The Small RNA World (TTPB5) – Teaching Guide

Overview – Small RNAs confer specificity to a set of pathways collectively called RNA-mediated silencing. Small interfering RNAs (siRNAs) ensure that silenced DNA remains silent and are important tools in the plant defensive arsenal. microRNAs (miRNAs) temporally and spatially regulate their targets, many of which are involved in developmental timing and patterning, nutrient uptake or stress responses. This lecture describes the production and actions of small RNAs in plants, and highlights some of the key studies in their discovery.

Learning Objectives
By the end of this lecture the student should be able to:
- Describe how miRNAs and siRNAs are produced
- State the functions of DCL and AGO proteins
- Describe the role of siRNAs in plant defenses against viruses
- Explain the difference between post-transcriptional gene silencing and transcriptional gene silencing, and how each is effected
- Describe three functions of miRNAs in plants

Study / exam questions (understanding and comprehension)
- Dicer has been described as acting like a molecular ruler – what does this description refer to?
- Gene silencing can be induced by a virus, a transgene, a hairpin-forming single-stranded RNA or a double stranded RNA. How can these different triggers initiate the same response?
- How could you test whether a viral protein acts as a suppressor of RNA silencing?
- True or False: Both miRNAs and siRNAs are involved in transcriptional and post-transcriptional gene silencing.
- How do the actions of plant and animal miRNAs differ?
- Which of the following statements is true:
  A. Many MIR genes can encode the same miRNA.
  B. Every MIR gene encodes a unique miRNA.
- How does the phenotype of the maize Corngrass1 mutant support the model that miR156 is an inhibitor of vegetative phase change?
- How can an mRNA be modified so that it is not a target for a miRNA without affecting the protein it encodes?
- Describe how a miRNA can contribute to developmental patterning.
- What is the experimental evidence for the movement of miR399 from shoot to root?
- What is a target mimic and how can it be used to modify gene expression?
Discussion Questions (engagement and connections)

- The central dogma of biology is that information flows from DNA to RNA to protein. Redraw the diagram below to incorporate the actions of small RNAs.

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\text{DNA} \rightarrow \text{RNA} \rightarrow \text{Protein}
\]

- What questions are being addressed in the experiments shown in slides 12 – 14 (from Ratcliff et al., 1997, and Hamilton and Baulcombe, 1999)? What do the results show?
- Plasmodesmata are regulated channels between cells. What kinds of molecules move between them, and how is the movement of molecules through plasmodesmata regulated?
- Matzke, M.A. and Matzke, A.J.M. (2004; doi:10.1371/journal.pbio.0020133) write a history of the key experiments that led to our understanding of the small RNA world. Draw a timeline showing the key papers and ideas cited in this paper. Can you identify five more papers published between 2004 and the present that you would describe as highly influential?
- RNA interference (RNAi) and artificial miRNAs can be used as experimental tools to identify gene function. Find a paper that uses one of these methods. What DNA construct did the authors make to induce silencing? How did they introduce the construct into plant tissues or cells? How did they assess whether the target gene(s) were silenced?
- Virus-induced gene silencing (VIGS) is another method that can be used to investigate gene function. What kinds of plant viruses are used for this method, and are different viruses needed for different types of plants? How are the viruses introduced and how are viral disease symptoms suppressed? (A recent and thorough review is found here: Senthil-Kumar, M. and Mysore, K.S. (2011) New dimensions for VIGS in plant functional genomics. Trends Plant Sci., doi:10.1016/j.tplants.2011.08.006).
- Describe a current model for the evolutionary origins of plant MIR genes. What evidence supports this model? What kinds of data or experiments would further test or support this model?
- There is accumulating evidence that miRNAs can move between cells and that this ability is important in some of their functions. How do miRNAs differ from other information-carrying molecules (such as hormones) that move between cells? Are there some situations in which a miRNA might be a more effective signal than other forms of signals?
- Many fruit trees do not begin to flower until they are several years old, making life difficult for their breeders! Propose a method to try to shorten the time a plant breeder has to wait between generations. Conversely, some trees are grown primarily for shade and not fruit. Propose a method that would prolong the period during which a tree does not produce flowers or fruit.
- Find the paper by Chitwood et al (2007) Pattern formation via small RNA mobility. Genes Dev 23: 549-554 (doi:10.1101/gad.177009). How did these authors show that small RNAs are mobile and contribute to developmental patterning?
- How do you explain the phenotype of the argonaute1 mutant shown in slide 5?
The paper by Schauer, S.E., Jacobsen, S.E., Meinke, D.W. and Ray, A. (2002). DICER-LIKE1: Blind men and elephants in Arabidopsis development. Trends Plant Sci. 7: 487-491 (doi:10.1016/S1360-1385(02)02355-5) describes the different ways that the dcl1 mutant was characterized. What phenotypes were described for the mutants identified as sin1, sus1 and caf? Why was the dcl1 mutant phenotype characterized in such diverse ways? Why is the title of this article appropriate for the story of the dcl1 mutant?

Find three examples of the use of siRNAs or miRNAs in plant biotechnology and for medicinal therapies.

Find two examples of the use of miRNA target mimics in research or therapy.

In what ways are ta-siRNAs similar and different from miRNAs and siRNAs?

Did the 2006 Nobel Committee get it right or wrong? Andrew Fire and Craig Mello were awarded the Nobel Prize in Physiology or Medicine in 2006 for their studies of RNA silencing in C. elegans, a recognition that some felt should have been shared with plant biologists (see for example doi:10.1038/443906a and http://www.sciencemag.org/content/314/5803/1242.2.full). What do you think? Do these prizes systematically overlook the contributions of plant scientists? How many plant scientist Nobel Laureates can you name?
Lecture synopsis
Introduction and overview (1 – 6)
Small RNAs are a pool of 21 – 24 nucleotide (nt) RNAs that function in gene regulation. They contribute to post-transcriptional gene silencing by affecting mRNA translation or stability. They contribute to transcriptional gene silencing through epigenetic modifications to chromatin. Small RNAs are produced from double-stranded precursors by the action of Dicer or Dicer-like (DCL) proteins, that measure and snip the RNA into short RNA duplexes. Small RNAs associate with ARGONAUTE (AGO) proteins through which they find and silence their targets. The two main categories of small RNAs are small-interfering RNAs (siRNAs) and microRNAs (miRNAs).

siRNAs – genomic defenders (7 – 52)
siRNAs protect the genome by suppressing invading viruses, silencing sources of aberrant transcripts, and silencing transposons and repetitive elements. They also help maintain some genes in an epigenetically silent state.

Virus-induced gene silencing (8 - 30)
Plants are susceptible to infections by viruses. Viruses reveal themselves through production of double-stranded RNAs or RNA hairpins during their replication. DCL processes dsRNA into siRNA, which interfere with viral replication. Furthermore, viral resistance spreads systemically to unexposed tissues, making them resistant to the infecting virus. After much speculation that the siRNAs themselves move into systemic tissues, it was finally demonstrated conclusively. The importance of the siRNA in viral resistance is also made evident by the fact that viruses have evolved mechanisms of enhanced pathogenicity by the production of suppressors of the RNA silencing machinery.

Silencing of transgenes (31 – 40)
In the 1990s, scientists were struggling to understand several strange effects that arose when introducing foreign genes into plants. One of these was called co-suppression, and involved the silencing of related genes by high level expression of an introduced gene. As an example, efforts to modify pigment color in petunia by expressing the gene encoding chalcone synthase (encoding a pigment biosynthesis gene) led to suppression of the endogenous gene, regardless of whether the introduced gene expressed the sense RNA strand or the antisense RNA strand. This effect was known as post-transcriptional gene silencing. A very similar observation was subsequently made in the nematode worm C. elegans, revealing that this is a universal property and not specific to animals.

Transcriptional gene silencing (41 – 52)
Another puzzling observation from studies of transgene interactions was the observation that in some cases an introduced gene could interfere with the transcription of a different gene; this was called transcriptional gene silencing and arises when siRNAs target the epigenetic silencing machinery to DNA. Further studies showed that transcriptional gene silencing in plants involves additional RNA Polymerases not found in animals. Genome-wide studies reveal that siRNAs with homology to transposons and other epigenetically silent regions of the genome are abundant. The picture has emerged that siRNAs are essential targets in suppressing unwanted transcription by targeting DNA methylases and histone modifying enzymes to DNA. A virus or a transgene that produces very high levels of RNA transcript or unusual RNA structures triggers the RNA silencing response.
microRNAs (miRNAs) - overview (53 - 63)
MicroRNAs are small RNAs that are encoded by MIR genes, but act in trans upon other genes. Thus, unlike siRNAs that silence their own progenitor, miRNAs silence something else. MIR genes produce transcripts with inverted duplications that fold-back upon themselves to form dsRNA templates for DCL processing. In plants, miRNAs bind tightly to a conserved region of their target and either cause the target mRNA to be degraded or interfere with its translation. Some miRNAs are highly conserved whereas others are present in one or a few plants. Many miRNAs are involved in regulating the expression of transcription factors, and they seem to be widely used as a way to fine-tune the action of their transcription factor targets in space and in time, as shown by a few examples below.

miRNAs and vegetative phase change (64 – 81)
Many plants exhibit a developmental change as they age, from juvenile to adult phase. Phase change can affect leaf size and shape and growth pattern, and also confers upon plants the ability to reproduce. The study of the genetic control of phase change has revealed that the activity of some of the key transcription factors is regulated by miRNAs. Because miRNAs bind their mRNA targets with high specificity, it is relatively easy to make a variant of the mRNA that is resistant to miRNA processing. As an example, the SPL transcription factor which promotes phase change is normally temporally regulated by miR156, which is abundantly expressed in juvenile tissues. Thus, SPL activity is delayed until miR156 is no longer produced. Altering the SPL mRNA so that it no longer is targeted by miR156 means that its activity is no longer delayed, and leads to an acceleration of phase change. The importance of miR156 in regulating phase change is also demonstrated in plants that constitutively express miR156 (leading to a delay in phase change) or do not produce it (leading to an acceleration of phase change). miR156 is a highly conserved miRNA that contributes to developmental transitions in other plants as well. Interestingly, the early studies that revealed miRNA action in C. elegans also involved regulation of developmental transitions.

miRNAs contribute to developmental patterning (82 - 88)
Another common function of miRNAs is in the control of developmental patterning. miR165/6 regulates the activity of the PHB transcription factor that determines leaf polarity and also the radial patterning in the root. It is thought that the ability of miRNAs to move between cells helps to establish and retain developmental patterns and gradients. The more we learn, the more we realize that miRNAs contribute enormously to the control of plant processes. More than ten different miRNAs are involved in leaf development, from initiation to senescence.

miRNAs and nutrient signaling (89 – 101)
Throughout a plant’s life it has to communicate vast amounts of information between the root and the shoot. Some of this information is conveyed by hormones and other small molecules, but we are now learning that miRNAs can also contribute to shoot-to-root communication. This is elegantly revealed in a study of the control of phosphate uptake by the roots, which has to be coordinated with the requirements for phosphate in the shoot. When a plant is starved for phosphate, it switches on its uptake machinery, but too much phosphate uptake is harmful, so this uptake is regulated. PHO2 turns off phosphate accumulation when it is not needed, and miR399 turns off PHO2 when phosphate is needed. miR399 accumulates when the plant is starved for phosphate. Grafting studies showed that PHO2 acts in the shoots, but
it is regulated by miR399 whether the miRNA is expressed in the shoot or root, demonstrating that the miRNA moves from shoot to root. Furthermore, the activity of miR399 is itself regulated by the presence of a target mimic, IPS1, which essentially fine-tunes the fine-tuning device.

**More types and classes of small RNAs (102 – 108)**
There are other forms of small RNAs, including trans-acting siRNAs (tasiRNAs). These act like siRNAs but are encoded by a gene called a *TAS* gene. Interestingly, the primary transcript of the *TAS* gene is copied into double-stranded RNA and then processed into several separate nested tasiRNAs, by the sequential movement of DCL along the transcript. Like miRNAs, tasiRNAs regulate the activities of their targets which include transcription factors. Nat-siRNAs are produced from a naturally occurring double stranded RNA that forms when two genes have overlapping transcripts.

**Applications and summary (109 – 110)**
Since their discovery, small RNAs have become important tools in research. The function of a gene can be investigated by introducing a construct that will produce a siRNA to target the endogenous gene. Applications of this technology include silencing genes that have undesirable consequences, like genes responsible for the production of toxins.
What are small RNAs?

The core of RNA silencing: Dicers and Argonautes

RNA silencing - overview

siRNAs – Genomic Defenders
siRNAs suppress viruses, silence sources of aberrant transcripts, and silence transposons and repetitive elements

Viral-induced gene silencing - overview

Double-stranded RNA viral replication intermediates are cleaved by dicer-like proteins (DLC) to make siRNAs

Key Experiment: Plants can recover from viral infection and become resistant

A plant that has been previously inoculated with a virus suppresses subsequent viral replication

Inoculated and systemic leaves accumulate small RNAs homologous to viral RNAs. These studies led to the hypothesis that the small RNAs themselves are responsible for viral resistance.

Model – production of siRNAs in an inoculated leaf causes siRNAs to accumulate in distal leaves, conferring resistance

How does RNA silencing spread systemically?

Studying silencing using GFP expression in leaves. Normally, the chlorophyll in leaves fluoresces red. A plant that is expressing GFP fluoresces green. Therefore, a leaf carrying a GFP gene will be green when the GFP gene is expressed but red when it is silenced.

Silencing can be induced by introduction of an inverted-repeat encoding DNA, that when transcribed produces an RNA that folds into a hairpin structure subject to processing to siRNA.

Introducing the IR-DNA into a leaf causes the GFP gene to be silenced, and this silencing spreads beyond the site of DNA introduction, indicating that the silencing spreads from cell to cell.

Plasmodesmata are connections between plant cells through which siRNA and other molecules can move (including proteins and viruses).

The silencing signal also moves through the phloem

Key experiment: What moves? Evidence that small RNAs are mobile within the plant.

Grafting an IR-GFP shoot onto a GFP root. Roots initiated after the graft are silenced in GFP expression, indicating that something has moved from shoot to root to silence expression.

Is the small RNA made in the shoot (site of double-stranded RNA) or in the root (site of silencing)? Test this using mutants deficient in dicer-like activity. A plant deficient in dcl2,3 and 4 does not silence GFP, because no siRNA is produced. When the root only is dcl2,3 and 4 deficient, GFP in the root is silenced. This experiment shows that siRNA produced in the shoot moves into the root. What moves? siRNA moves.

Move evidence for the movement of siRNA. Duplex siRNA was bombarded into a single leaf cell, but silencing spread beyond the cell that took up siRNA.

Signal amplification: A small amount of viral RNA can be amplified through RNA-dependent RNA polymerase action – there is not a stoichiometric relationship between viral RNA and siRNA.

siRNA production mutants are more susceptible to viral disease, providing further evidence that siRNAs are important in plant defense against viruses

It’s an arms race! As expected, viruses have evolved suppressor proteins that
Experimental evidence that viral suppressor proteins interfere with the plants silencing mechanism.

siRNAs are involved in more than viral defenses; mutants in siRNA production are more susceptible to bacterial pathogens.

**Summary of viral-induced gene silencing. Plants defend against viruses by producing siRNAs that interfere with viral replication and move systemically to confer resistance. Some viruses make suppressor proteins to evade siRNA silencing.**

Silencing of transgenes – studies of transgene silencing have been important in our understanding of siRNA silencing

**Key experiment:** In studies of petunia, introduction of the chalcone-synthase gene \((CHS)\) in the sense or antisense orientation led to silencing of the endogenous gene as well as the transgenes.

**Key experiment:** A similar study in the nematode \(C.\ elegans\) showed that double-stranded RNA is the strongest trigger for gene silencing

**Transcriptional gene silencing** - The previous examples involved post-transcriptional gene silencing, by preventing mRNA from being translated. However, siRNAs can also interfere with transcription.

**Key experiment:** Two transgenes in the same plant can interfere with each other’s expression. The silencing is correlated with DNA methylation of the silenced locus.

Mechanism of transcriptional gene silencing: Silenced genes can be silenced by DNA methylation or histone modification (epigenetic marks). Angiosperms have two additional RNA polymerases that contribute to gene silencing. A loss-of-function mutant of RNA Polymerase IV is deficient in gene silencing. Most siRNAs are produced from transposons and repetitive DNA and help to silence these loci. Furthermore, siRNAs can function non-cell-autonomously, as shown by the epigenetic reprogramming that takes place during gametogenesis.

**siRNAs – Summary – siRNAs are produced from double stranded RNAs and can silence genes transcriptionally or post-transcriptionally**

**MicroRNAs** – miRNAs are produced from \(MIR\) genes and silence other genes. They have particular roles in regulating developmental processes temporally and spatially

**Origins of miRNAs: processing from \(MIR\) genes, evolutionary origins**

**miRNAs and vegetative phase change.** Many plants show morphological differences as they move from juvenile to adult phase, and this transition is regulated by miRNAs. Results from maize and Arabidopsis studies are shown. Early \(C.\ elegans\) studies also involved developmental progression.

**miRNAs and developmental patterning.** Leaf development including leaf abaxial / adaxial polarity is regulated by miRNAs, and root radial patterning is too.

**miRNAs and nutrient signaling.** Phosphorous uptake in the root is under the control of the \(PHO2\) gene. \(PHO2\) expression in the root prevents excessive phosphate uptake. \(PHO2\) activity is regulated by miR399, which can be translocated from shoot to root. Activity of miR399 is regulated by a target mimic.

**Other kinds of small RNAs include tasiRNAs (trans-acting siRNAs) and nat-siRNAs (natural cis-acting siRNAs)**

**Applications of small RNA technologies – eliminate toxins and allergens, confer resistance to viruses and parasitic nematodes**

**Small RNAs contribute to regulation and defense of the genome**