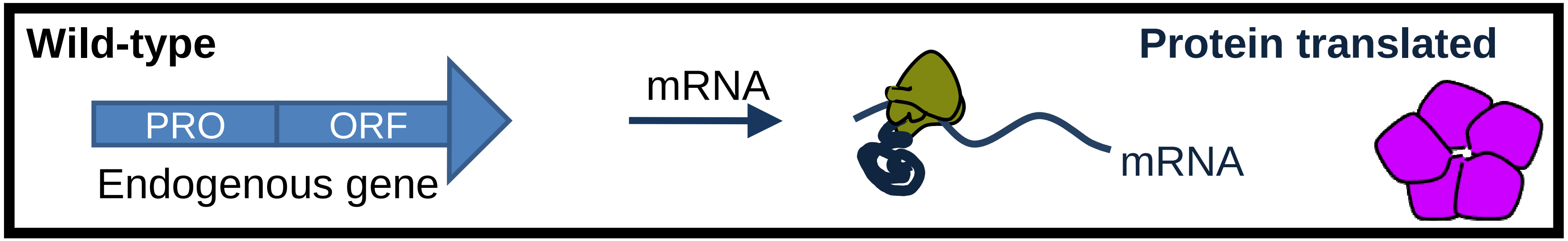
Transgene RNA

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



Wild-type

Protein translated

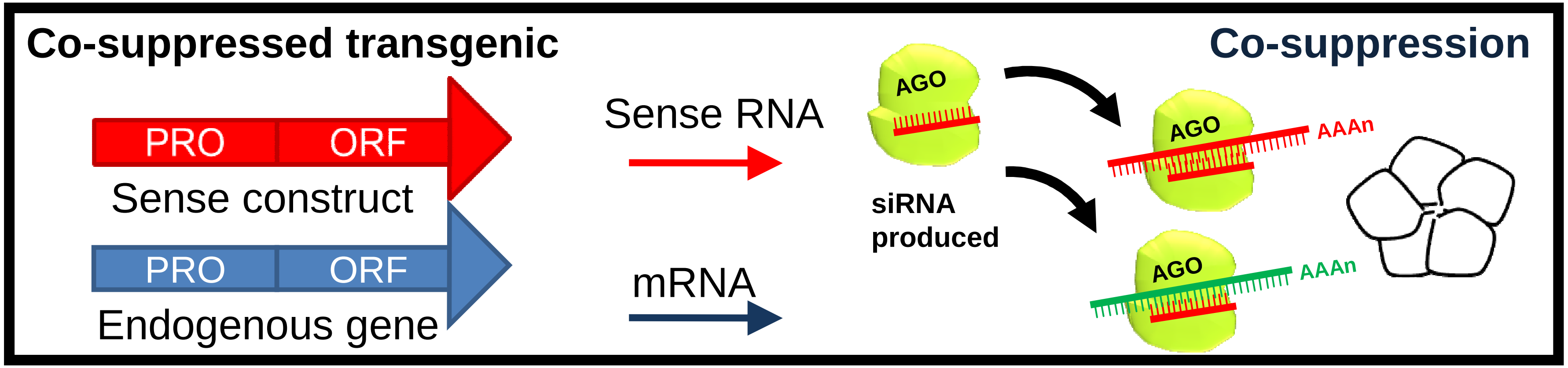
PRO

ORF

Endogenous gene

mRNA

mRNA



Co-suppressed transgenic

Co-suppression

PRO

ORF

Sense construct

PRO

ORF

Endogenous gene

Sense RNA

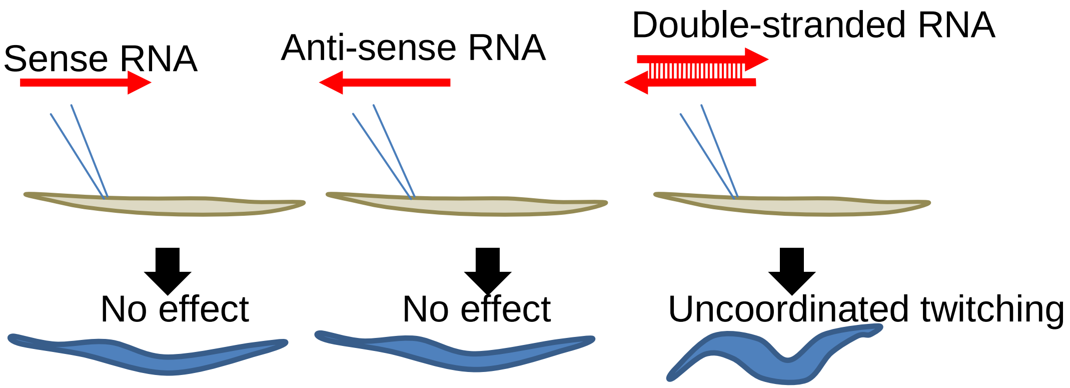
mRNA

siRNA produced

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

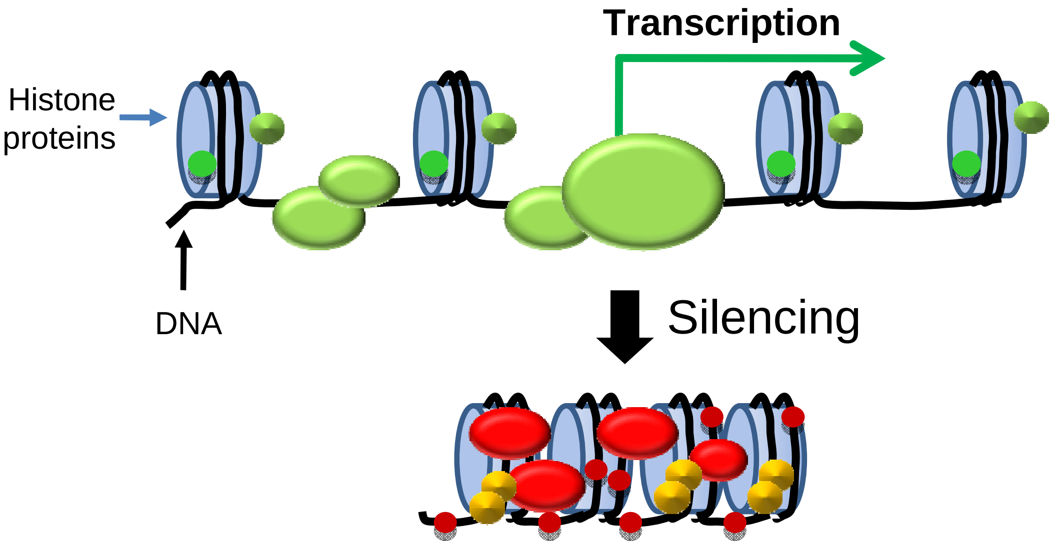
Sense, antisense or double-stranded RNAs homologous to the *unc-22* gene were introduced into worms. Silencing of *unc-22* causes loss of muscle control – hence its name, "*uncoordinated*".



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.

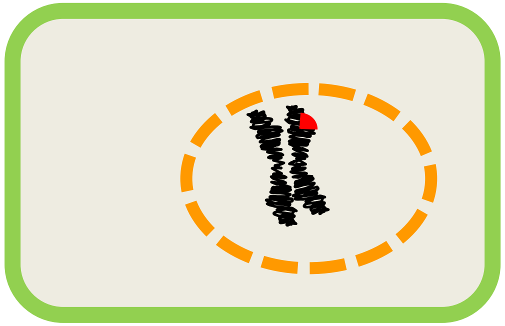
Transcriptional gene silencing

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.

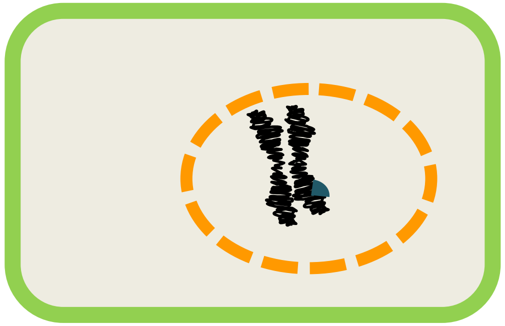
Transcriptional gene silencing



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.

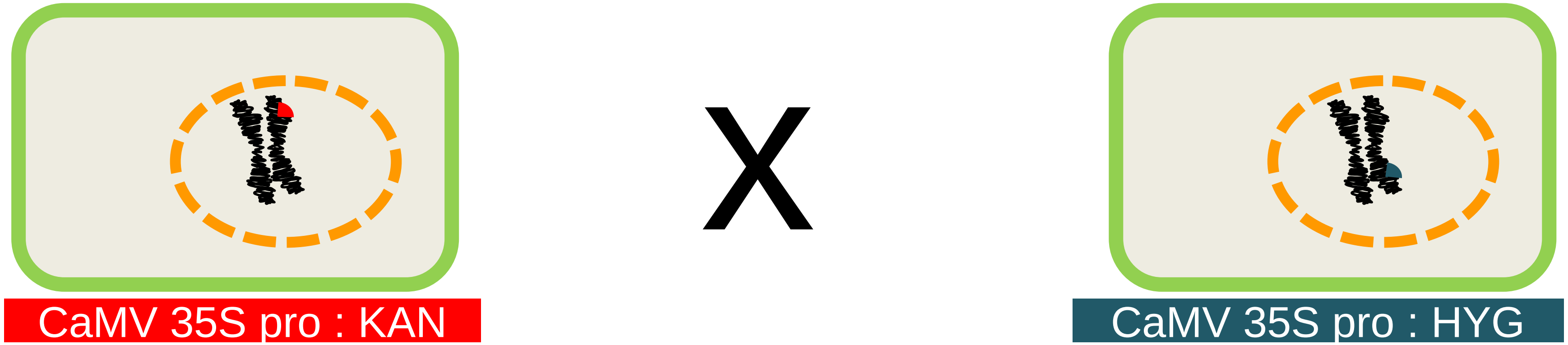


CaMV 35S pro : HYG

Expression of a gene that confers resistance to the antibiotic hygromycin

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.

Transcriptional gene silencing

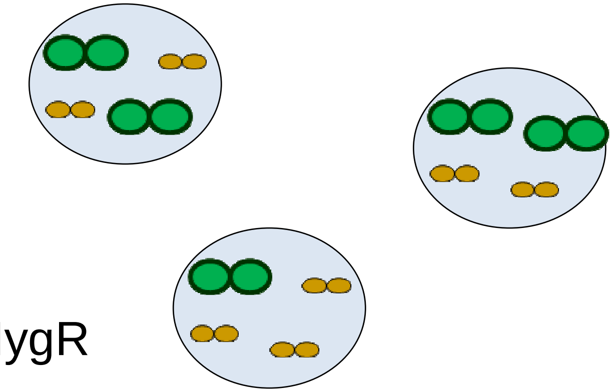


Expected Results

Selection on kanamycin only: 50% KanR

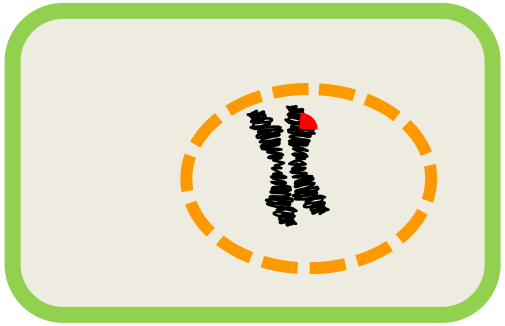
Selection on hygromycin only: 50% HygR

Selection on Kan + Hyg: 25% KanR and HygR



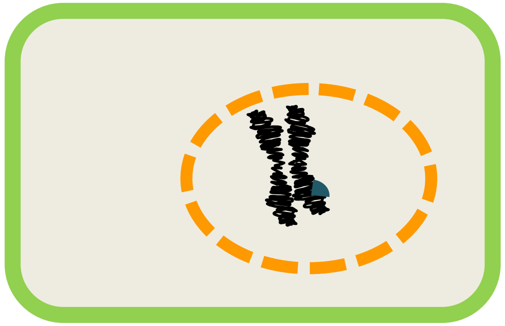
Based on Matzke, M., Primig, M., Trnovsky, J., Matzke, A. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed plants. EMBO J. 8: 643-649.

Transcriptional gene silencing



CaMV 35S pro : KAN

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



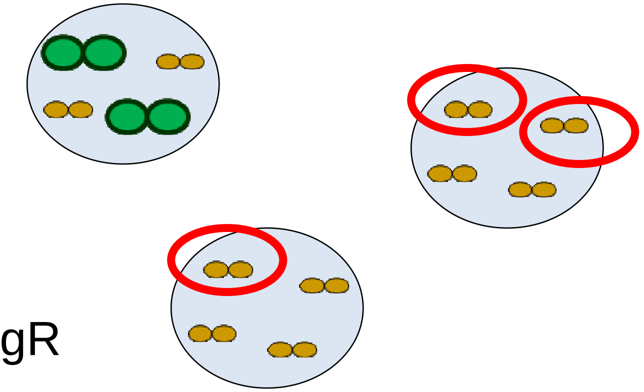
CaMV 35S pro : HYG

<u>Observed</u> Results

Selection on kanamycin only: 50% KanR

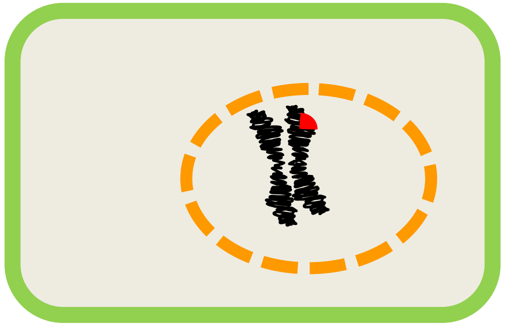
Selection on hygromycin only: 0% HygR

Selection on Kan + Hyg: 0% KanR and HygR



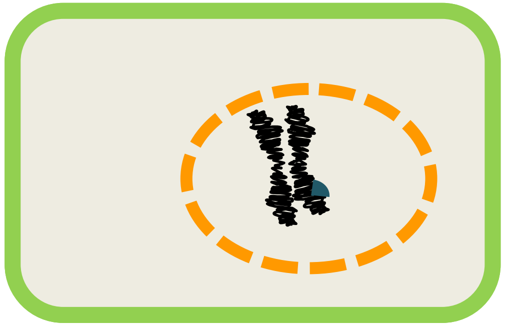
Based on Matzke, M., Primig, M., Trnovsky, J., Matzke, A. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed plants. EMBO J. 8: 643-649.

Transcriptional gene silencing

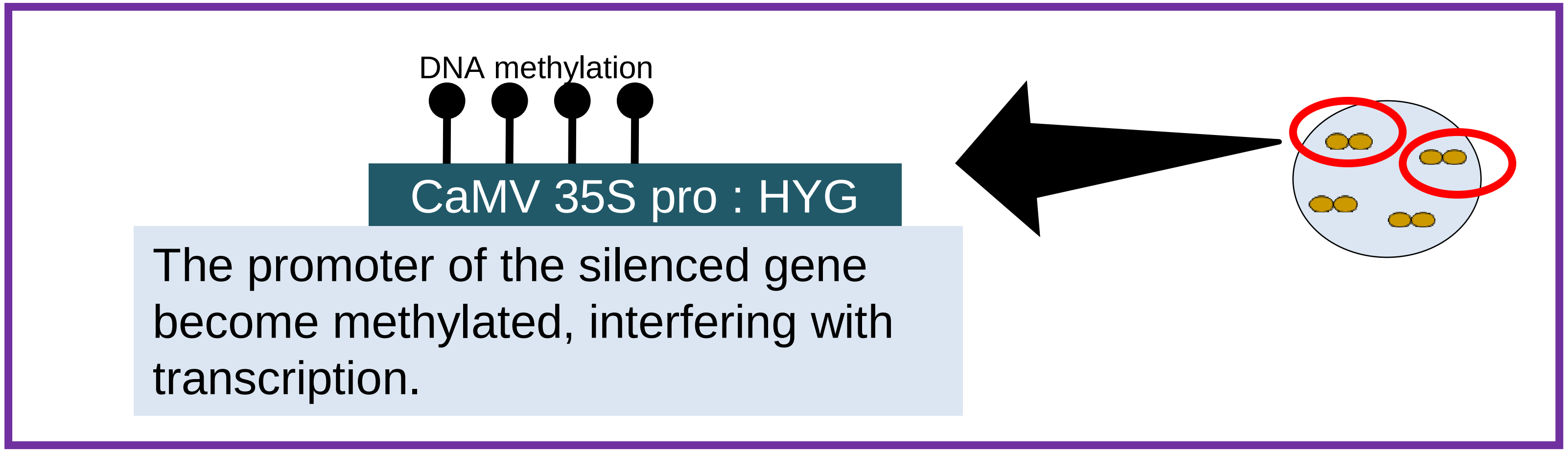


CaMV 35S pro : KAN

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



CaMV 35S pro : HYG



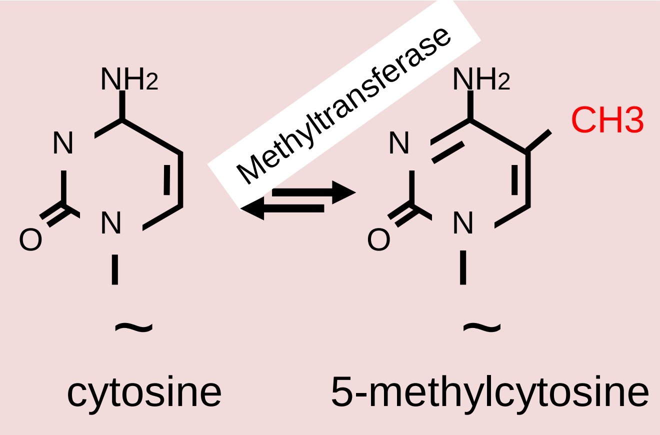
DNA methylation

CaMV 35S pro : HYG

The promoter of the silenced gene become methylated, interfering with transcription.

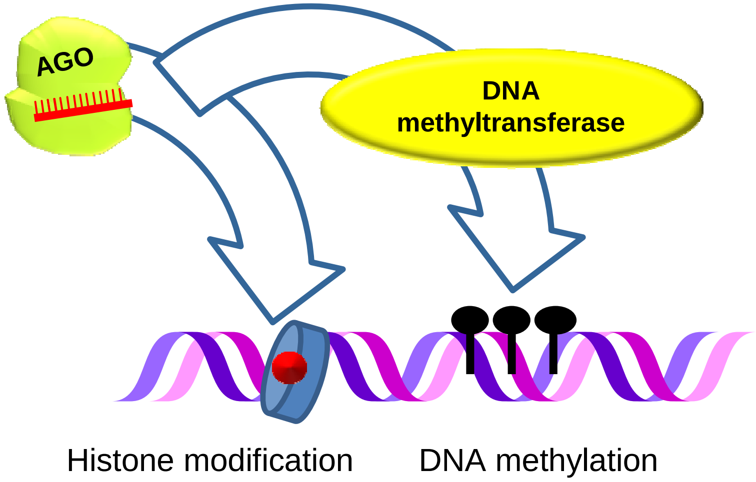
Based on Matzke, M., Primig, M., Trnovsky, J., Matzke, A. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed plants. EMBO J. 8: 643-649.

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).



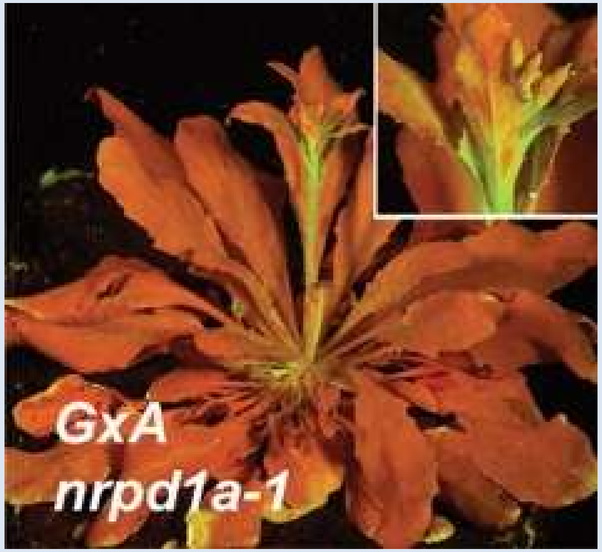
Plants have additional RNA Polymerase complexes that contribute to silencing

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

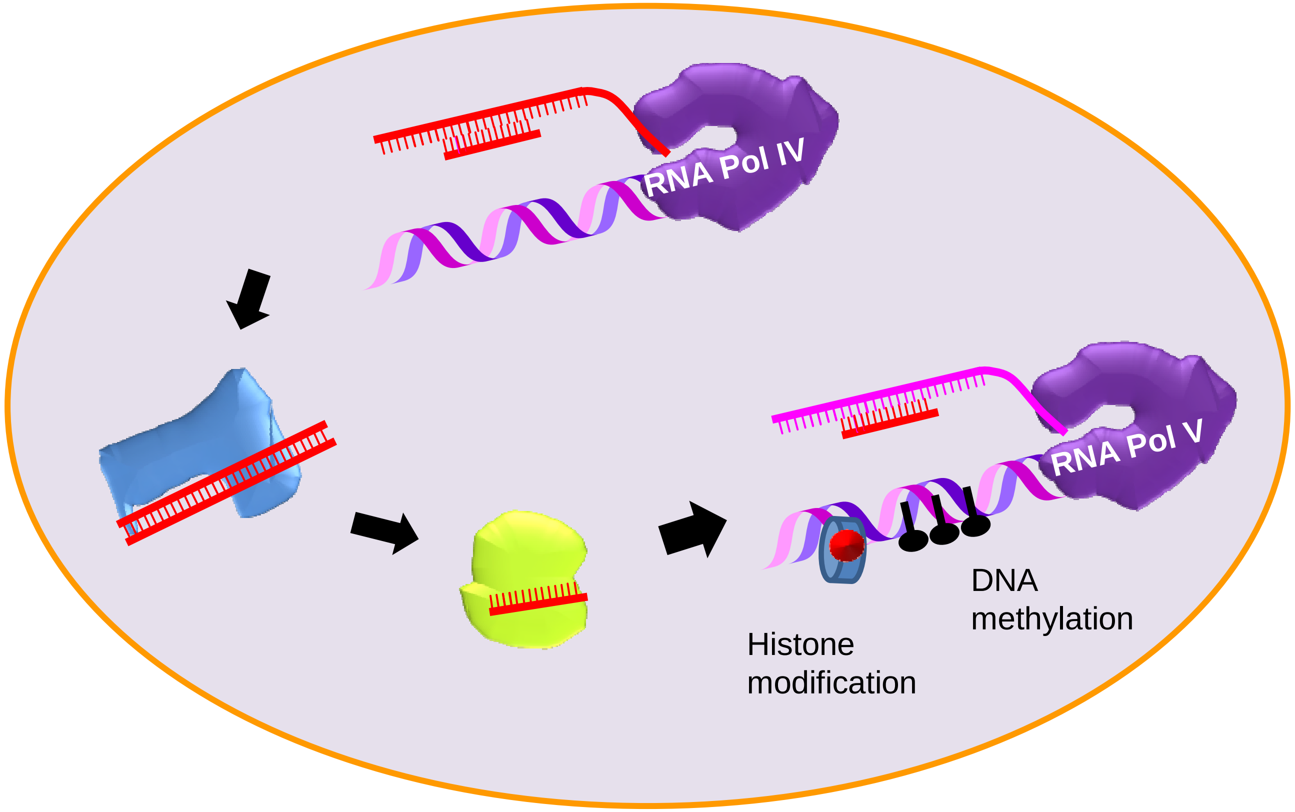
Loss-of-function mutant *nrpd1a-1*. *NRPD1A* encodes a subunit of RNA Polymerase IV.



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

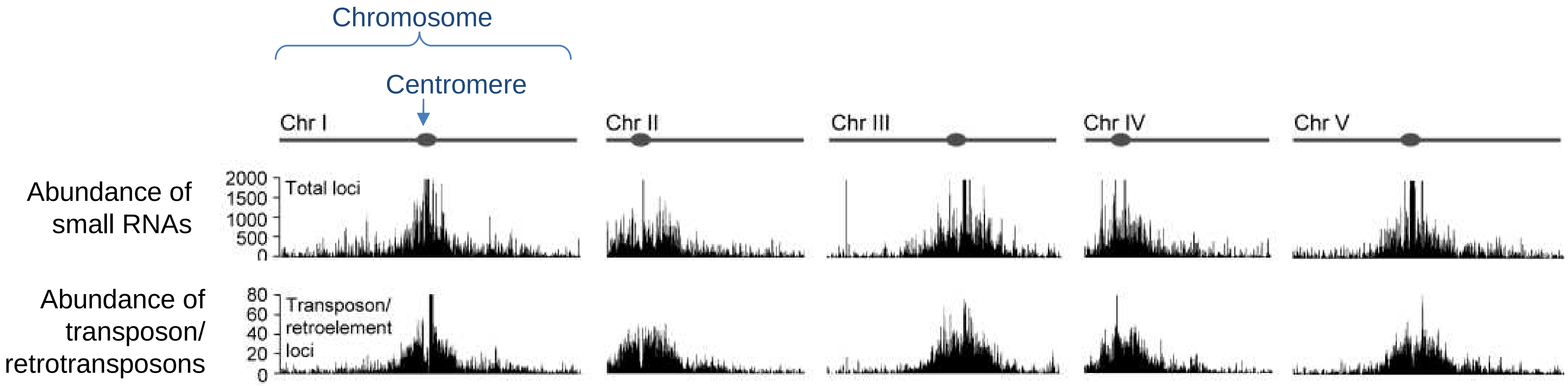
From Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005) RNA polymerase IV directs silencing of endogenous DNA. Science 308: 118–120. Reprinted with permission from AAAS.

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

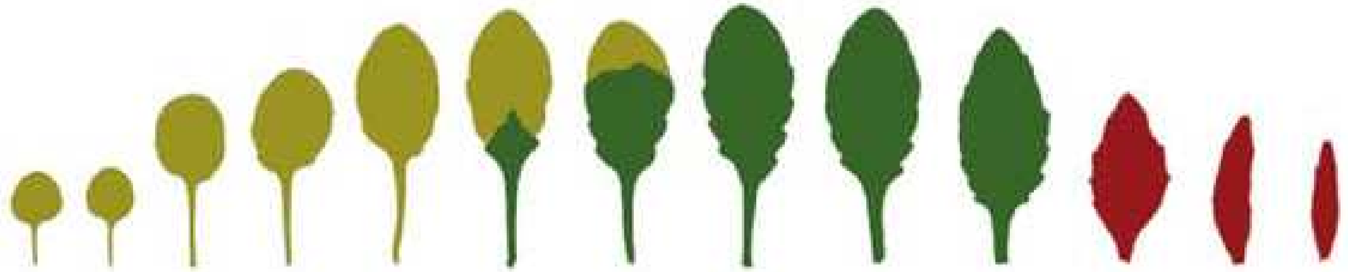
The siRNAs associate with AGO proteins and form silencing complexes

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

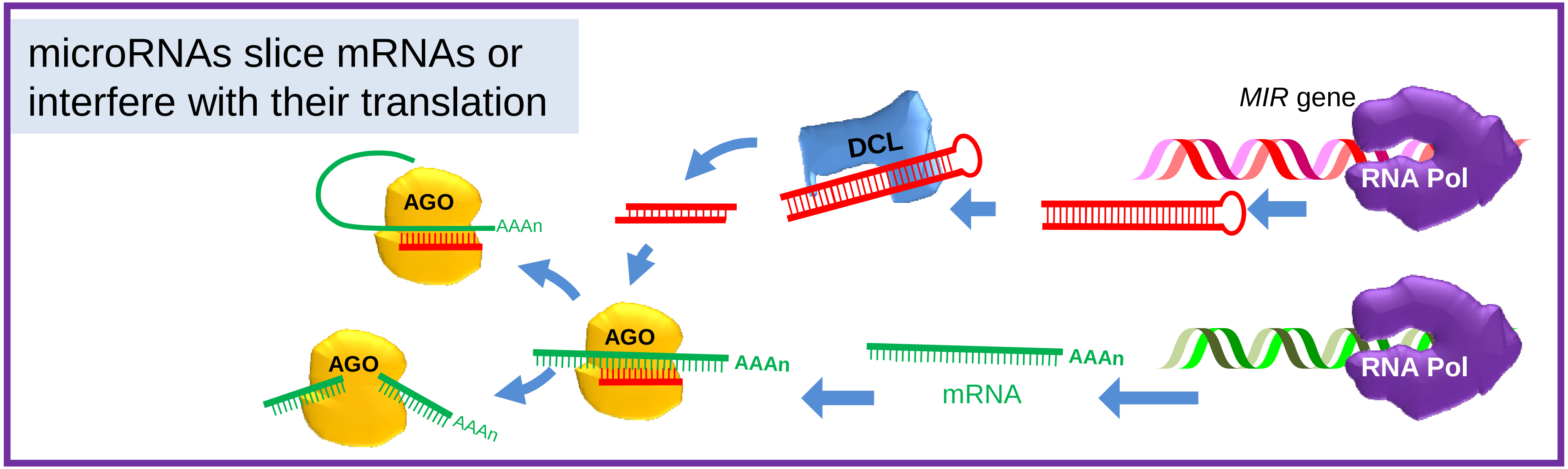
The silencing complexes can also act on chromatin, silencing their targets by DNA methylation or histone modification

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

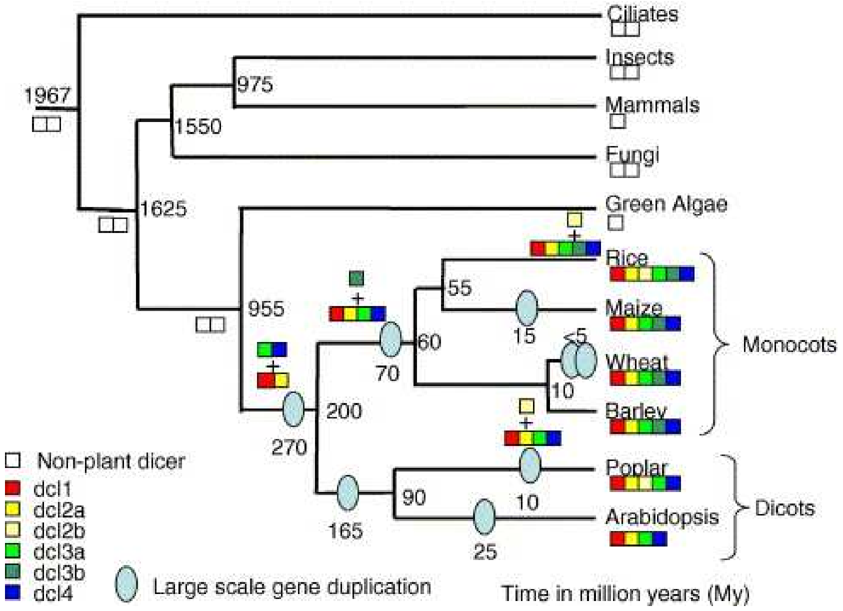


microRNAs slice mRNAs or interfere with their translation

miRNAs and siRNAs are processed by related but different DCL proteins

AtDCL1 produces **miRNA**

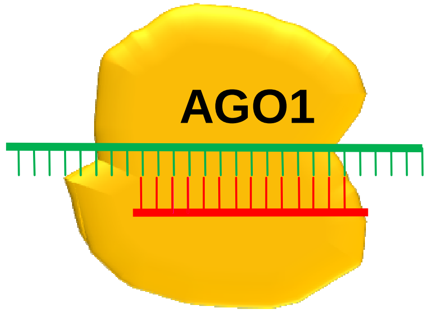
AtDCL2 - 4 produce **siRNA**



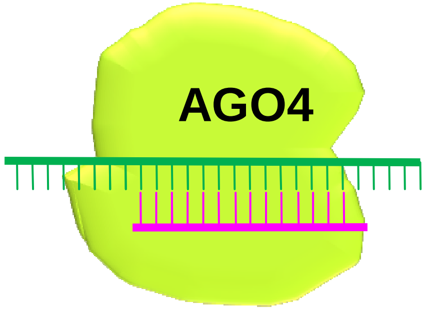
Plants have 4 or more DCL proteins, more than found in other organisms. The amplification of DCL proteins is thought to allow plants great flexibility in pathogen defense responses.

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.

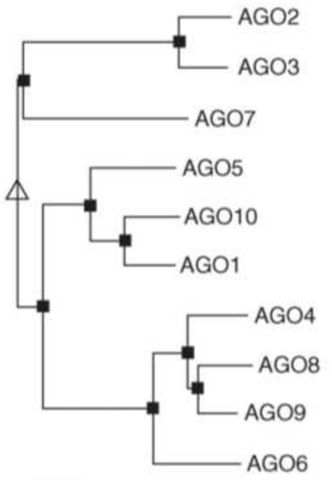
miRNAs and siRNAs associate with several AGO proteins



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.

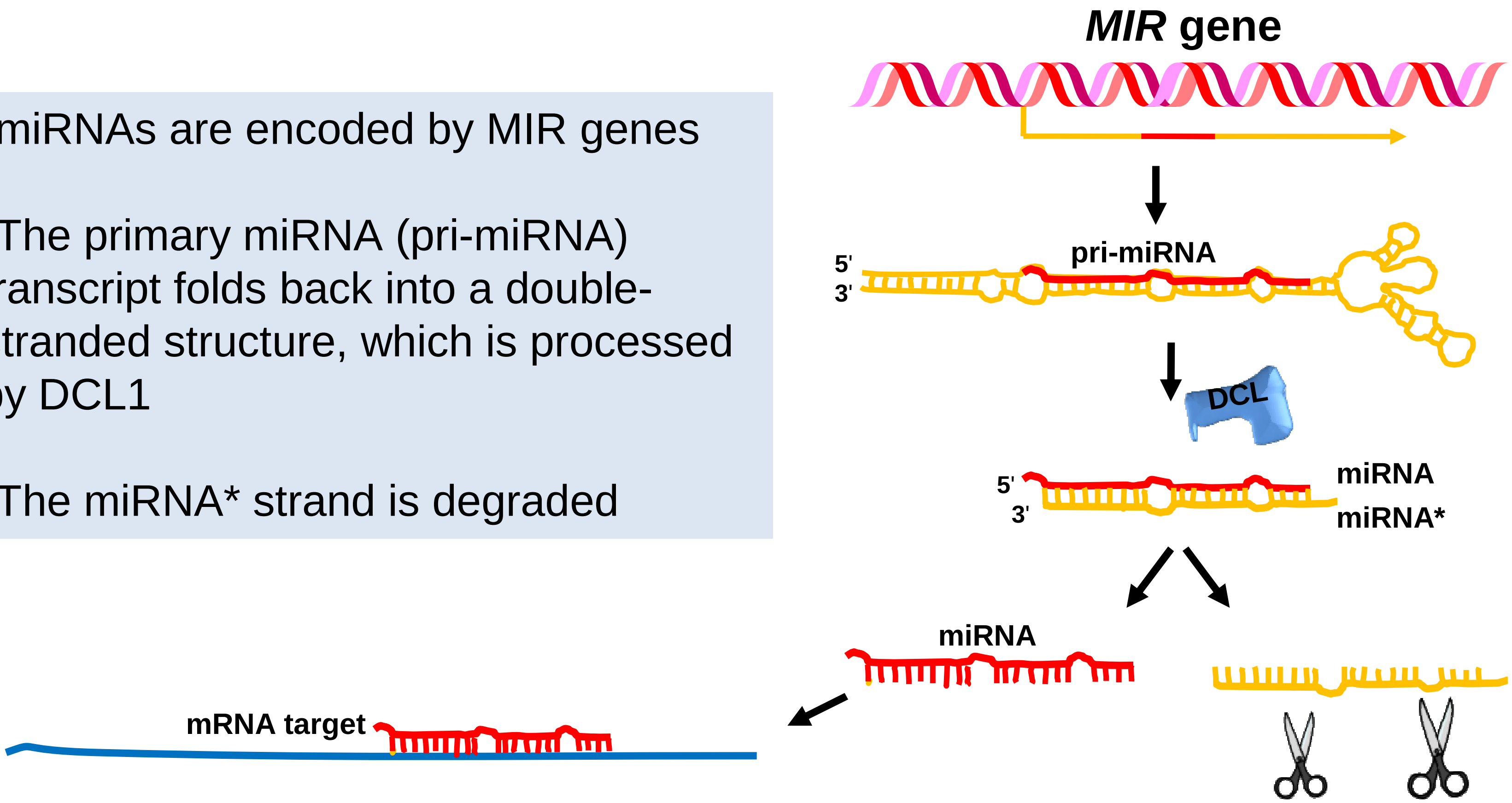
Reprinted from Vaucheret, H. (2008) Plant ARGONAUTES. Trends Plant Sci. 13: 350-358 with permission from El

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

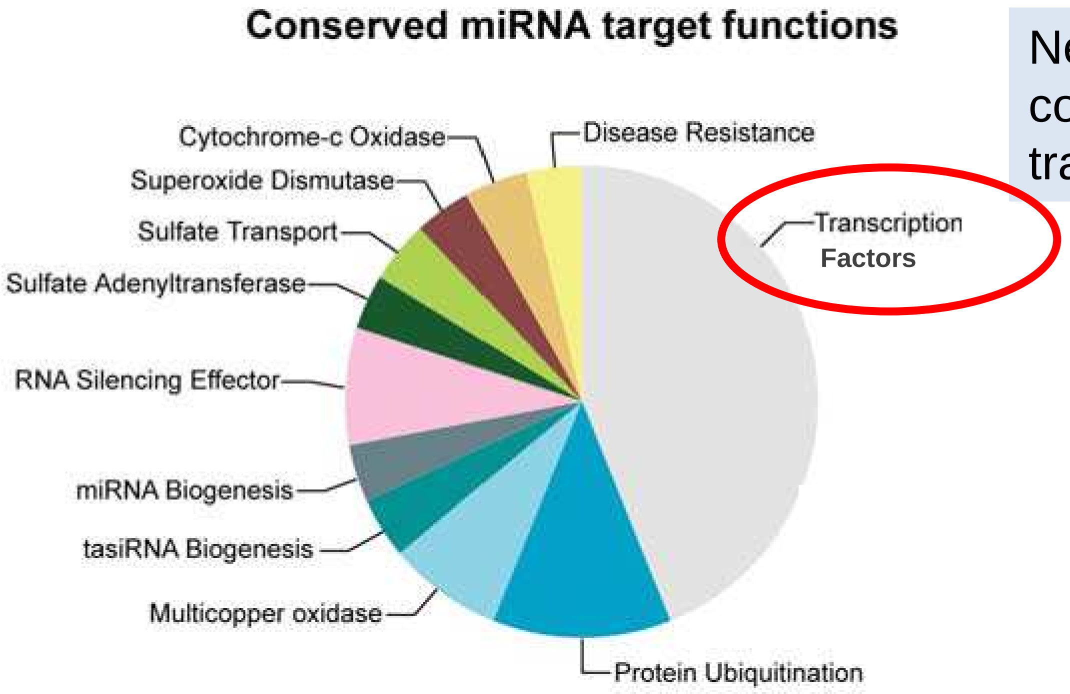
- miRNAs are encoded by MIR genes

- The primary miRNA (pri-miRNA) transcript folds back into a double-stranded structure, which is processed by DCL1

- The miRNA* strand is degraded



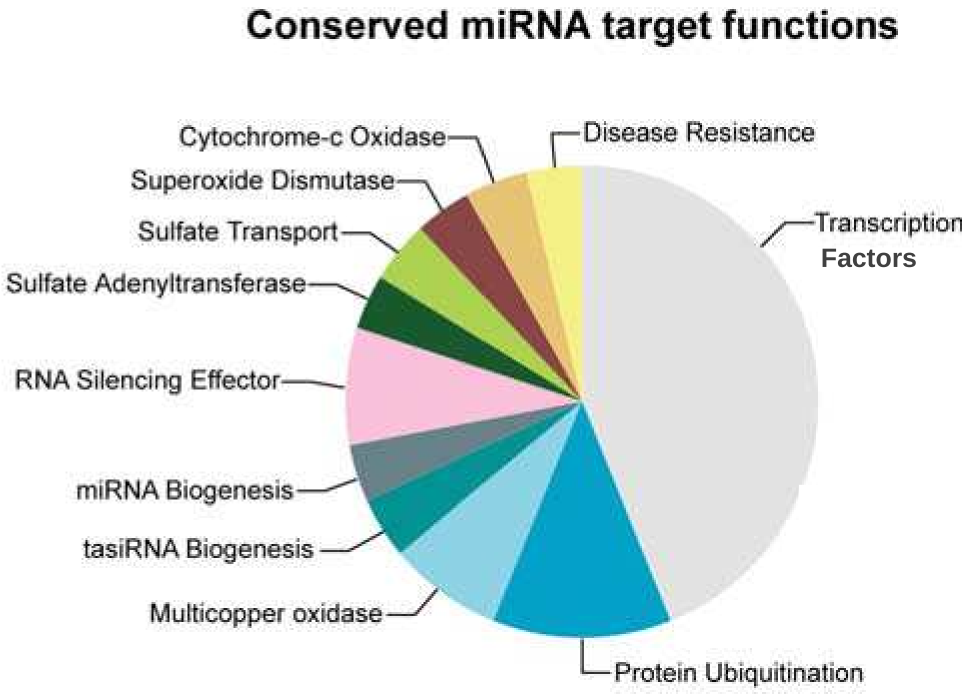
Some miRNAs are highly conserved and important gene regulators



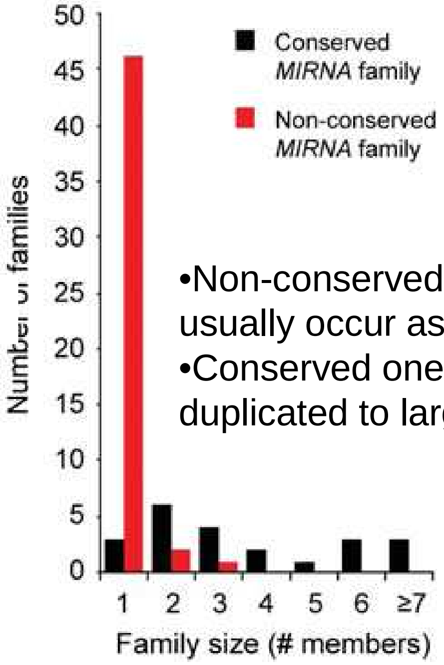
Nearly half of the targets of conserved miRNAs are transcription factors.

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.



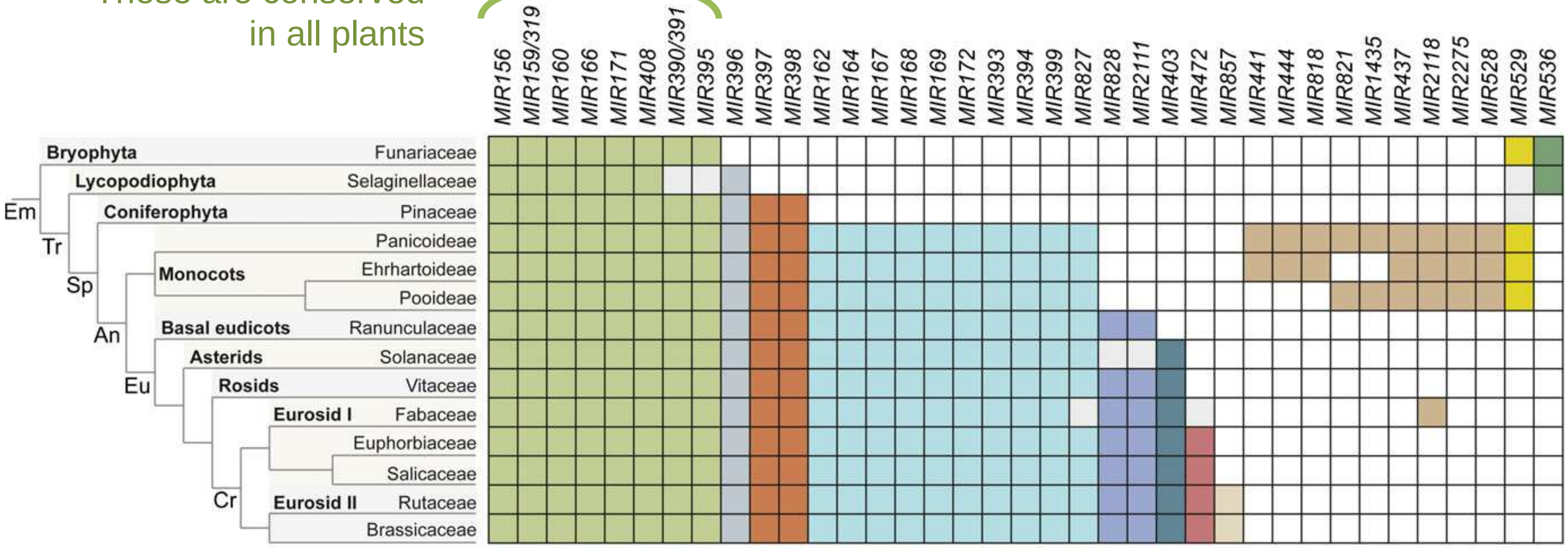
- Non-conserved *MIRNA* families usually occur as single genes
- Conserved ones have often duplicated to larger gene families

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

These are conserved in all angiosperms

These are conserved in all plants



		MIR156	*MIR159/319*	*MIR160*	*MIR166*	*MIR171*	*MIR408*	*MIR390/391*	*MIR395*	*MIR396*	*MIR397*	*MIR398*	*MIR162*	*MIR164*	*MIR167*	*MIR168*	*MIR169*	*MIR172*	*MIR393*	*MIR394*	*MIR399*	*MIR827*	*MIR828*	*MIR2111*	*MIR403*	*MIR472*	*MIR857*	*MIR441*	*MIR444*	*MIR818*	*MIR821*	*MIR1435*	*MIR437*	*MIR2118*	*MIR2275*	*MIR528*	*MIR529*	*MIR536*
Bryophyta	Funariaceae	■	■	■	■	■	■	■	■																												■	■
Lycopodiophyta	Selaginellaceae	■	■	■	■	■	■	░	░	■																											░	■
Coniferophyta	Pinaceae	■	■	■	■	■	■	■	■	■	■	■																									░	
Monocots	Panicoideae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■						■	■	■	■	■	■	■	■	■	■	
	Ehrhartoideae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■						■	■	■			■	■	■	■	■	
	Pooideae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■								■	■	■	■	■	■	■	■	
Basal eudicots	Ranunculaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■														
Asterids	Solanaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	░	░	■													
Rosids	Vitaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■													
Eurosid I	Fabaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	░	■	■	■	░								■				
	Euphorbiaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
	Salicaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
Eurosid II	Rutaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■											
	Brassicaceae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■											

Em = Embryophyta Tr = Tracheophyta Sp = Spermatophyta An = Angiosperms Eu = Eudicots Cr = Core rosids

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

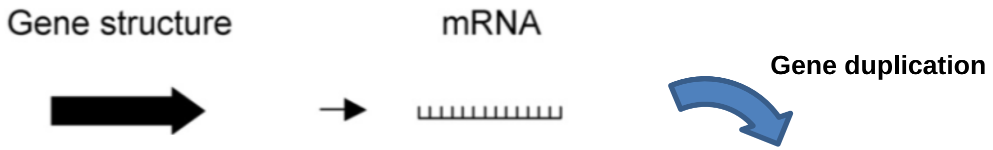
Reprinted from Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002) MicroRNAs in plants. Genes Dev. 16: 1616–1626.

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

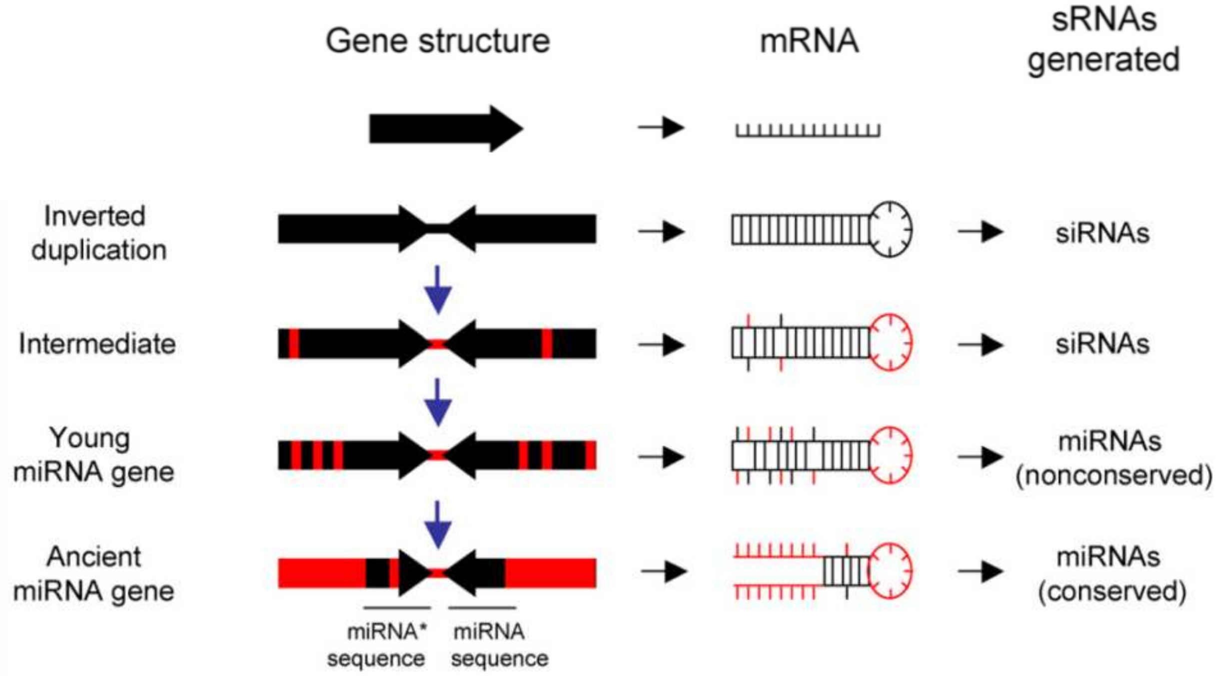
Adapted from Willmann, M.R., and Poethig, R.S. (2007) Conservation and evolution of miRNA regulatory programs in plant development. Curr. Opin. Plant Biol. 10: 503–511..

Plant miRNAs are thought to be distantly related to their targets



Plant miRNAs are thought to be derived from their target sequences following gene duplication, inverted duplication and divergence.

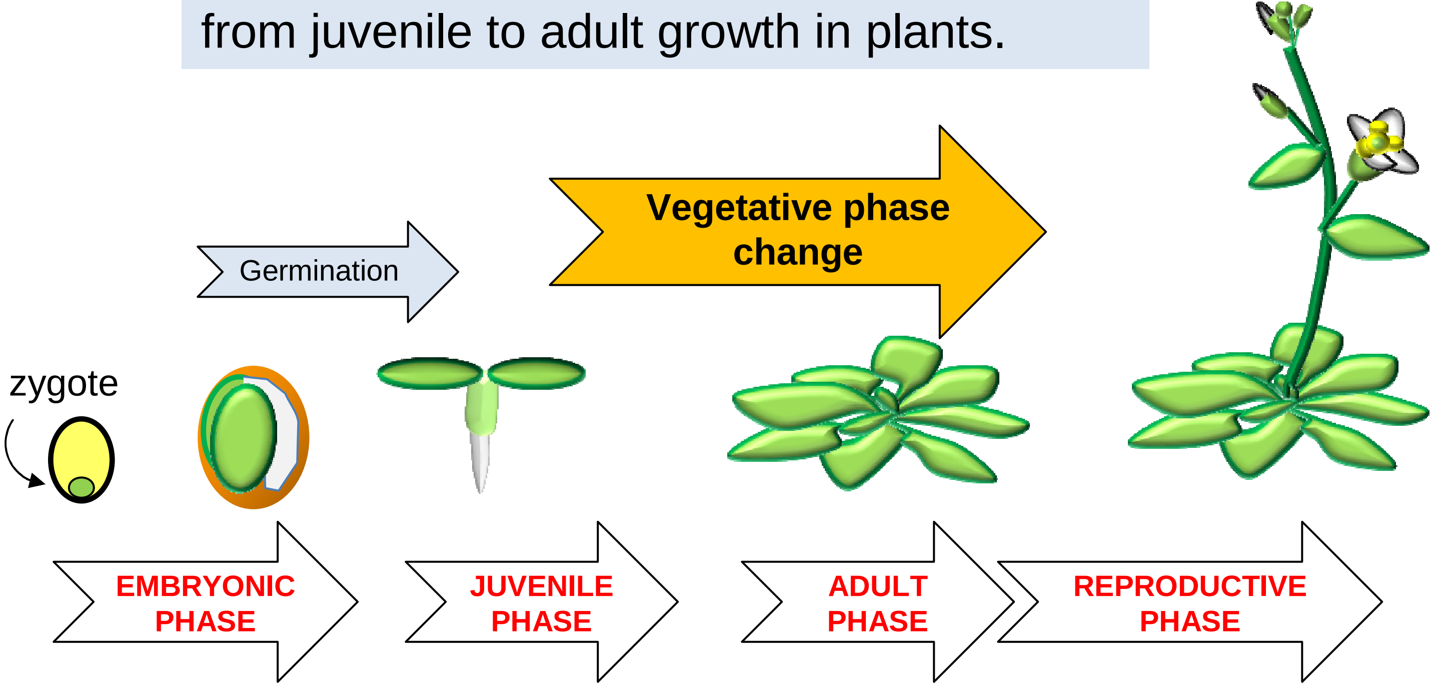
Only some miRNAs confer selective advantage and are retained and further duplicated.



Reprinted 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 with permission from Elsevier.

miRNAs and vegetative phase change

Vegetative phase change is the transition from juvenile to adult growth in plants.



Germination

Vegetative phase change

zygote

EMBRYONIC PHASE

JUVENILE PHASE

ADULT PHASE

REPRODUCTIVE PHASE

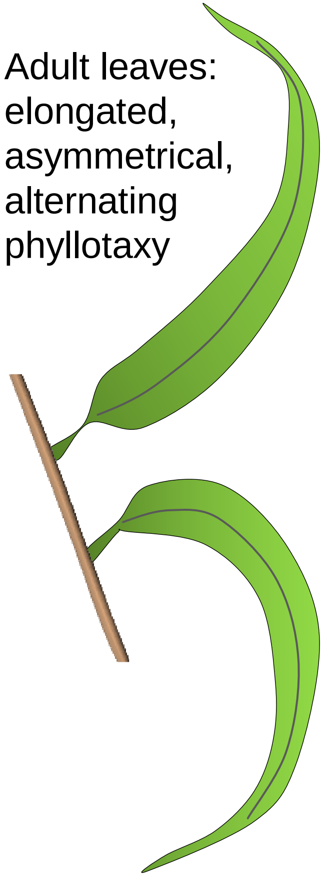
Vegetative phase change affects morphology and reproductive competence

Some cacti have very different juvenile and adult growth patterns.

Photos courtesy of James Mauseth

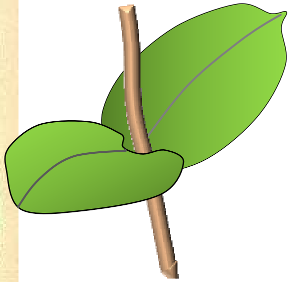
Phase change can affect leaf shape, phyllotaxy, and trichome patterns

Adult leaves:
elongated,
asymmetrical,
alternating
phyllotaxy



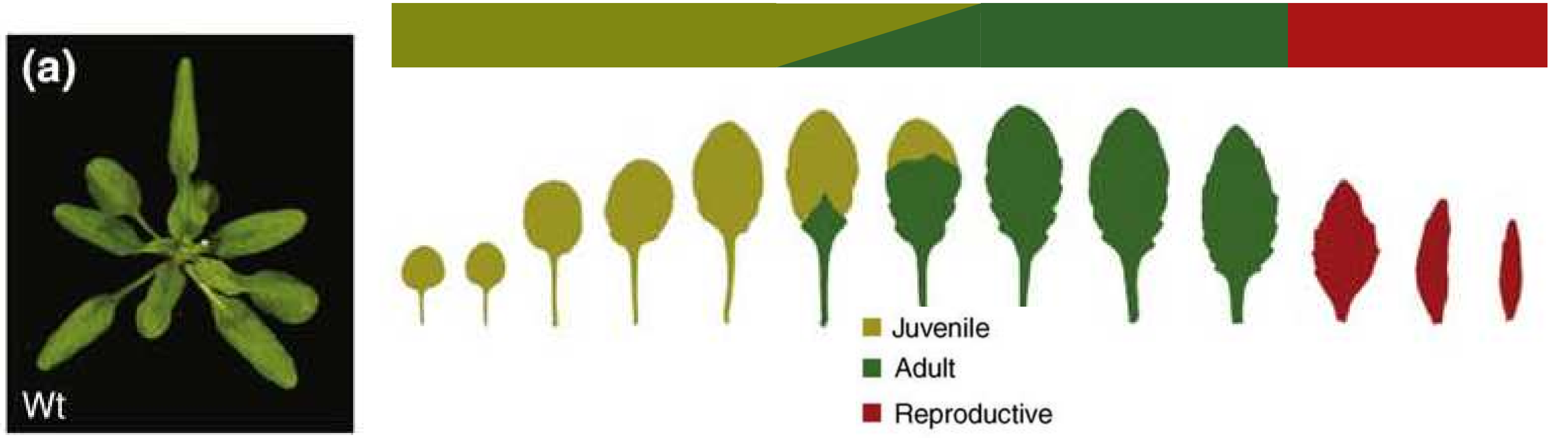
Eucalyptus globulus

Juvenile leaves:
rounded,
symmetrical,
opposite
phyllotaxy



Eucalyptus leaves are strongly dimorphic, as are leaves of holly and ivy. In other plants including Arabidopsis and maize the change is more subtle.

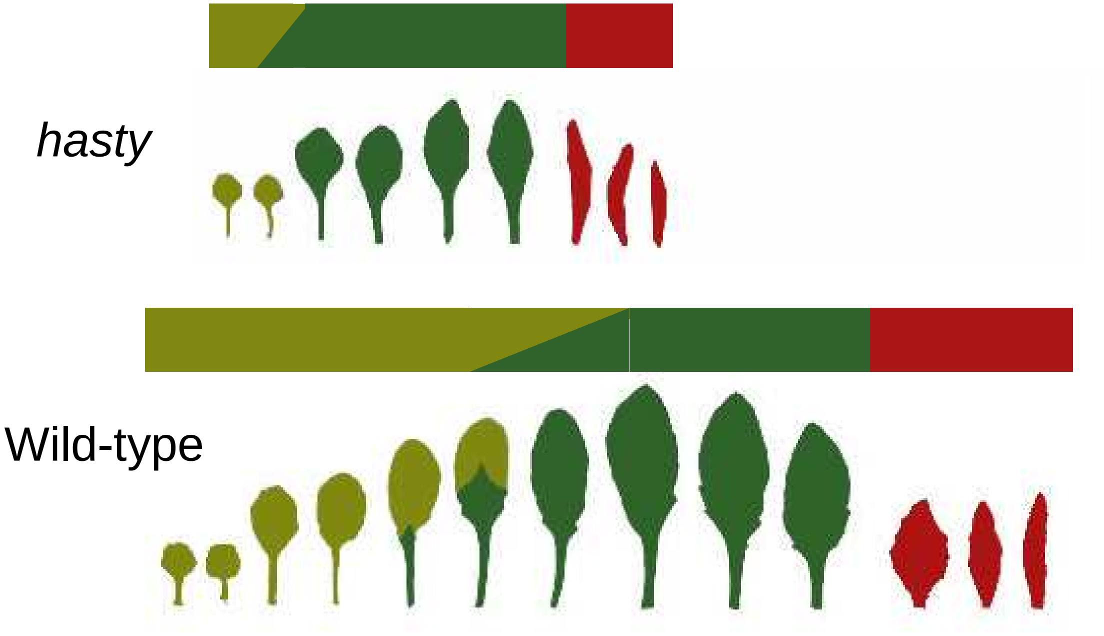
In Arabidopsis, phase change affects leaf shape and trichome patterning



Juvenile leaves are rounder, less serrated, and have trichomes only on the upper (adaxial) surface; adult leaves also have trichomes on the lower (abaxial) surface.

Reprinted from Poethig, R.S. (2009) Small RNAs and developmental timing in plants. Curr. Opin. Genet. Devel. 19: 374-378, with permission from Elsevier.

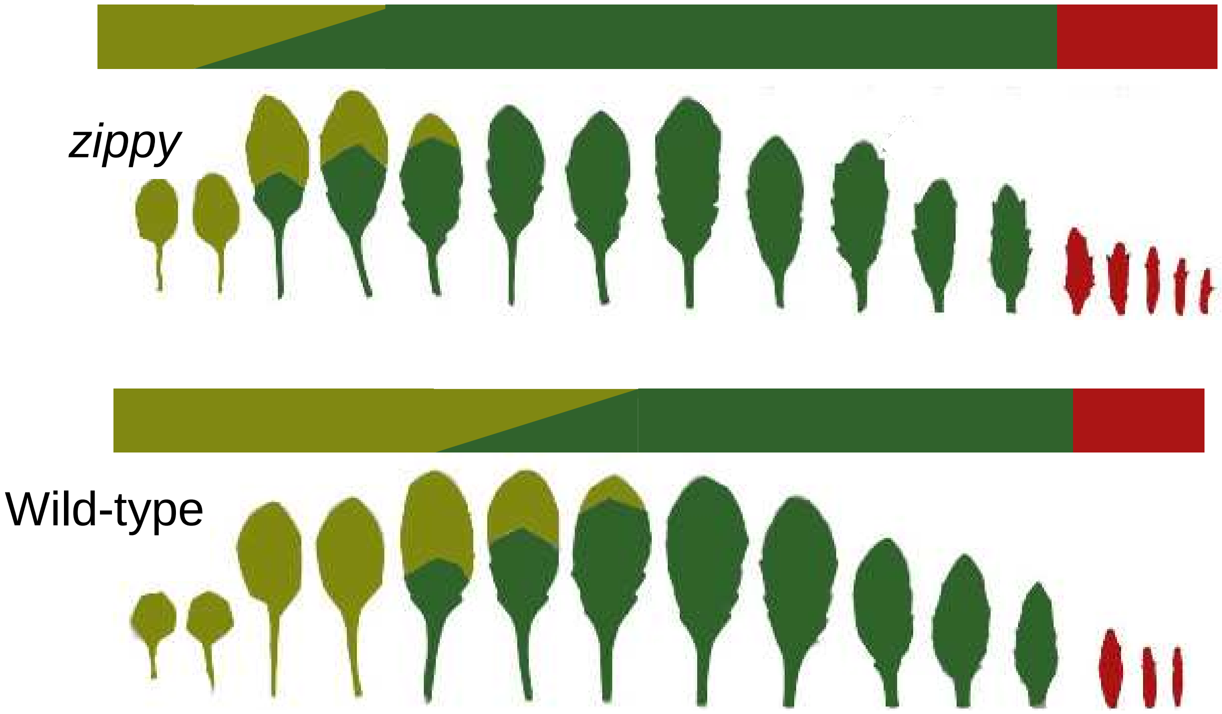
Phase change is specified by miRNAs



HASTY, with a shortened juvenile phase, encodes a protein needed for miRNA export from nucleus to cytoplasm.

Reprinted with permission from Bollman, K.M. Aukerman, M.J., Park, M.-Y., Hunter, C., Berardini, T.Z., and Poethig, R.S. (2003) HASTY, the *Arabidopsis* ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. Development 130: 1493-1504.

Phase change is specified by miRNAs



Loss-of-function *zippy* mutants prematurely express adult vegetative traits. *ZIPPY* encodes an ARGONAUTE protein, AGO7.

Reprinted from Hunter, C., Sun, H., and Poethig, R.S. (2003) The *Arabidopsis* heterochronic gene *ZIPPY* is an *ARGONAUTE* family member. Curr. Biol. 13: 1734–1739, with permission from Elsevier.

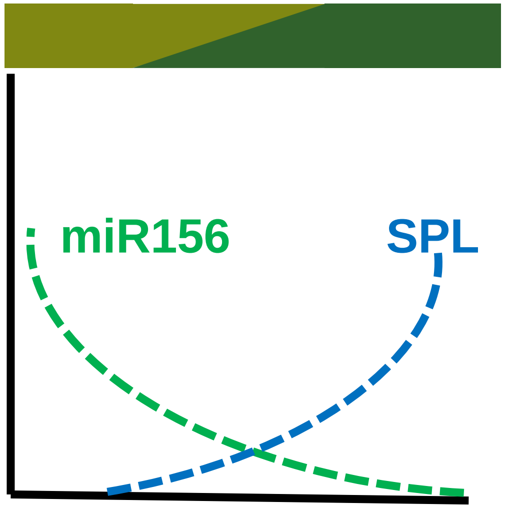
miR156 overexpression prolongs juvenile phase in Arabidopsis

Reprinted from Poethig, R.S. (2009) Small RNAs and developmental timing in plants. Curr. Opin. Genet. Devel. 19: 374-378, with permission from Elsevier.

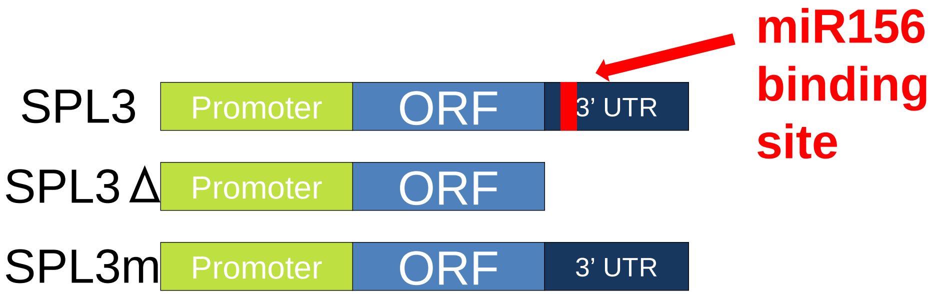
miR156 targets *SPL* genes, promoters of phase change

The SPL genes are a family of transcription factors that are miR156 targets.

In wild-type plants, miR156 expression decreases with plant age, allowing SPL to accumulate and promote phase change.



miR156-resistant SPL promotes precocious phase change

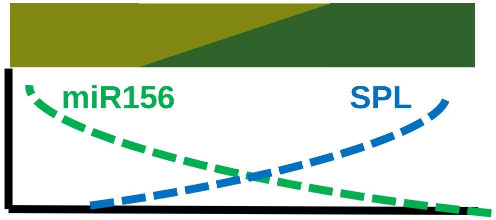

SPL3 | Promoter | ORF | 3’ UTR

SPL3 Δ | Promoter | ORF

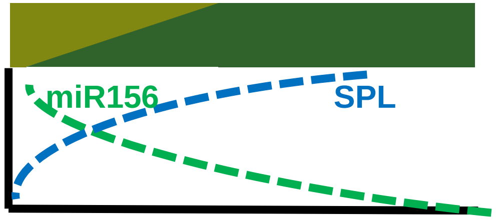
SPL3m | Promoter | ORF | 3’ UTR

miR156 binding site

Wild-type

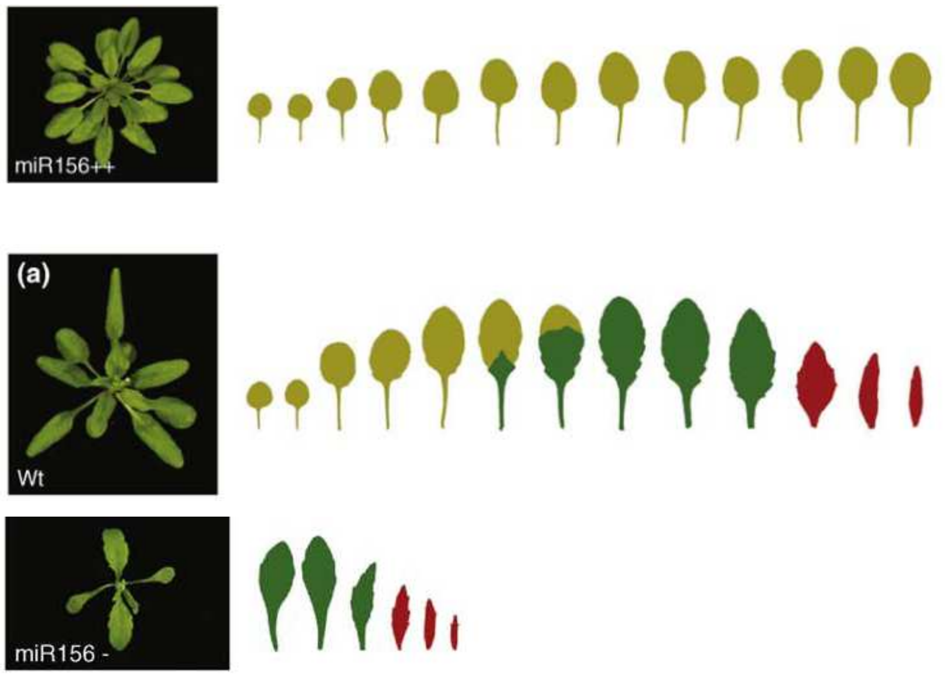


miR156-resistant



Reproduced with permission from Wu, G., and Poethig, R.S. (2006) Temporal regulation of shoot development in *Ara*
thaliana by miR156 and its target SPL3. Development **133**: 3539–3547.

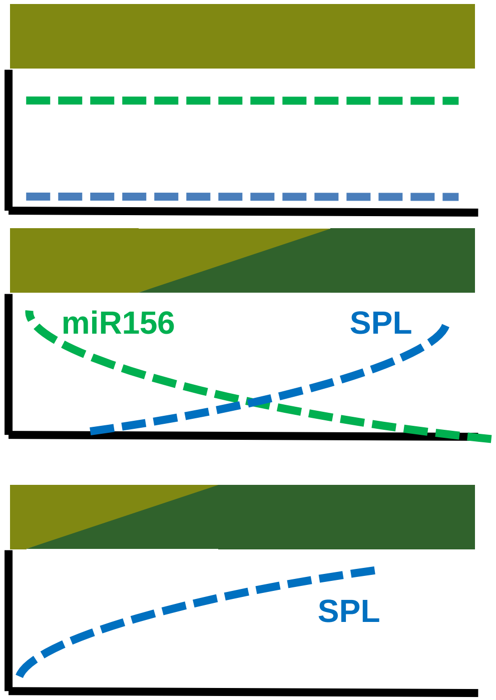
miR156 loss-of-function promotes precocious phase change



miR156
OX

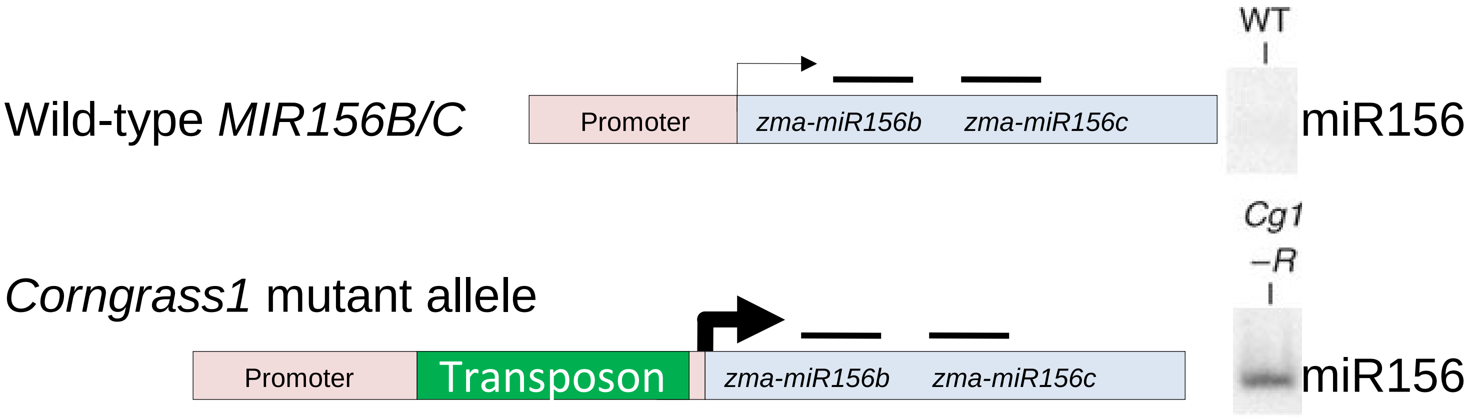
Wild-
type

miR156-
loss-of-
function



Reprinted from Poethig, R.S. (2009) Small RNAs and developmental timing in plants. Curr. Opin. Genet. Devel. 19: 374-378, with permission from Elsevier.

The function of miR156 in promoting developmental change is conserved



Wild-type *MIR156B/C*

Promoter

zma-miR156b *zma-miR156c*

WT

miR156

Corngrass1 mutant allele

Promoter

Transposon

zma-miR156b *zma-miR156c*

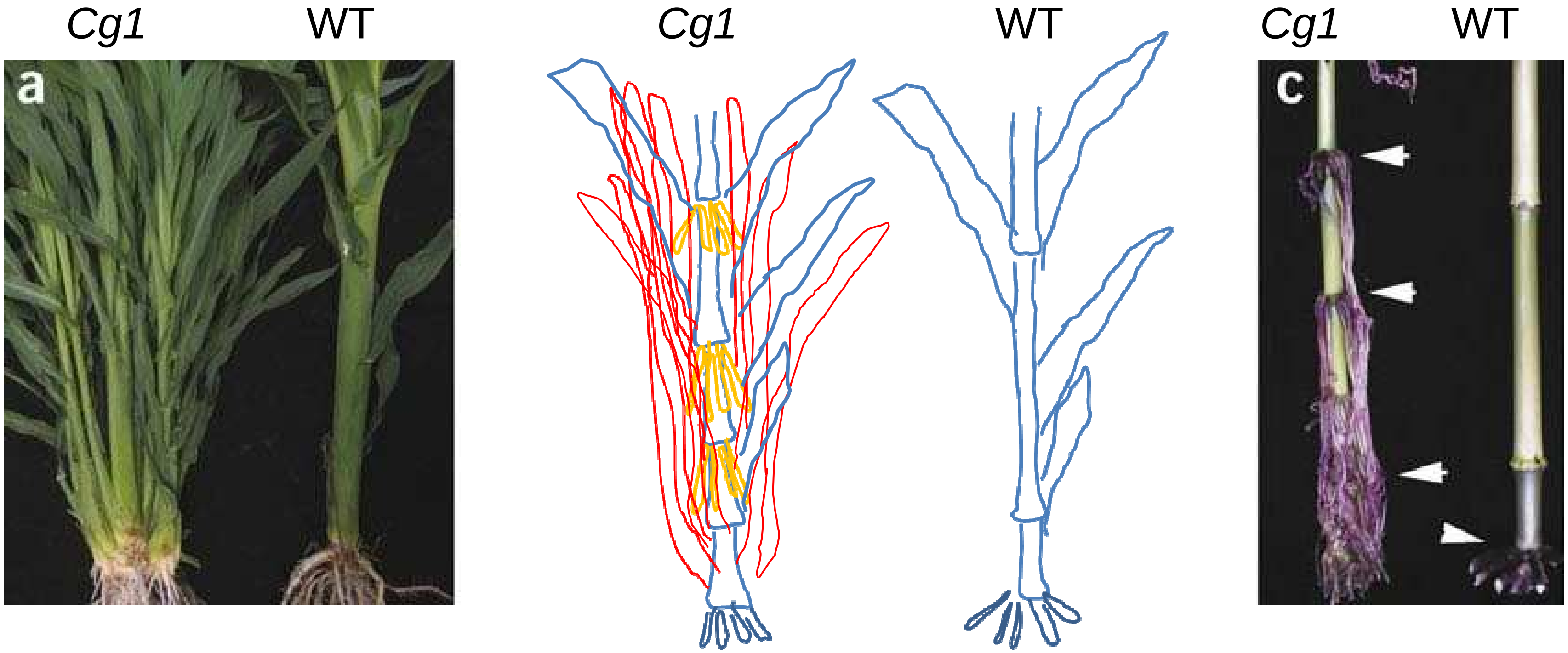
Cg1
–R

miR156

In the *Zea mays Corngrass1* mutant (*Cg1*), a transposon inserted upstream of the *MIR156B/C* locus increases its transcription level, causing more miR156 to accumulate.

Reprinted by permission from Macmillan Publishers, Ltd. Copyright 2007. Chuck, G., Cigan, A.M., Saeteurn, K., and Hake, S. (2007) The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nat. Genet. 39: 544–549.

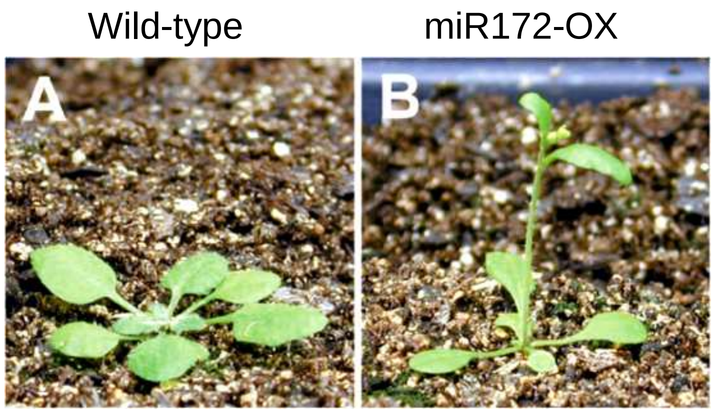
Elevated expression of miR156 in *Cg1* prolongs the juvenile phase



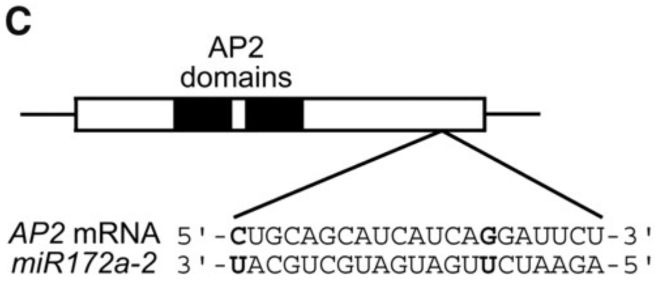
Adult *Corngrass1* plants express juvenile traits of tillers (red) in leaf axils and roots (yellow) from leaf nodes.

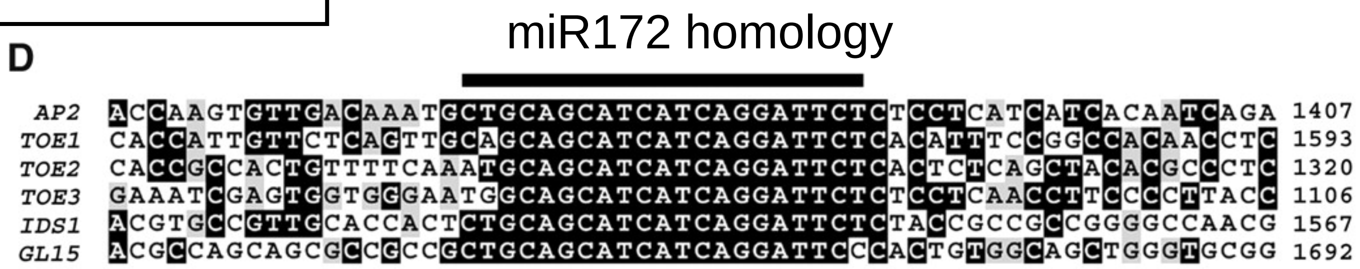
Reprinted by permission from Macmillan Publishers, Ltd. Copyright 2007. Chuck, G., Cigan, A.M., Saeteurn, K., and Hake, S. (2007) The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nat. Genet. 39: 544–549.

miR172 promotes flowering in Arabidopsis by targeting AP2-like transcription factors



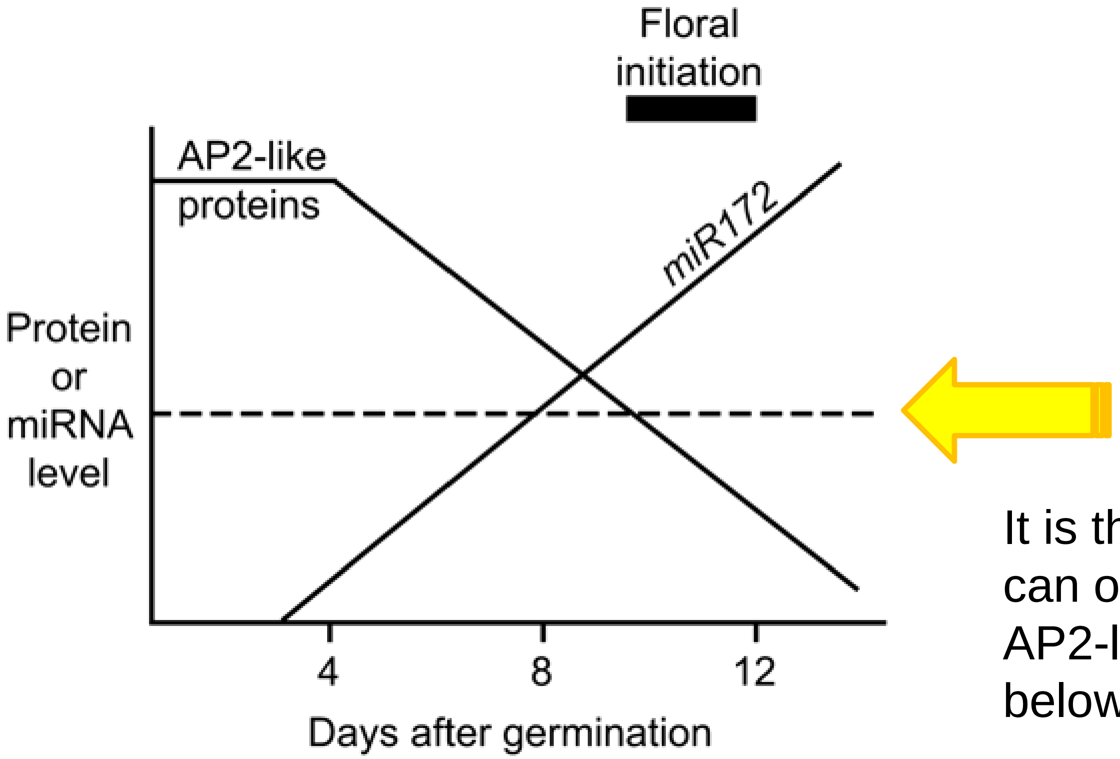
Arabidopsis plants overexpressing miR172 flower early.





Aukerman, M.J., and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-Like target genes Plant Cell 15: 2730-2741.

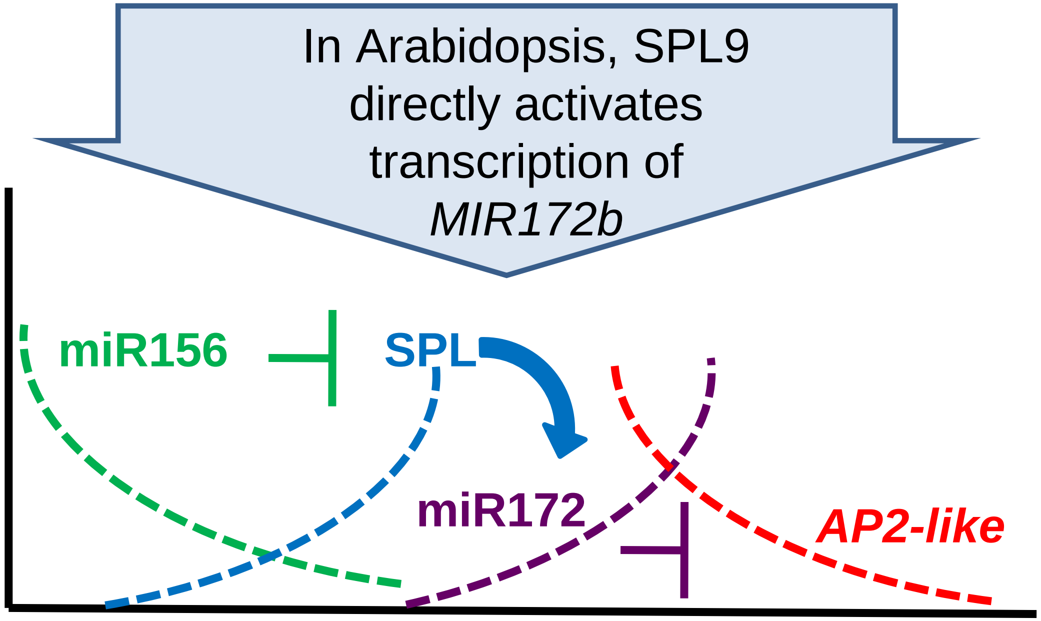
miR172 expression temporally regulates AP2-like proteins



It is thought that floral initiation can occur when the level of AP2-like floral inhibitors drops below a certain level.

Aukerman, M.J., and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-Like target genes Plant Cell 15: 2730-2741.

Phase change may involve a temporal cascade of miRNAs and transcription factors

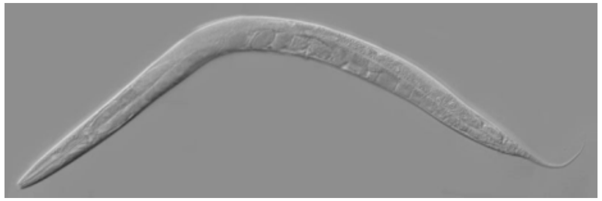


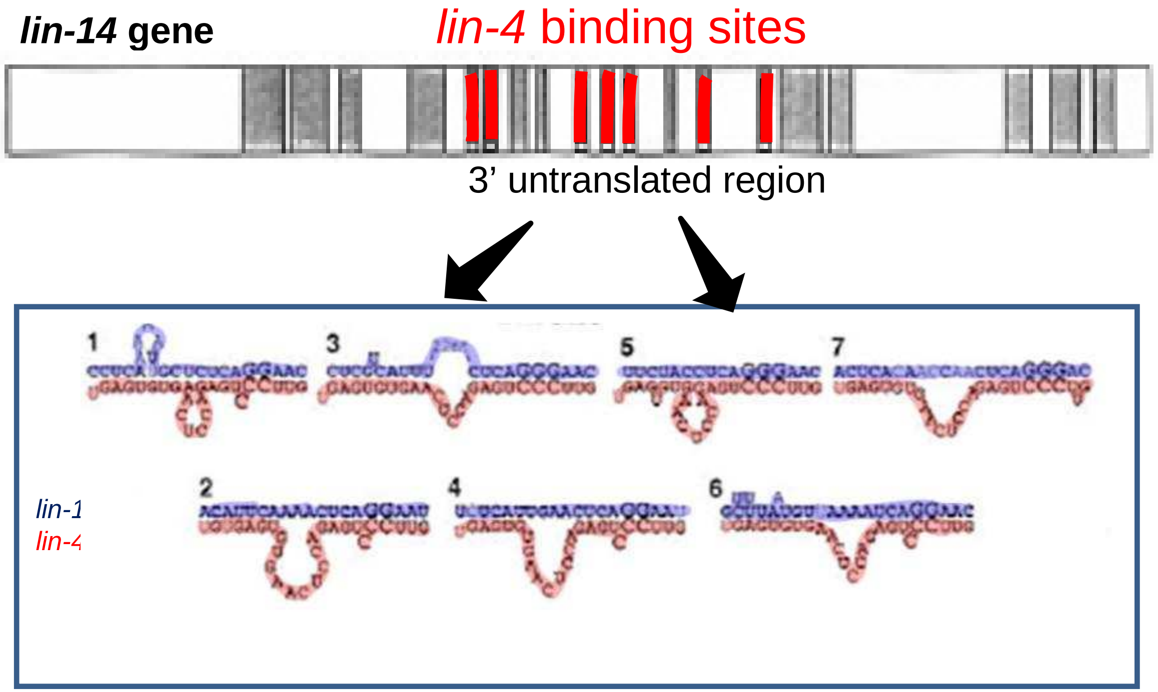
In Arabidopsis, SPL9 directly activates transcription of *MIR172b*

miRNAs regulate developmental timing in other organisms

miRNAs were discovered in studies of developmental progressions in the nematode *C. elegans*.

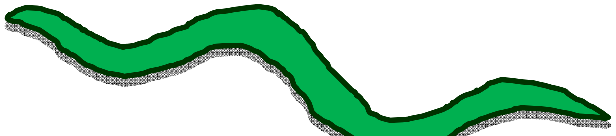
A miRNA encoded by *lin-4* is required for proper larval development.



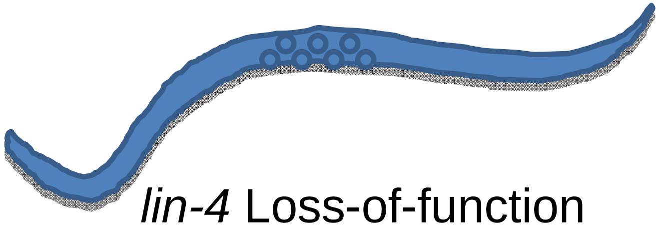


Lee, R.C., Feinbaum, R.L., and Ambrose, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75: 843–845. Wightman, B., Ha, I., and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. Cell 75: 855–862.

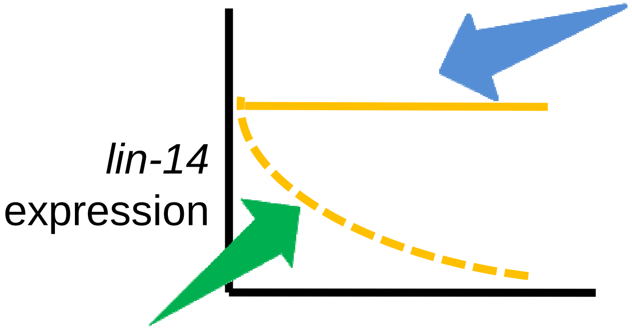
Downregulation of *lin-14* by *lin-4* is necessary for normal development



Wild-type *C. elegans*



lin-4 Loss-of-function



lin-4 loss-of-function causes *lin-14* expression to remain high.

In wild-type worms, *lin-14* is expressed early and then shut off.

***lin-4* is a negative regulator of *lin-14*.**

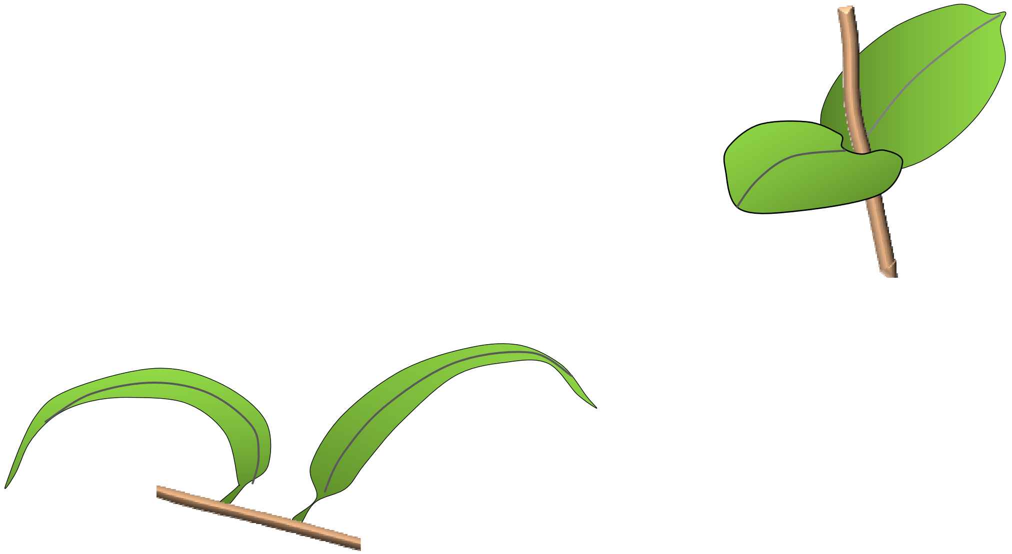
Lee, R.C., Feinbaum, R.L., and Ambrose, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75: 843–845.
Wightman, B., Ha, I., and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. Cell 75: 855–862.

miRNAs and phase change - summary

- Vegetative phase change affects morphology and reproductive competence

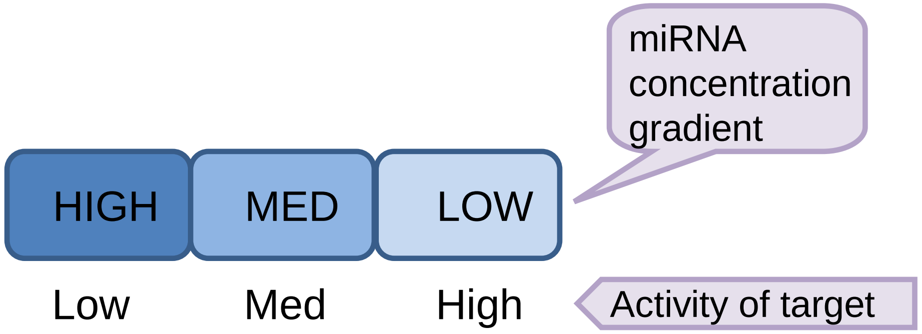
- miRNAs contribute to the temporal control of gene expression and phase change

- In the nematode *C. elegans*, *lin-4* silencing of *lin-14* is required for developmental progression



miRNAs contribute to developmental patterning

miRNA distribution patterns can spatially restrict activity of their targets



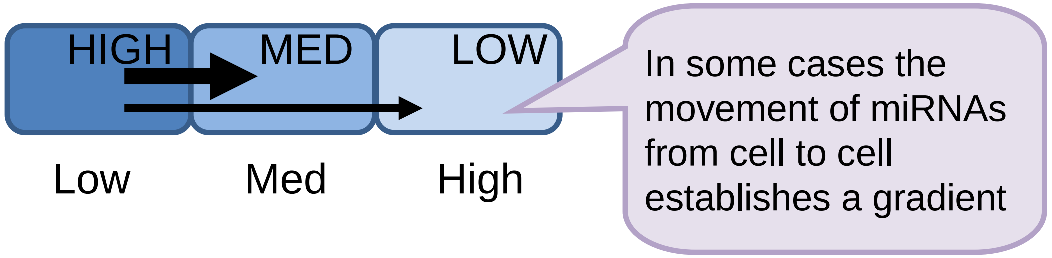
HIGH MED LOW

miRNA concentration gradient

Low Med High

Activity of target

miRNAs can move between cells to spatially restrict activity of their targets



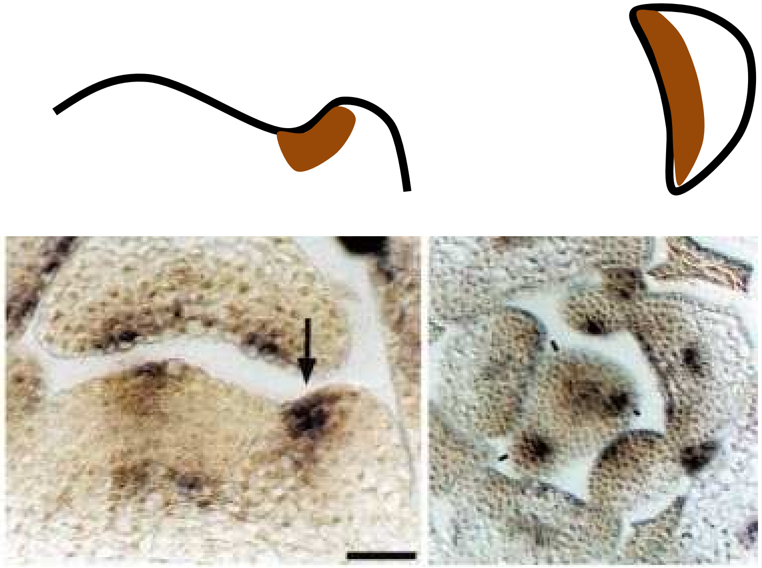
HIGH MED LOW

Low Med High

In some cases the movement of miRNAs from cell to cell establishes a gradient

The *phb-1d* mutation affects *PHB* mRNA distribution

In wild-type plants, *PHB* expression is restricted to the adaxial side of the leaves



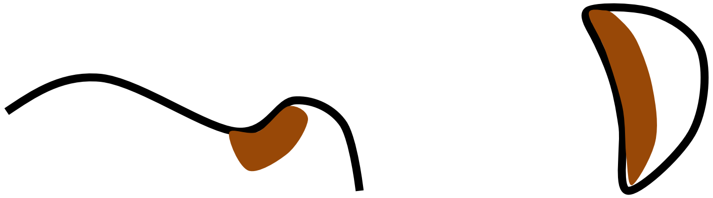
Longitudinal section Cross section

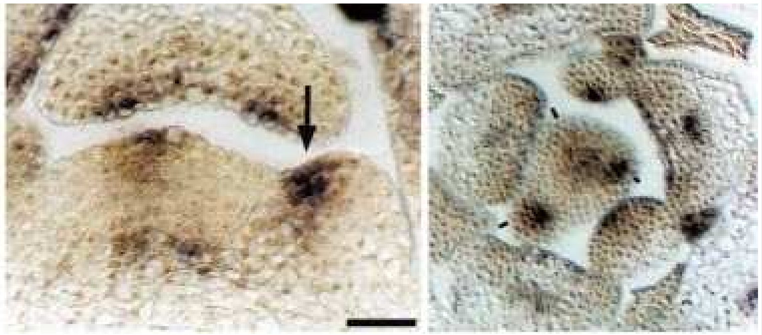
PHB and PHV encode two related HD-ZIPIII transcription factor genes

Reprinted by permission from Macmillan Publishers, Ltd: NATURE. McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shootsNature 411: 709-713, copyright 2001.

The *phb-1d* mutation affects *PHB* mRNA distribution

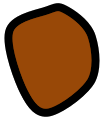
In wild-type plants, *PHB* expression is restricted to the adaxial side of the leaves





Longitudinal section Cross section

In gain-of-function *phb-1d* mutants, *PHB* is expressed everywhere, resulting in adaxialized, radially symmetric leaves.

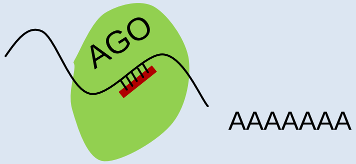


Scanning electron micrograph

Cross section

Reprinted by permission from Macmillan Publishers, Ltd: NATURE. McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shootsNature 411: 709-713, copyright 2001.

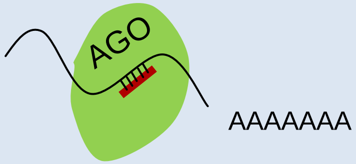
PHB expression is regulated by a miRNA



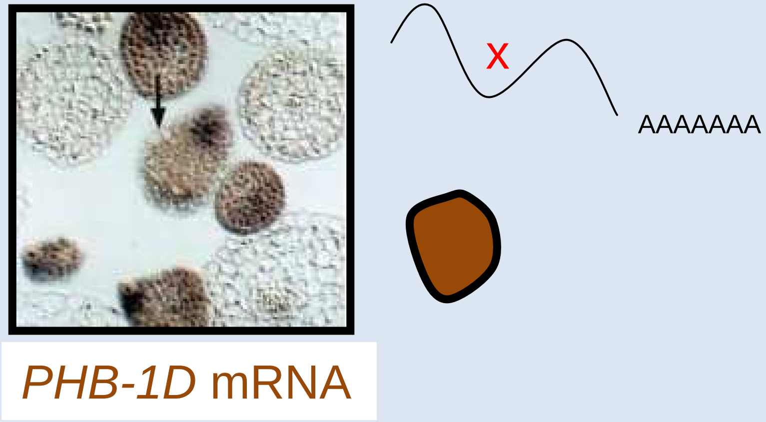
In wild-type plants, miR166 binds to the *PHB* mRNA and degrades it on the abaxial side of the leaf primordium.

Reprinted by permission from Macmillan Publishers, Ltd: NATURE. Kidner, C.A. and Martienssen, R.A. (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 428: 81-84, copyright 2004; McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. Nature 411: 709-713, copyright 2001.

Control of PHB expression by miRNA



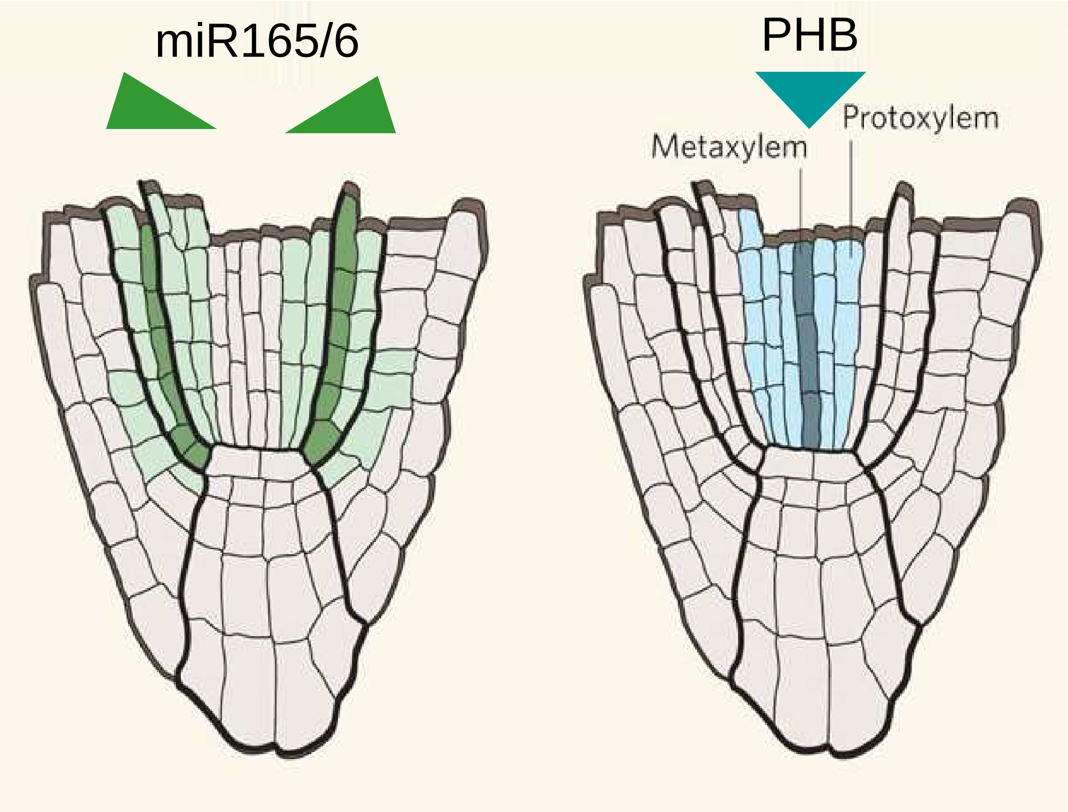
In wild-type plants, miR166 binds to the *PHB* mRNA and degrades it on the abaxial side of the leaf primordium.

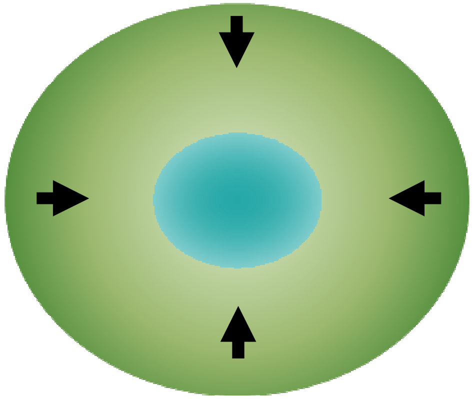


In *phb-1d* plants, base changes in the *PHB* mRNA prevent miR166 from binding to it, allowing it to accumulate throughout the leaf primordium.

Reprinted by permission from Macmillan Publishers, Ltd: NATURE. Kidner, C.A. and Martienssen, R.A. (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 428: 81-84, copyright 2004; McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. Nature 411: 709-713, copyright 2001.

In roots, miR165/6 moves from endodermis into vascular cylinder

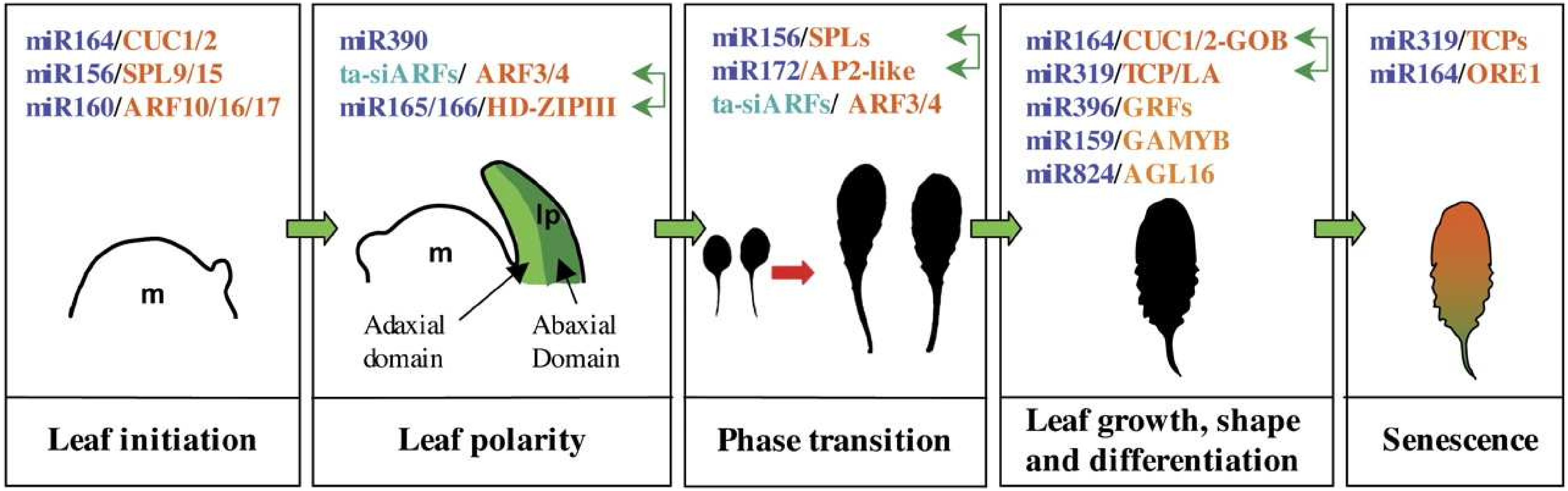




Movement of miR165/6 inwards from the endodermis in which it is produced helps to establish the radial pattern of the root

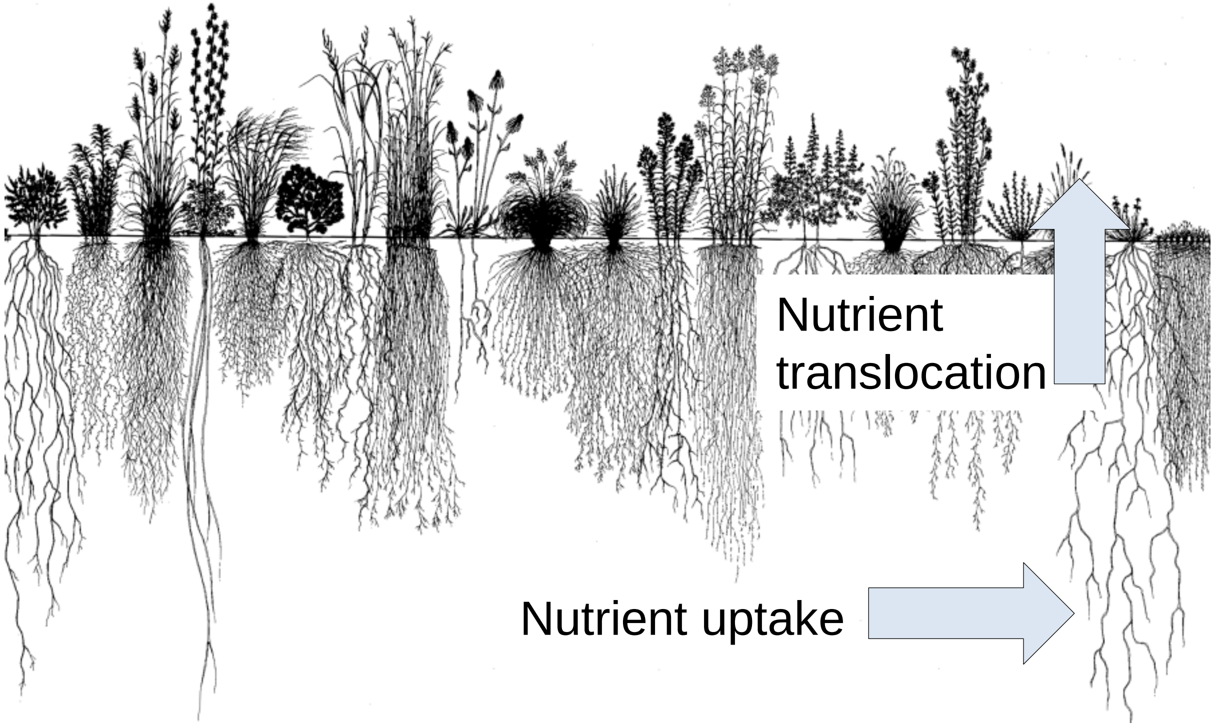
Reprinted by permission from Macmillan Publshers Ltd. Scheres, B. (2010). Developmental biology: Roots respond to an inner calling. Nature 465: 299-300; Carlsbecker, A. et al., (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465: 316-321.

Leaves are modulated by miRNA activity throughout develoment



Pulido, A., and Laufs, P. (2010). Co-ordination of developmental processes by small RNAs during leaf development. J.Exp.Bot. 61: 1277-1291, by permission from Oxford University Press.

miRNAs and nutrient signalling

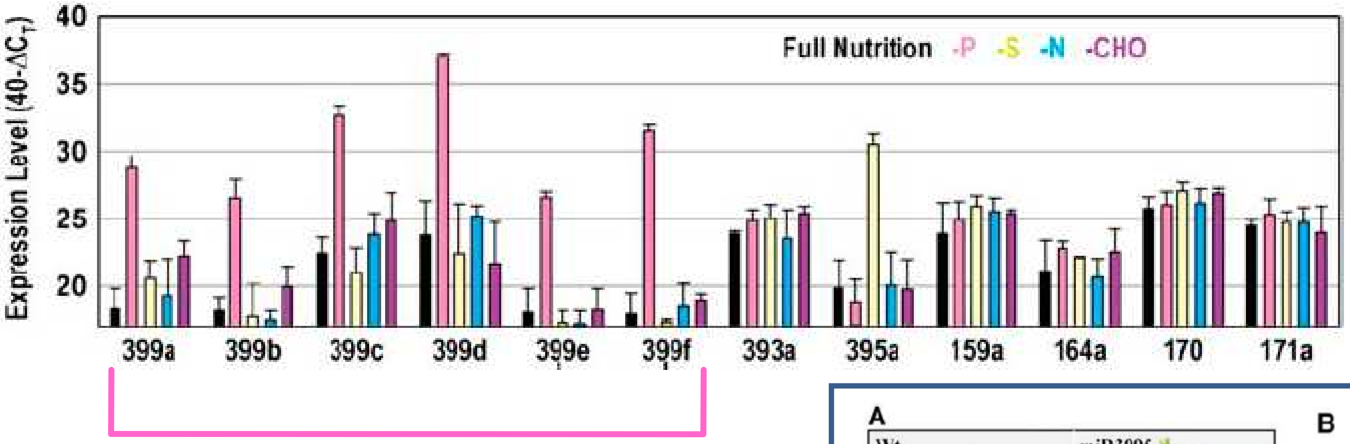


Plants take up nutrients from soil. The shoot sends signals to the root to indicate nutrient demands.

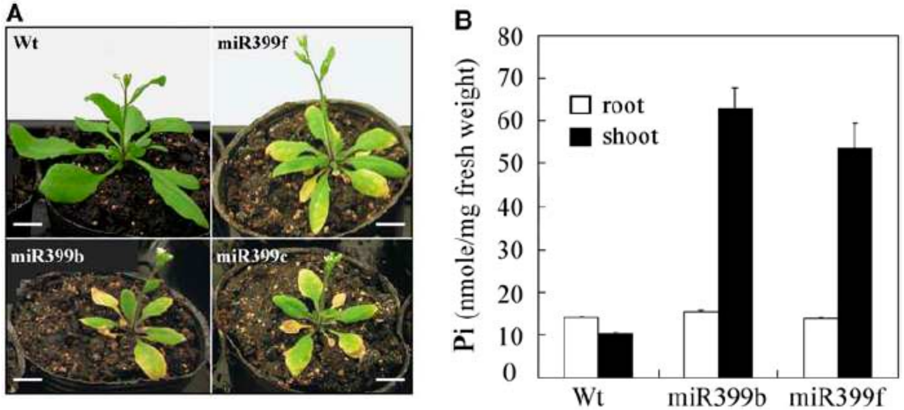
miRNAs move from shoot to root in phloem and regulate nutrient uptake.

© Heidi Natura of the Conservation Research Institute.

miR399 is induced upon phosphate starvation



Genes encoding miR399 are specifically induced by phosphate starvation.

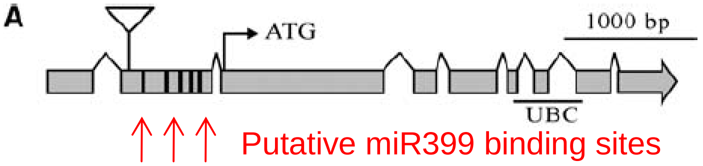


Plants that overexpress miR399 overaccumulate phosphate

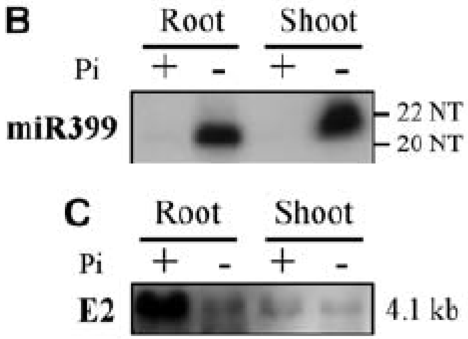
Bari, R., Pant, B.D., Stitt, M. and Scheible, W.-R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol. 141: 988–999; Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F. and Su, C.L. (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell 18: 412–421.

A ubiquitin-conjugating E2 is a target for miR399

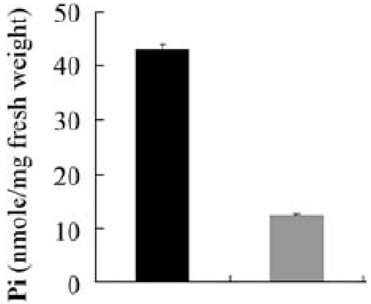
E2 gene structure



Putative miR399 binding sites



When miR399 increases, E2 expression decreases

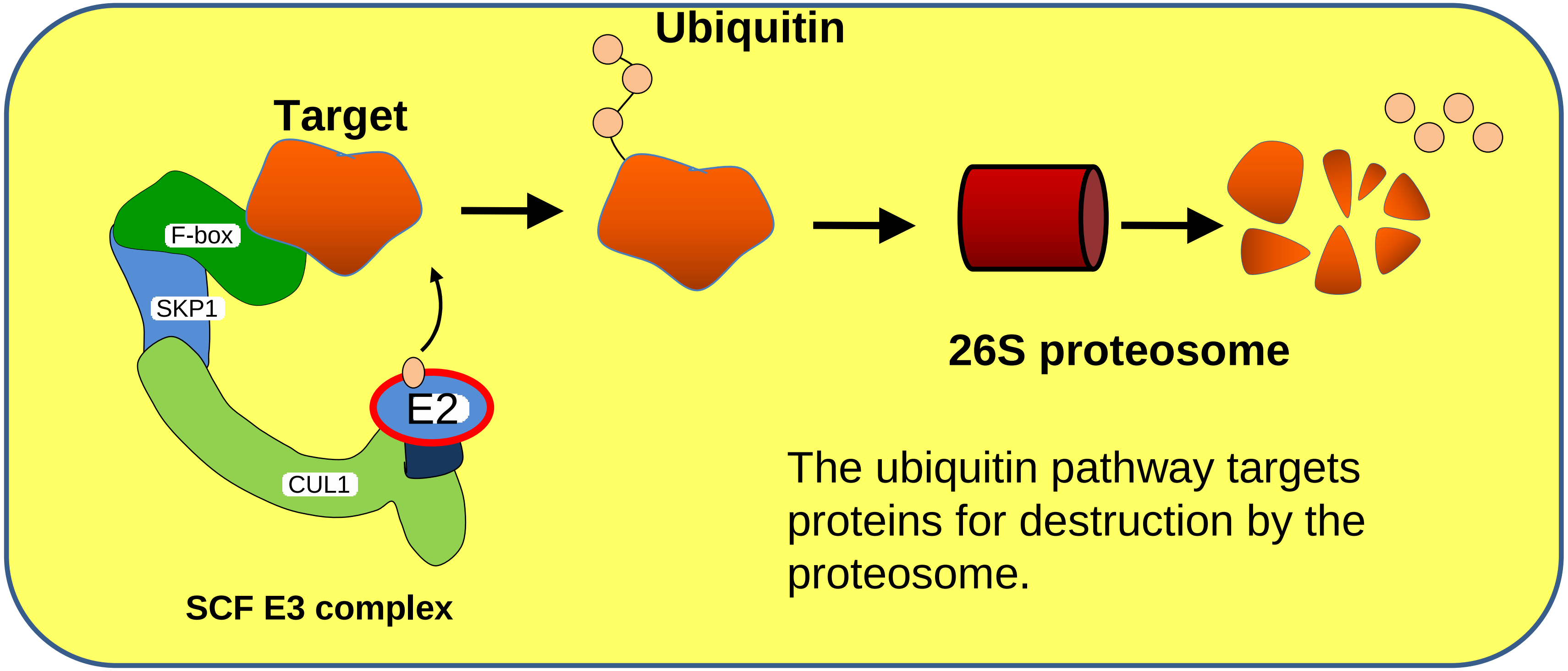


Loss-of-function of E2 (aka *pho2*) (left) causes phosphate overaccumulation
.

miR399 ⊣ E2 ⊣ Phosphate accumulation

Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F. and Su, C.L. (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell 18: 412–421.

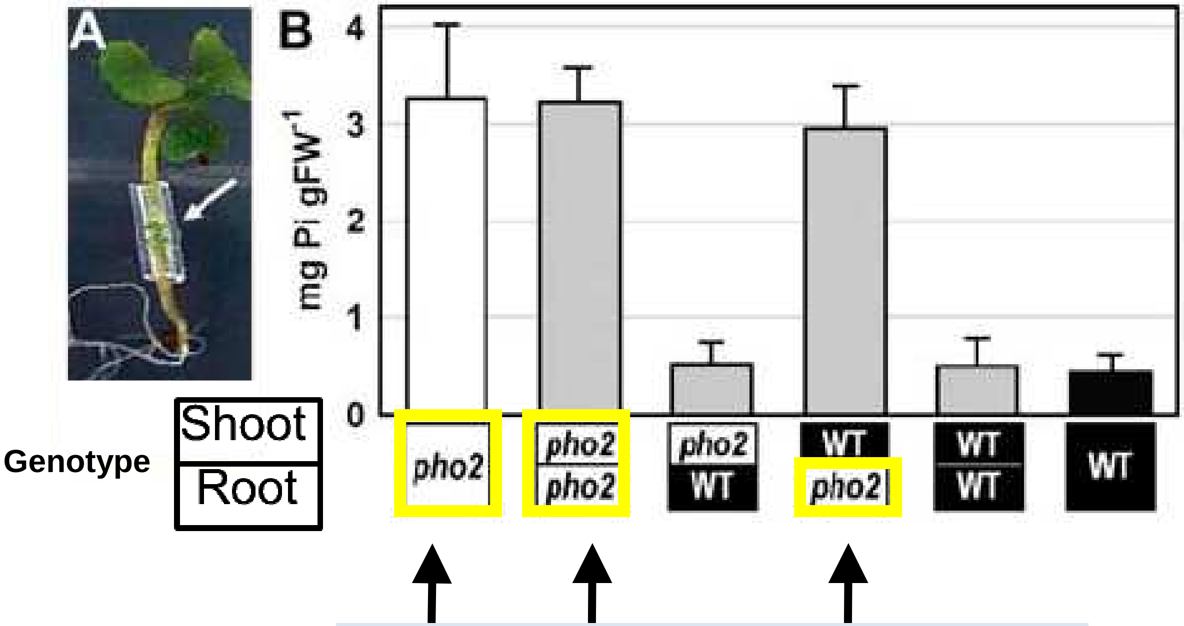
E2 is a component of the ubiquitin proteolysis pathway



The ubiquitin pathway targets proteins for destruction by the proteosome.

Adapted from Vierstra, R.D. (2009) The ubiquitin–26S proteasome system at the nexus of plant biology. Nat. Rev. Mol. Cell Biol. 10: 385-397.

The E2 conjugase functions in the root, not the shoot

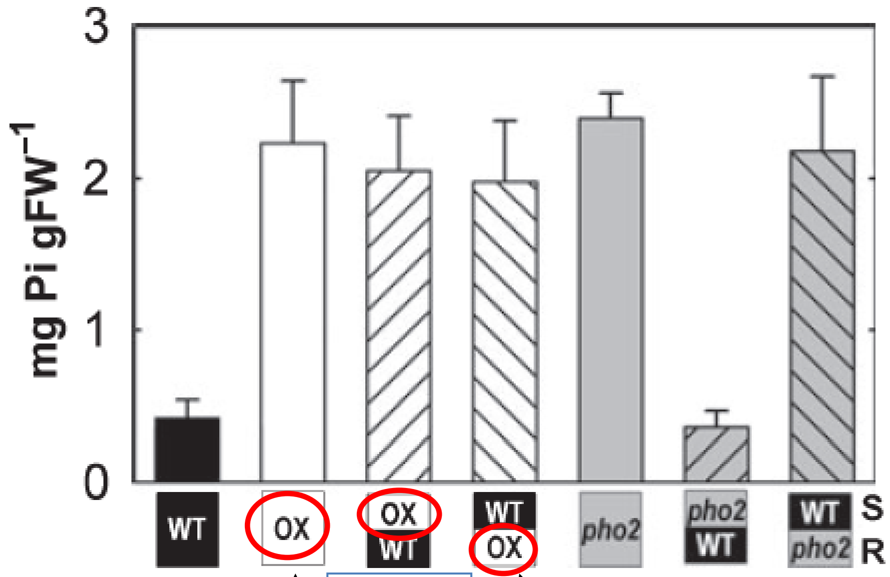


Micrografting of *pho2* and wild-type shoots and roots demonstrates that PHO2 function in the roots is sufficient for normal phosphate uptake.

Phosphate overaccumulates only in plants with mutant roots.

Bari, R., Pant, B.D., Stitt, M. and Scheible, W.-R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol. 141: 988–999

By contrast, overexpression of miR399 in the shoot or root causes increased phosphate accumulation



miR399 overexpression in shoot + root

shoot only

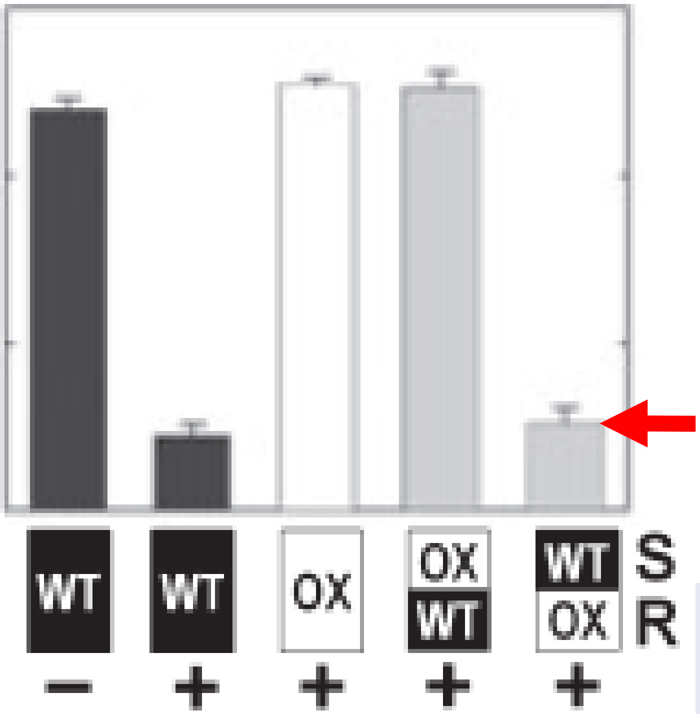
root only

How can overexpression of miR399 in the shoot affect E2 activity in the root?

Pant, B.D., Buhtz, A., Kehr, J., Scheible, W.-R. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. 5

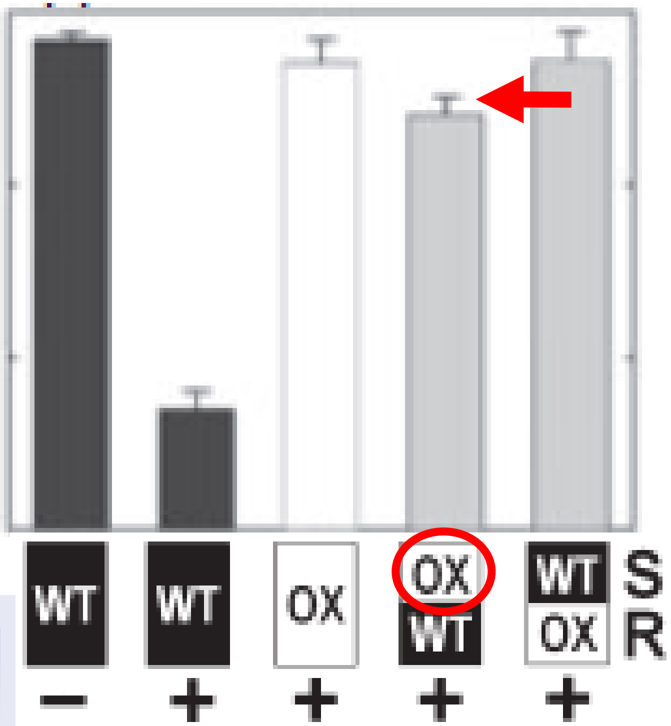
miR399 is translocated from shoot to root, but not root to shoot

miR399 levels in shoot



When miR399 is overexpressed in the root there is no increase in shoot levels.

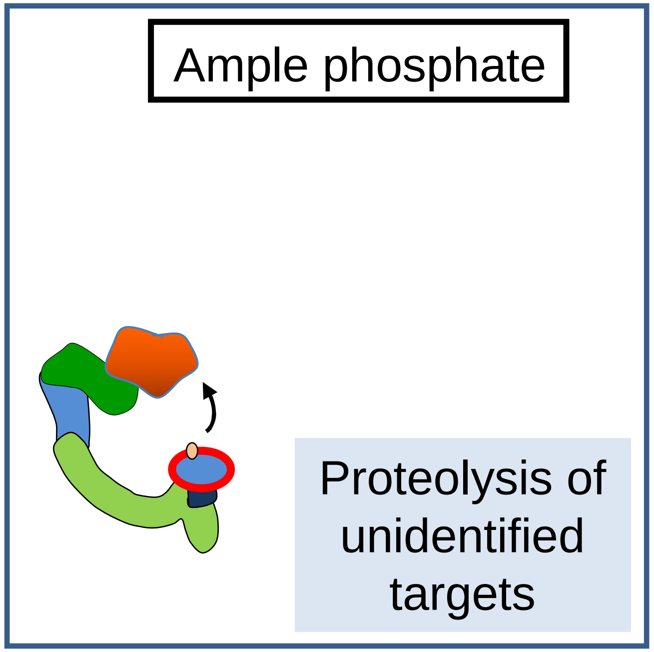
miR399 levels in root



When miR399 is overexpressed in the shoot, the level in the root ***increases***.

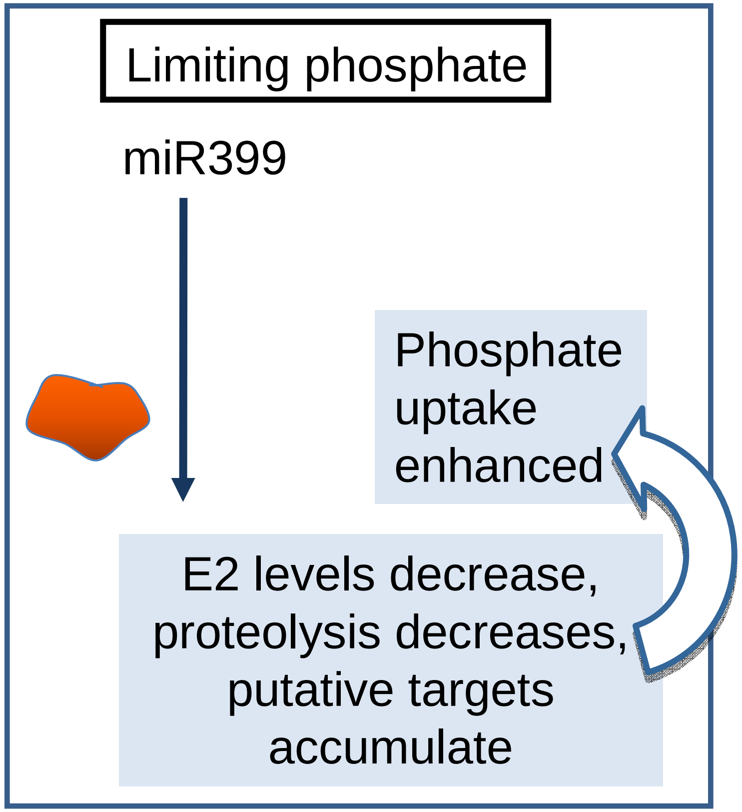
Pant, B.D., Buhtz, A., Kehr, J., Scheible, W.-R. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. 5

miR399 is a phloem-mobile systemic signal



Ample phosphate

Proteolysis of unidentified targets



Limiting phosphate

miR399

Phosphate uptake enhanced

E2 levels decrease, proteolysis decreases, putative targets accumulate

But wait – there’s more..........

miR399 activity is regulated by a *target mimic*

miR399 GUCCCGUUUAGAGGAAACCGU
PHO2   UUGGGCAAAUCUCCUUUGGCA

miR399 is highly complementary to its target, *PHO2*

miR399 activity is regulated by a *target mimic*

miR399 GUCCCGUUUAGAGGAAACCGU
PHO2   UUGGGCAAAUCUCCUUUGGCA

miR399 is highly complementary to its target, *PHO2*

IPS1 binds miR399 but has additional nucleotides at the cleavage site, so it is not cleaved

miR399 GUCCCGUUGAGAGGAAACCGU 5’
IPS1   UUGGGCAACUUUCCUUUGGCA
CUA

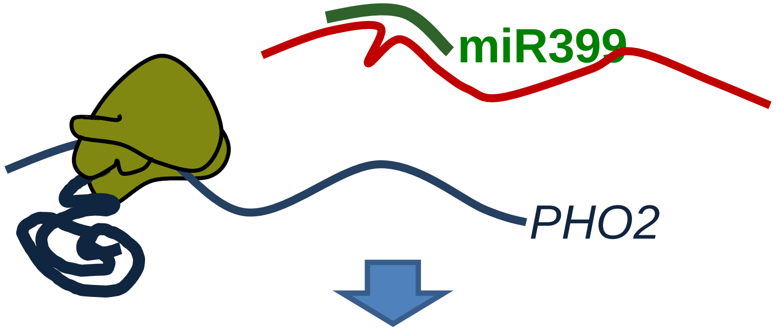
Redrawn from Franco-Zorrilla, J. M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., and Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39: 1033–1037.

miR399 activity is reduced when *IPS1* is present

No *IPS1*

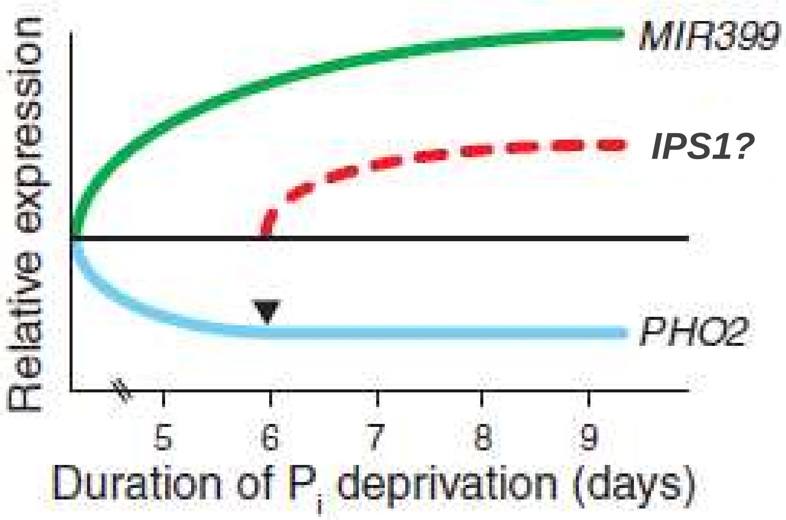
miR399 binds to and cleaves *PHO2.*
No PHO2 made, no phosphate uptake

IPS1 present



miR399 sequestered.
PHO2 translated, phosphate uptake

The target mimic *IPS1* may modulate miR399 function



During phosphate deprivation, miR399 is induced, inhibiting production of *PHO2*.
After a few days, *ISP1* expression is induced, sequestering miR399 and allowing some *PHO2* expression.

Reprinted by permission of Macmillan Publishers, Inc. Chitwood, D.H., and Timmermans, M.C.P. (2007) Nat. Genet. 39: 935-936

miRNAs and nutrient uptake-summary

- The rate of nutrient uptake in the roots is regulated by signals from the shoot

- *PHO2* encodes a ubiquitin-conjugating E2 that presumably contributes to directed proteolysis

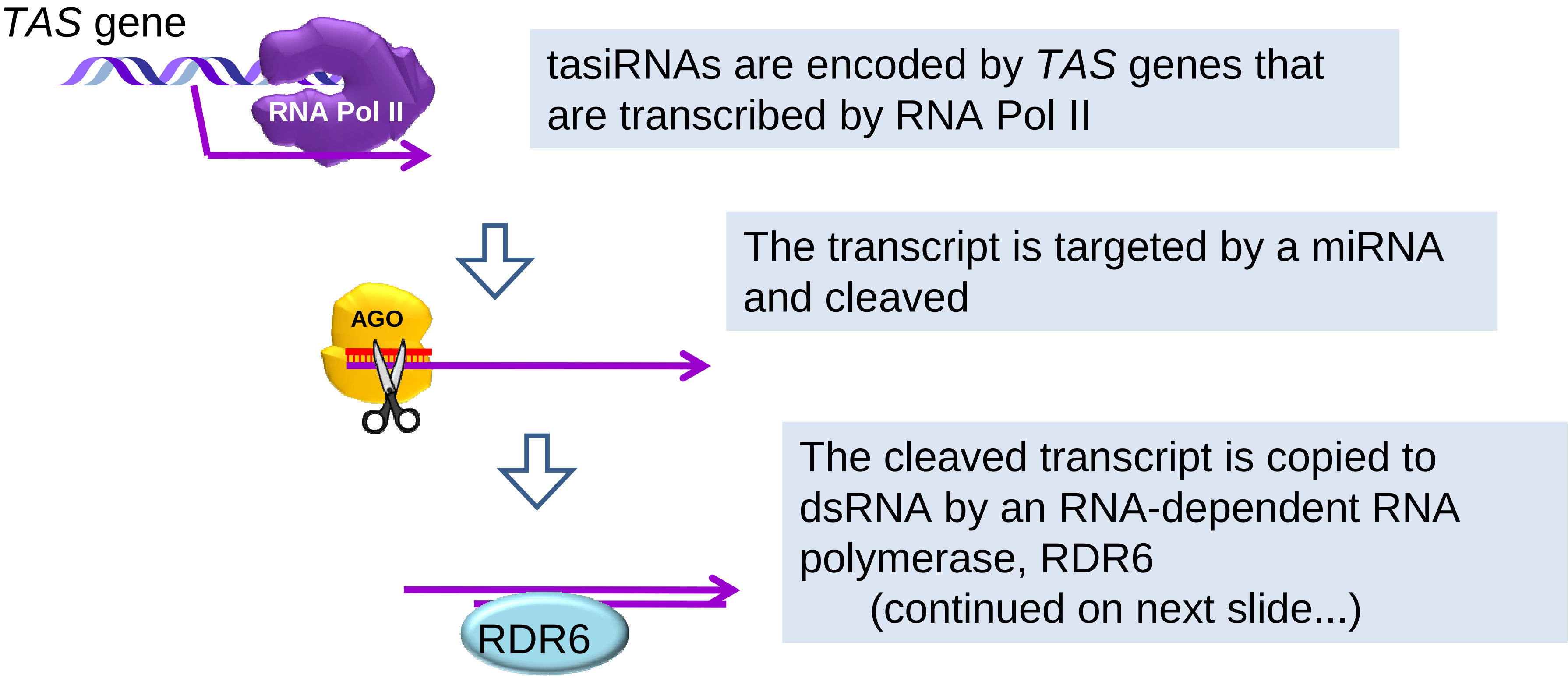
- PHO2 levels are regulated by miR399 which accumulates upon phosphate starvation

- Starvation-induced miR399 moves from shoot to root, regulating phosphate uptake through PHO2

- *PHO2* expression is also controlled by *IPS1*, a target mimic of miR399

tasiRNAs

tasiRNAs – trans-acting siRNAS
Encoded by *TAS* genes
Primary transcript processing initiated by miRNA



tasiRNAs are encoded by *TAS* genes that are transcribed by RNA Pol II

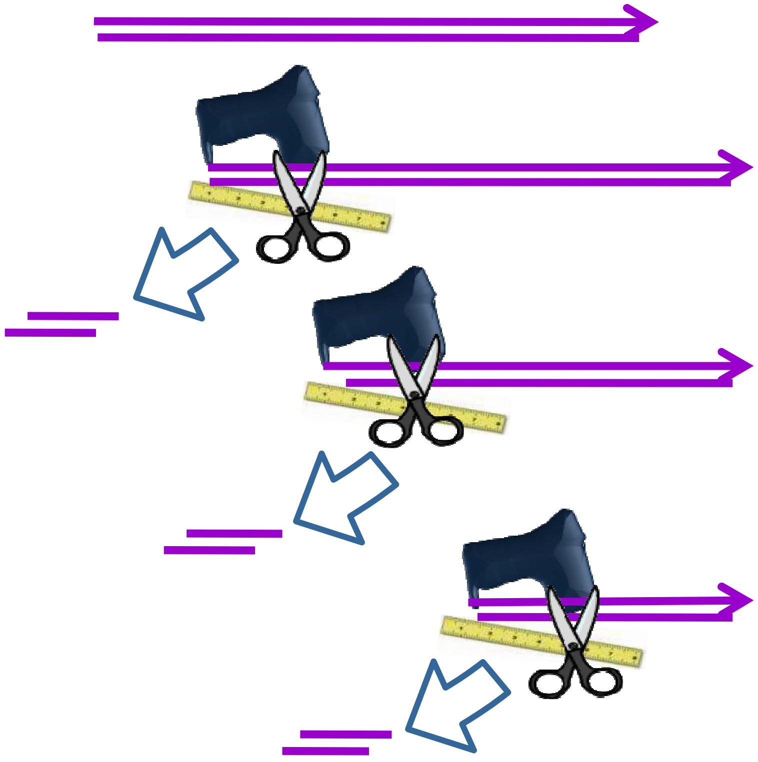
The transcript is targeted by a miRNA and cleaved

The cleaved transcript is copied to dsRNA by an RNA-dependent RNA polymerase, RDR6

(continued on next slide...)

tasiRNA biogenesis

The dsRNA is cleaved by DCL4 into a series of shorter dsRNAs, releasing many tasiRNAs from a single *TAS* gene.



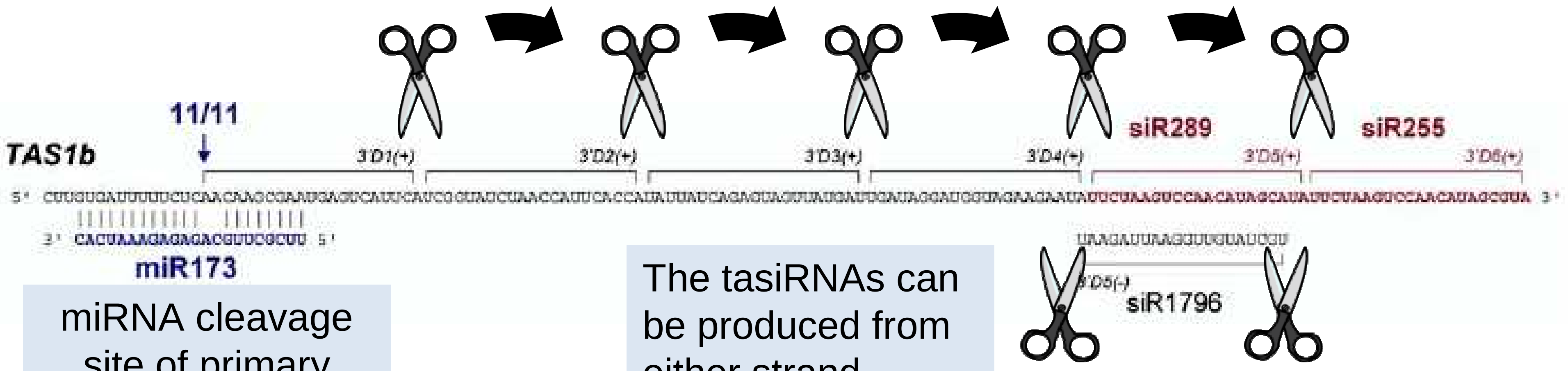
Arabidopsis has four families of *TAS* genes

•*TAS1 and TAS2* tasiRNAs target pentatricopeptide repeat genes.

•*TAS3* tasiRNAs target ARF transcription factors.

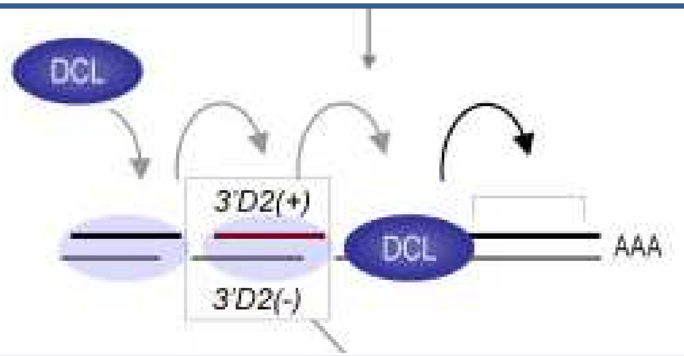
•*TAS4* tasiRNAs target MYB transcription factors

Several “phased” tasiRNAs are derived from each *TAS* gene



miRNA cleavage site of primary transcript

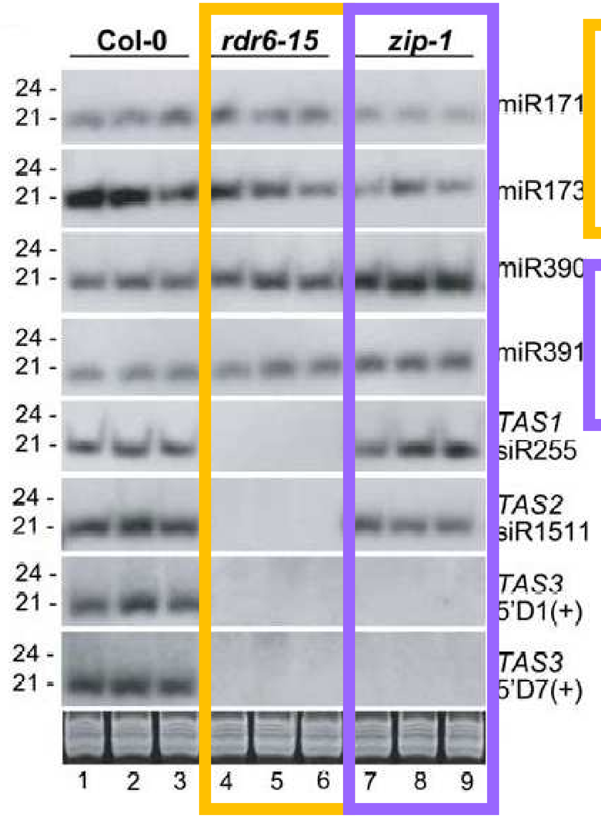
The tasiRNAs can be produced from either strand.



DCL4 moves along the dsRNA, measuring and cutting

Reprinted from Allen, E., Xie, Z., Gustafson, A M., and Carrington, J.C. (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121: 207-221, with permission from Elsevier.

Mutations that affect tasiRNA production affect phase change

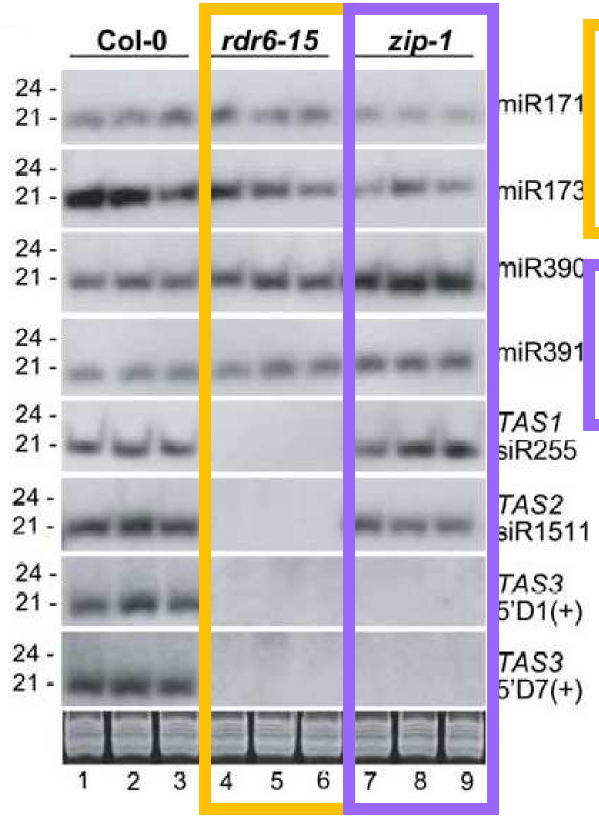


rdr6-15 mutation eliminates tasiRNA production

zip-1 eliminates *TAS3* tasiRNA production

Reprinted from Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., and Carrington, J.C. (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. Curr. Biol. 16: 939–944 with permission from Elsevier.

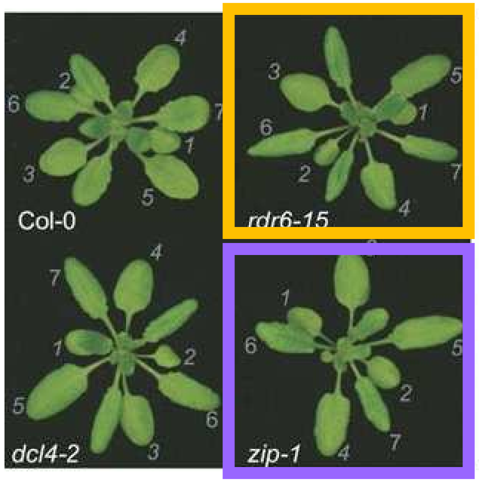
Mutations that affect tasiRNA production affect phase change



rdr6-15 mutation eliminates tasiRNA production

zip-1 eliminates *TAS3* tasiRNA production

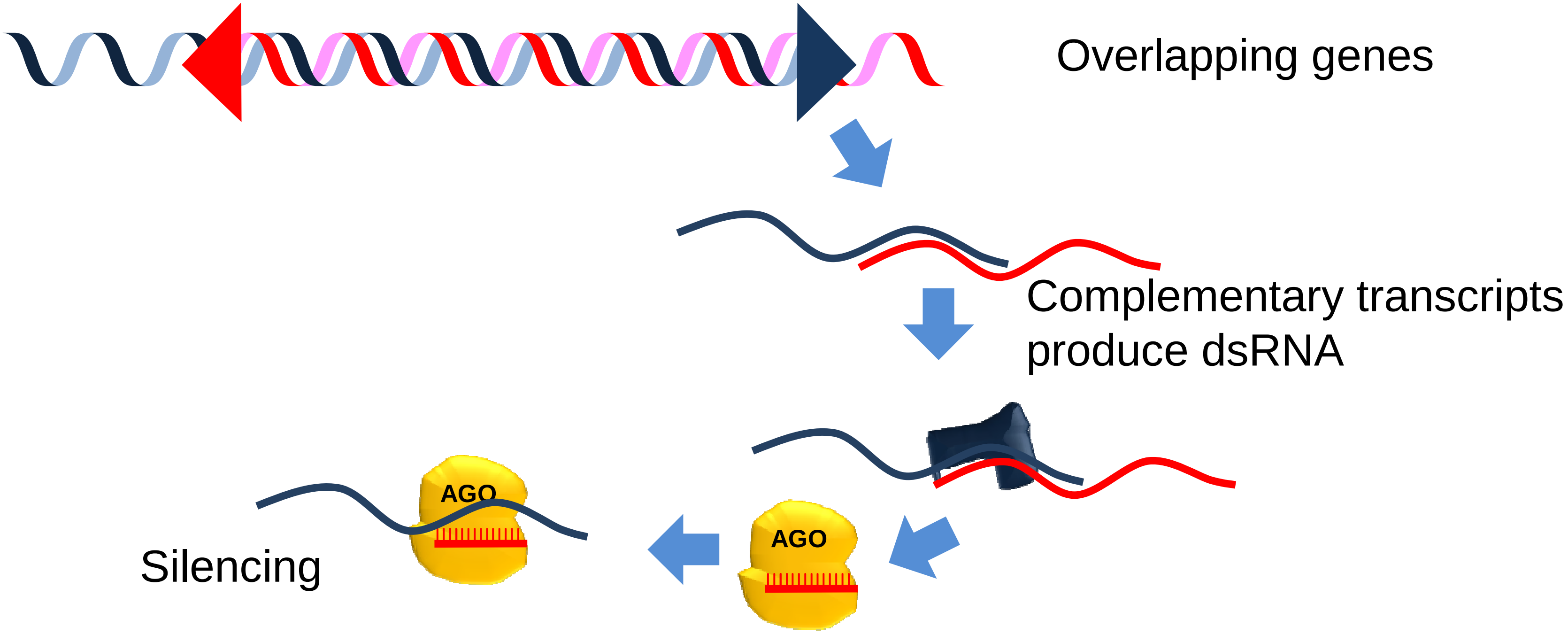
Both mutations, as well as *dcl4* and *tas3*, accelerate phase change



Reprinted from Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., and Carrington, J.C. (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. Curr. Biol. 16: 939–944 with permission from Elsevier.

nat-siRNAs

Nat-siRNAs – Natural *cis*-acting siRNAs
Derived from overlapping transcripts
Involved in abiotic and biotic stresses


Overlapping genes

Complementary transcripts produce dsRNA

Silencing

Redrawn from Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas Jr. A., Zhu, J.-K., Staskawicz, B.J., and Jin, H. (2006) A pathogen-inducible endogenous siRNA in plant immunity. Proc. Natl. Acad. Sci. USA 103: 18002–18007.

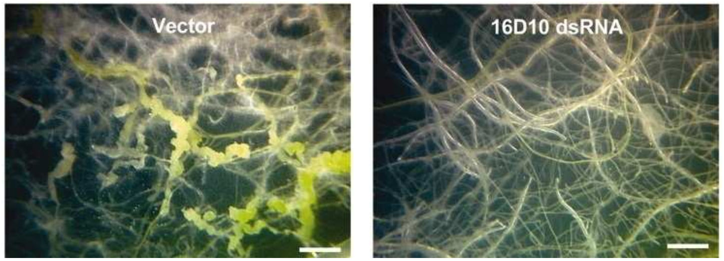
Applications of small RNA technologies

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

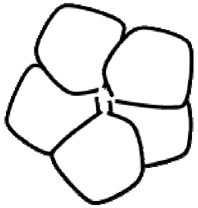
RNAi-inducing – no infection

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

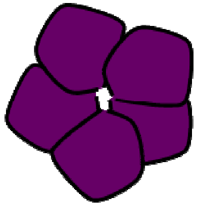
Huang, G., Allen, R., Davis, E.L., Baum, T.J., and Hussey, R.S. (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proc. Natl. Acad. Sci. USA 103: 14302–14306.

Conclusions

Small RNAs contribute to the regulation and defense of the genome, and confer silencing specificity through base-pairing



siRNA targets include repetitive-rich heterochromatin, transposons, viruses or other pathogens



miRNAs and tasiRNAs targets include regulatory genes affecting developmental timing or patterning, nutrient homeostasis and stress responses