miRNAs in plants

Introduction

In the previous lessons, you have been introduced to small non-coding RNAs (sRNAs) as important regulators of cellular functions, focusing on miRNAs in animals. Here, we want to focus on microRNAs (miRNAs) in plants. Currently, there are nearly 1'000 plant *miRNA* genes deposited in the miRBase registry, which are involved in virtually all aspects of plant biology. As their animal counterparts, plant miRNAs are 20- to 24-nucleotide RNAs that regulate the expression of their targets post-transcriptionally. However, you will see that there are several differences, such as the location of *miRNA* genes in the genome, the length of pre-miRNAs, as well as the degree of sequence complementarity and the resulting modes of action. We will discuss how miRNAs are expressed in plants and by which mechanisms their expression can be regulated. Finally, we will look at how plants use different circuits to achieve tissue-specific or temporal regulation of miRNA targets.

Plant-miRNA biogenesis

Like in animals, plant miRNAs are transcribed from genomic DNA. However, while in animals, many *miRNA* genes are found within introns or even exons, *miRNA* genes of plants are in their vast majority intergenic, i.e., located in non-coding regions between the protein-coding genes (see figure 4-1). Like animal *miRNA* genes, they are transcribed by DNA-dependent RNA-polymerase II, the same polymerase that also transcribes protein-coding genes into mRNA, producing a primary miRNA (pri-miRNA) that is capped and poly-adenylated (as many pri-miRNAs in animals). In the nucleus, the pri-miRNA undergoes two processing steps catalyzed by the RNase III Dicer-like 1 (DCL1), with the first one being the formation of the stem-loop precursor (pre-miRNA). Plant pre-miRNAs are much more variable in size than their animal counterparts (usually about 70 nt long) and might form stems more than 2 kb long. The second excision by DCL1 forms the mature miRNA duplex.

This is different to miRNA biogenesis in animals, where nuclear processing of the pri-to-pre-miRNA is mediated by Drosha and processing into the mature miRNA duplex is catalyzed by Dicer in the cytoplasm. In plants, the mature miRNA duplex is exported into the cytoplasm and both strands are 2'-O-methylated at the 3' end by *HEN1* to protect the duplex from oligo-uridylation and degradation by exonucleases. One of the two miRNA strands, the so-called guide-miRNA strand, which contains the sequence complementary to the target sequence, is then incorporated into Argonaute (AGO) proteins. In principle, both strands can contain the guide sequence necessary for target recognition. The mechanism by which the correct guide-miRNA strand is selected is not yet formally resolved in plants but occurs most likely as for animal miRNAs (see lesson 1). AGO loaded with the guide-miRNA forms - together with other proteins - the RNA-induced silencing complex (RISC) to mediate target-gene specific silencing. Of the ten AGO proteins found in *Arabidopsis*, AGO1 is the main effector of miRNAs. Some miRNAs are also effected by AGO10, which is in the same genetic clade as AGO1.

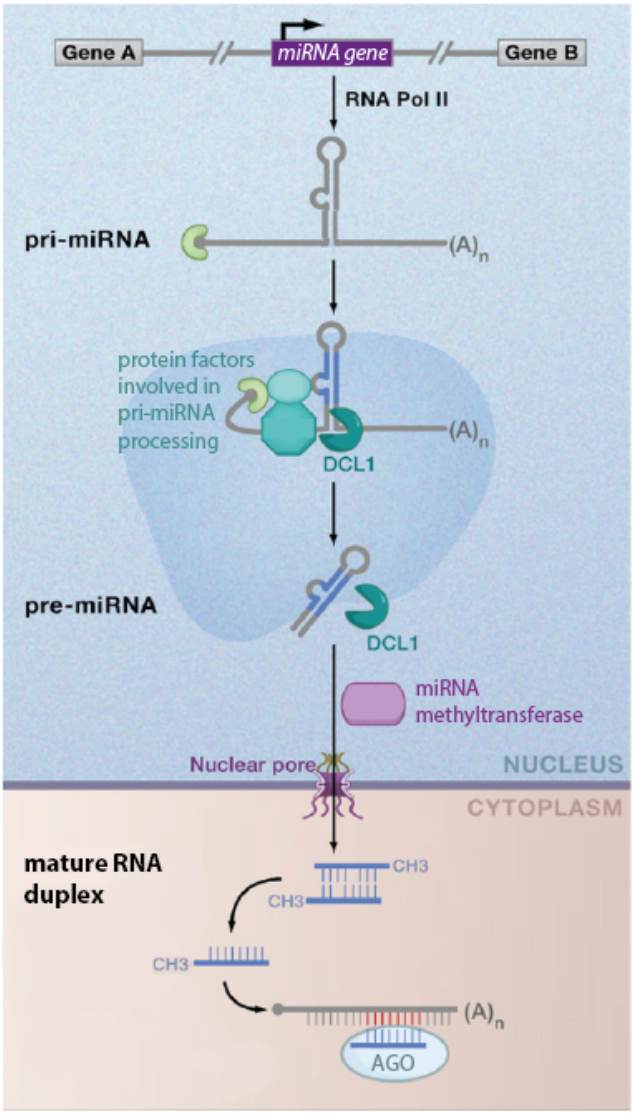

Figure 4-1 Biogenesis of miRNAs in plants. miRNA genes, which are usually located intergenically, are transcribed by RNA Polymerase II (RNA Pol II) and processed into mature miRNA duplexes by Dicer-like 1 (DCL1) and other processing factors in the nucleus. They are exported into the cytoplasm, methylated to prevent degradation, and the guide strand binds to Argonaute (AGO) forming the RISC. In plants, 10 Argonaute proteins are predicted to exist. (Adapted from O. Voinnet, *Cell*, 2009)

Slicing or translational inhibition - two different modes of silencing

Now that we have introduced the general mechanism of how miRNAs are produced in plants, let's look at how they function. Perhaps the most prominent difference between animal and plant miRNAs is the degree of complementarity to their targets: Typically, animal miRNAs are thought to regulate their targets through imperfect complementarity to sites found in 3'-untranslated regions (3' UTR), which mostly leads to translational inhibition and only rarely to slicing, i.e., endonucleolytic cleavage of the target mRNA. Plant miRNAs, in contrast, largely regulate their targets by binding to highly complementary target sites in coding regions (see figure 4-2). This has two consequences for plant miRNAs: i) they often function through mRNA degradation, clearing the target mRNA irreversibly and ii) they are predicted to have a much more limited number of mRNA targets than their animal counterparts, because they require near-perfect matching with their target. An advantage is that computer-based target identification is very straightforward, because it is much less prone to false positives than in animals, in which conservation of the seed-complementary sequence (which is just six nucleotides long) is the main criterion.

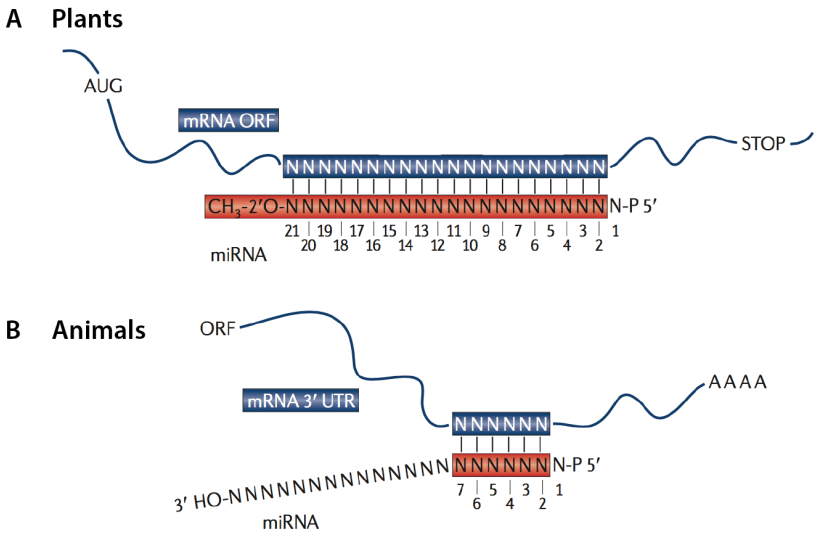

Figure 4-2 Difference in sequence complementarity between plant and animal miRNAs and their targets. (A) Plant miRNAs mostly bind to target sites in coding regions with high complementarity, often leading to slicing (RNA degradation). **(B)** Animal miRNAs typically bind to sites in 3′ UTRs of their targets with imperfect complementarity, resulting in translational inhibition. (Adapted from E. Huntzinger and E. Izaurralde, *Nat. Rev. Genet.*, 2011)

To better understand how miRNA-mediated mRNA degradation works, let's look at how target cleavage is achieved mechanistically: After binding of the RISC to a target, AGO1 can cleave the target mRNA between nucleotide 10 and 11, opposite the guide-miRNA strand. The mRNA fragments resulting from the endonucleolytic cleavage are degraded from the newly generated 3' and 5' ends (see figure 4-3B).

However, despite high sequence complementarity to their targets, plant miRNAs were found to often function by translational inhibition. Prof. Voinnet and his colleagues performed a forward-genetics screen in *Arabidopsis* and looked for mutants that have defects in miRNA-induced silencing processes. Specifically, they used a transgenic strain that constitutively expressed a green-fluorescent-protein (GFP) reporter construct, where GFP was fused upstream of the sequence containing a certain miRNA (miR-171) target site (see figure 4-4A). If you remember, this is quite similar in essence to what researchers initially did in *Drosophila* to validate the first few fly miRNA targets identified (see lesson 1) using sensor constructs *in vivo*.

Upon expression of the miR-171 GFP sensor construct, a fusion mRNA between the *GFP* gene and the miRNA target sequence is made. Because the researchers knew miR-171 is expressed in virtually all parts of the plants, they predicted that these plants would, under normal conditions, not show green fluorescence, because the presence of the miRNA target site in the fusion mRNA would prevent translation of this RNA due to miR-171 binding and silencing. Now, after mutagenizing with ethyl methanesulfonate, the researchers could identify *Arabidopsis* mutants that exhibited bright-green fluorescence, indicating either a defect in miR-171 biogenesis or activity (see figure 4-4B). In some of the mutants they identified, the lack of miRNA function could be traced to defective miRNA biogenesis (e.g., mutation in *dcl1*) or stability (e.g., mutation in *HEN1*) since analysis by gel electrophoresis showed that little mature miRNAs of 21 to 24 nucleotides in length, including miR-171, accumulated in these mutants. However, the researchers also identified six mutants, in which i) mature miRNAs were produced and ii) green fluorescence could be observed indicating that these plants were miRNA-activity deficient (*mad* mutants).

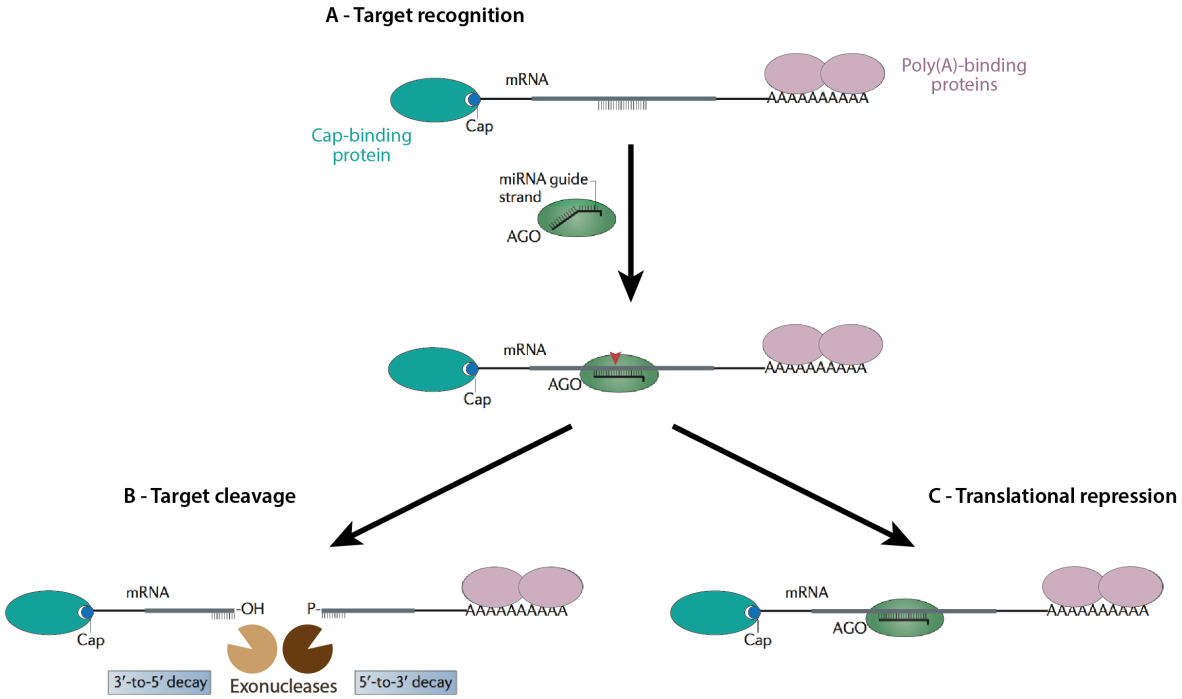

Figure 4-3 Mechanism of miRNA-mediated gene silencing in plants. (A) Plant miRNAs that are bound to Argonaute (AGO) recognize mRNA targets containing fully or nearly complementary binding sites, which are predominantly located in the ORF. mRNA ends are protected by a 5′ cap and a 3′ poly(A) tail, which are recognized by cytoplasmic proteins (in blue and pink) that facilitate translation. AGO can cleave the mRNA in the base-paired region (between nucleotides 10 and 11, opposite the miRNA strand, at the red arrowhead). **(B)** The mRNA fragments resulting from the endonucleolytic cleavage are degraded from the newly generated 3′ and 5′ ends. **(C)** Alternatively, cleavage by AGO is prevented and the mRNA target is translationally repressed by an unknown mechanism. (Adapted from E. Huntzinger and E. Izaurralde, *Nat. Rev. Genet.*, 2011)

Characterization of these *mad* mutants led to a surprising result: While in all of them, GFP protein was strongly expressed (see figure 4-4C, lower panel), the GFP mRNA was only detectable in four of the mutants (see figure 4-4C, upper panel). From this observation, we can conclude that mutants *mad1* to *mad4* were unable to degrade GFP mRNA, explaining the fluorescence. However, in *mad5* and *mad6*, miRNA-guided mRNA degradation appeared to occur normally as indicated by the fact that the decrease in GFP mRNA was comparable to that seen in the parental transgenic line before mutagenesis (see figure 4-4C, upper panel); yet both mutants failed to silence the GFP-mRNA fusion at the translational level, since the GFP protein could still be detected in these mutants (see figure 4-4C, lower panel). This indicates that silencing of GFP expression is due to two distinct mechanisms, which can both be mediated by the same miRNA (here miR-171): i) mRNA slicing and degradation leading to reduced mRNA levels and ii) inhibition of protein production from the remaining mRNA, which was not sliced and degraded. Apparently, *mad5* and *mad6* are defective in the second process. You have already encountered several different mechanisms of how translation can be inhibited by animal miRNAs in lesson 1 and it is believed that similar processes may account for this mode of regulation in plants as well.

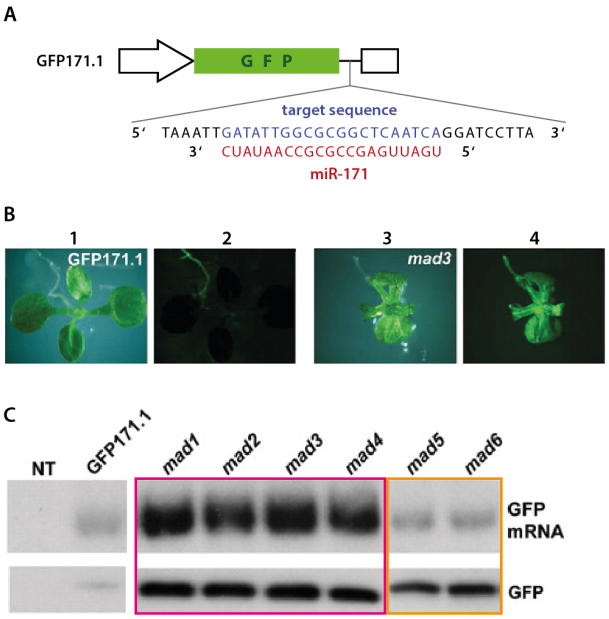

Figure 4-4 Discovery of miRNA-action deficient mutants (*mad* mutants). **(A)** Schematic representation of the construct GFP171.1 encoding GFP containing a miR-171 target site (blue). The miR-171 target site is perfectly complementary to miR-171 (red). **(B)** Images 1 and 2 show a transgenic plant (GFP171.1) with normal miRNA-mediated silencing (1: transmitted light. 2: blue light excitation). Here, GFP mRNA is silenced by miR-171 and no fluorescence is visible under blue light. Images 3 and 4 show plants mutant for the miRNA *mad3* after 15 days of growth (3: transmitted light. 4: blue light excitation). Since GFP expression is not silenced, the plant fluoresces under blue light. **(C)** Gel electrophoresis analysis of GFP mRNA (top) and GFP protein (bottom) in non-transgenic plants (control, NT), GFP171.1 (transgenic plant before mutagenesis), and *mad* mutants *1* to *6*. The mutants *mad1* to *4* (pink) are deficient in silencing, while *mad5* and *6* (orange) are defective in silencing by translational inhibition. (Adapted from P. Brodersen *et al.*, *Science*, 2008)

From the example above, we can learn several things: First, in plants, miRNA-mediated silencing functions by target-mRNA slicing and degradation as well as translational inhibition. Second, one single miRNA (e.g., miR-171) can result in a combination of the two types of silencing. In fact, this mode of action is expected to be true for most plant miRNAs, but with varying involvement of each process depending on the miRNA:target pair considered. Third, since the miRNA used in this study showed perfect complementarity to its target and triggered both silencing mechanisms, one cannot infer from the sequence complementarity, whether the target will be sliced or translationally inhibited. Moreover, neither the position of binding to the target (coding region or 5'/3' UTR) nor the degree of pairing appears to be predictive of the prevalence of one silencing process over the other. There is recent evidence that translational inhibition is promoted by protein factors that interact with AGO, but the factors influencing the mechanism how targets are silenced still remain to be elucidated.

Now, the question arises: Which mode of action is especially well suited for the regulation of which cellular process? The slicing and degradation of mRNA targets is irreversible and could provide a switch that is required for permanent decisions such as cell fate acquisition during embryogenesis or in adult stem cell niches found in plant meristems. One argument in favor of this hypothesis is that mutations perturbing the silencing process by mRNA slicing and degradation have severe consequences for plant development as do mutations in factors required for miRNA biogenesis (e.g., *dcl1* mutant).

Translational inhibition, on the other hand, is reversible and could, for example, be well suited to coordinate stress-responsive gene expression. There are several examples of miRNAs that inhibit negative regulators of stress responses by binding to their mRNA. Adaptation to stress is therefore achieved by repressing the stress-response repressor. Reversibility of this process would ensure that the translation of the negative regulators resumes immediately after the stress is gone. This would reduce the fitness costs of a prolonged stress-response activation. Ultimately, processes that are regulated by only target degradation or translational inhibition are expected to be rare, since the flexibility that arises from combining both mechanisms is very advantageous for an organism.

Modulating plant miRNA activity

As with every regulatory mechanism in cells of multicellular organisms, we have to ask: How is it possible that, despite all cells having the same genetic makeup, different cell types show different activities of genes and their products? The activity of various miRNAs must therefore be modulated differentially. In principle, we can expect this to occur on three different levels: Transcription of *miRNA* genes, miRNA processing, and activity of mature miRNAs as part of the RISC complex.

Regulation of *plant-miRNA* gene transcription

In contrast to some of their animal counterparts, plant *miRNA* genes usually are independent transcription units with their own promoters and regulatory elements. Just like for protein-coding genes, their transcription is modulated by transcription factors. Depending on the combination of transcription factors that bind to the regulatory regions of *miRNA* genes, highly specific expression pattern in different cell types can thus be achieved.

Let's look at one example: miR-166 is an important, evolutionarily conserved regulator in plant development. It is encoded by seven distinct genes (*mir166a-g*) producing different miRNA primary transcripts (pri-mRNAs) that, however, all produce nearly identical mature miRNAs after processing, only differing by one or two nucleotides. Interestingly, studies showed that not all *mir166* paralogs are expressed equally in different parts of a plant. Specifically, researchers looked at differential transcription patterns in the maize shoot apex, the aerial stem cell niche of plants from which new leaves and stems are growing continuously (see figure 4-5A). This tissue consists of different substructures, and in each of the substructures, different *mir166* paralogs are actively transcribed (see figure 4-5B and C). This high degree of cell specificity is likely due to different regulatory elements in the promoters of the different paralogs.

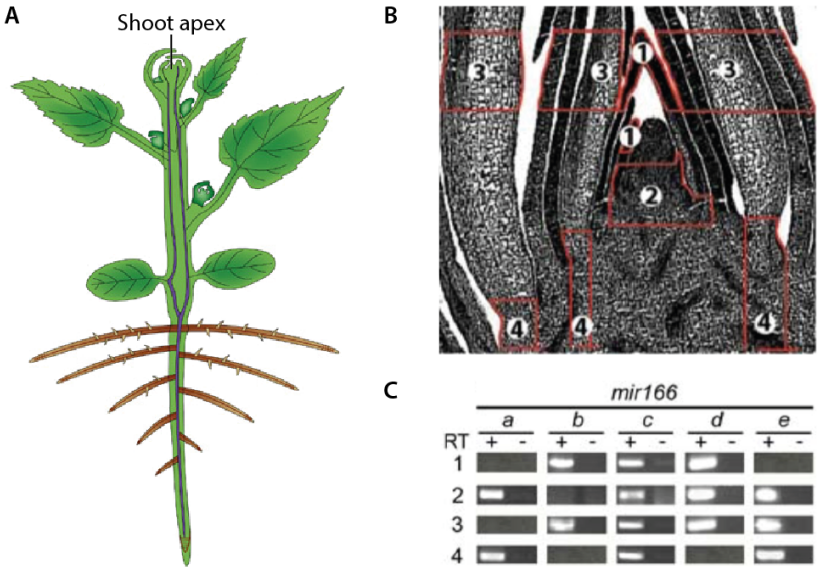

Figure 4-5 Differential transcriptional regulation of *mir-166* family members in maize shoot apex. (A) Schematic illustration of a whole plant, in which the shoot apex is indicated. **(B)** Sections through a maize shoot apex indicating four different substructures (1-4). **(C)** Reverse-transcriptase PCR (RT-PCR) analysis of the genes *mir-166a* to *mir-166e* and subsequent separation by gel electrophoresis shows that these *mir-166* family members exhibit distinct expression profiles within the shoot apex. For each gene, a control without RT (-) is shown in addition to the analyzed sample. (A: Adapted from M. Tsiantis and A. Hay, *Nat. Rev. Genet.*, 2003, B and C: Adapted from F.T.S. Nogueira *et al.*, *PLOS*, 2009)

Regulating miRNA processing and activity

In the beginning of this lesson, we have introduced the key players of plant miRNA biogenesis and activity: Dicer-like 1 (DCL1) is the main enzyme that processes pre-miRNAs to yield mature miRNAs in plants. AGO, which incorporates guide miRNA strands and carries out the silencing reactions, is the key miRNA effector protein. It is therefore not surprising that regulation of miRNA processing and activity involves regulation of DCL1 and AGO. Interestingly, this regulation is mediated by two miRNAs, miR-162 and miR-168, that specifically target the DCL1 and AGO mRNA, respectively, and thus provide an important feedback regulation (see figure 4-6).

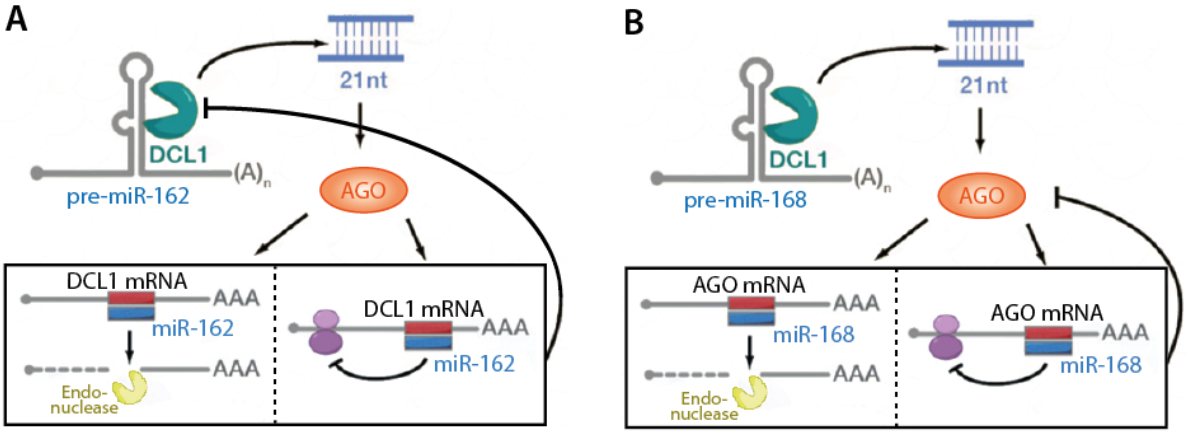

Figure 4-6 Regulation of plant miRNA processing and activity. Both Dicer-like 1 (DCL1, **A**) and Argonaute (AGO, **B**) undergo feedback regulation mediated by miR-162 and miR-168, respectively. miRNA-loaded AGO silences the mRNA encoding DCL1 and AGO, thereby globally regulating miRNA-mediated silencing. (Adapted from O. Voinnet, *Cell*, 2009)

Another possibility to modulate miRNA action temporarily involves their binding to inert targets, which makes them unavailable for silencing their cognate targets. This mechanism is used, for example, as a response to phosphate starvation: If a plant has too little phosphate, a complex stress response is turned on that promotes phosphate uptake, but also closely monitors that the phosphate level does not rise above toxic levels. Therefore, positive as well as negative regulators of phosphate uptake are expressed as a response to phosphate starvation. The protein PHO2 is such a negative regulator. PHO2 is inhibited by a certain miRNA (miR-399), which is expressed in response to phosphate starvation until phosphate levels have risen again. The activity of miR-399 itself is regulated via another mechanism: IPS1, a non-protein-coding RNA is also transcribed as a response to low phosphate. It has a target site for miR-399, just like the PHO2 mRNA, but unlike PHO2, this target site displays a central mismatch that prevents the slicing reaction and therefore sequesters miR-399-bound RISC complexes for as long as IPS1 is transcribed in the cell (see figure 4-7). IPS1 therefore modulates miR-399 activity temporarily through so-called "target mimicry". Target mimicry was later discovered to also regulate animal miRNA activity. In mammals, circular RNAs displaying dozens of miRNA target sites were recently discovered, that very efficiently divert cellular miRNAs away from their normal target mRNAs. The ways of transient miRNA regulation described here can also be used in genetic studies by engineering RNAs that mimic a target and temporarily knock down miRNAs.

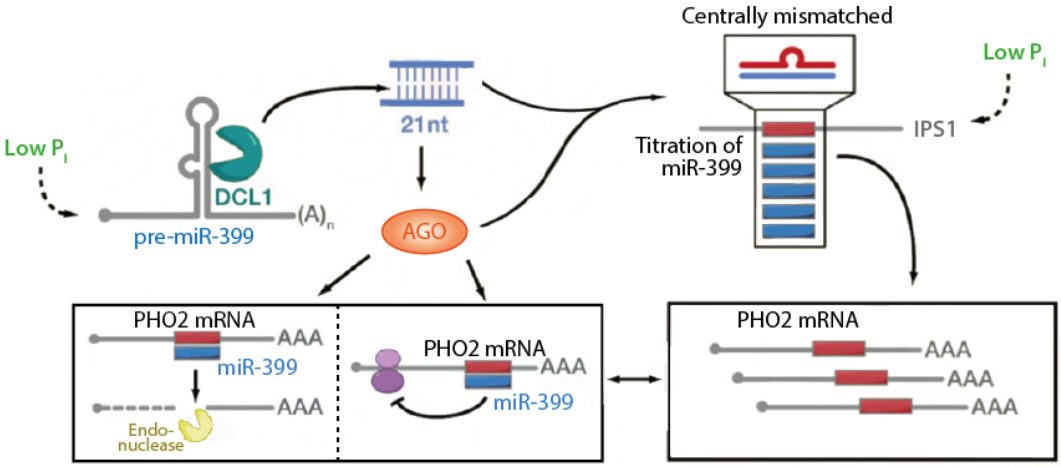

Figure 4-7 Regulation of plant miRNA activity by "target mimicry". As a response to phosphate starvation, miR-399, PHO2, and IPS1 are expressed. PHO2 is a negative regulator of phosphate uptake and is silenced by miR-399. Fine-tuning of this silencing reaction is achieved by IPS1, a non-protein-coding RNA with a miR-399 target sequence that sequesters miR-399-loaded RISC. Due to a central mismatch in the base pairing between IPS1 and miR-399, cleavage of IPS1 by Argonaute is prevented. (Adapted from O. Voinnet, *Cell*, 2009)

Plant miRNA-directed regulation

Now that we have discussed how miRNAs can be regulated on different levels, let's look at how miRNA in turn regulate cellular processes. During this section, always keep in mind the mechanisms by which different levels of miRNA expression and activity can be modulated, because they are the basis of the regulatory circuits we will discuss.

In multicellular organisms such as plants, it is often necessary that certain gene products are only active in a specific tissue but not in another (spatial restriction) or at a specific time (temporal regulation). How can such patterns be achieved by miRNA-mediated silencing? Before we look at actual examples, let's consider two hypothetical domains - A and B - within a developing plant organ. In spatial restriction, we want a certain protein target to be translated in domain A, but not in domain B. An easy way to achieve this is to express a miRNA that specifically binds the target mRNA and silences it in domain B, but not in domain A (see figure 4-8A). In this scenario, if the organism was unable to produce the miRNA, the target protein would be present in both domains.

In temporal regulation, we look at only one domain, in which a certain target protein is not produced at one point in time, but is actively translated at another. To achieve this, a gradient of miRNA expression generates an opposing gradient of its target over time (see figure 4-8B). Although target transcription stays constant, decreasing miRNA expression results in increased target translation, and vice versa. In the absence of miRNA expression, the target would be translated all the time.

Keep in mind that in both scenarios, silencing can be achieved by either cleavage of target mRNA, translational inhibition, or a combination of the two mechanisms.

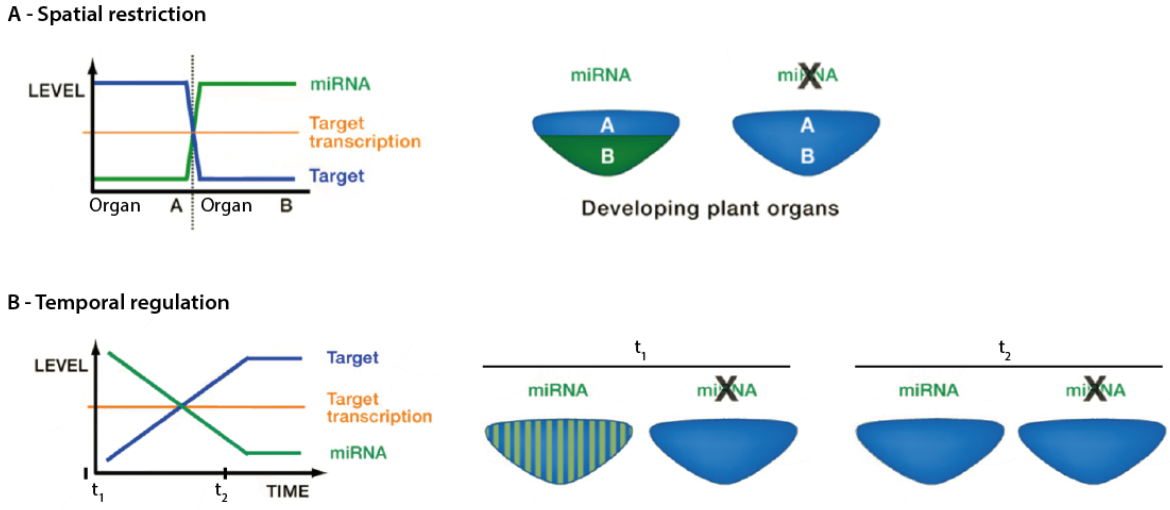

Figure 4-8 Diagram of regulatory circuits mediated by plant miRNA. (A) Spatial restriction of target (blue) accumulation in two developing plant organs A and B. Although target transcription is the same in both organs, the target accumulates in organ A where the miRNA is not present and is depleted from organ B where miRNA is expressed. In absence of miRNA, the target is translated in both domains. **(B)** In temporal regulation, a difference in target accumulation at two points in time can be achieved by generating a gradient of miRNA expression that results in an opposing gradient of its target. In the absence of miRNA, the target is translated at both time points. (Adapted from O. Voinnet, *Cell*, 2009)

Let's look at one example of how spatial restriction is achieved by miRNA-mediated silencing in *Arabidopsis*. The protein CUP-SHAPED COTYLEDON2 (CUC2) influences plant morphology, particularly the serration of leaves (tooth-like indentations) and how the sepals of flowers are joined during development. CUC2 is regulated by the miRNA miR-164. In wild-type *Arabidopsis* flowers, miR-164 is expressed in the central part (when looking from the top, figure 4-9A, area indicated by the blue oval), resulting in an absence of CUC2 protein in this region. In plants containing a mutation in *cuc2* that is not responsive to miR-164, the CUC2 protein is also now present in the central part of the flower (4-9B). That means that normally, miR-164 constrains CUC2 expression to the outer part of the flower. In miR-164-resistant *cuc2* mutants, however, CUC2 is produced even in the presence of miR-164. You can see from this example that the resistance of a target to its miRNA can result in the same phenotype as a loss-of-function mutation of the miRNA itself (see figure 4-8A above).

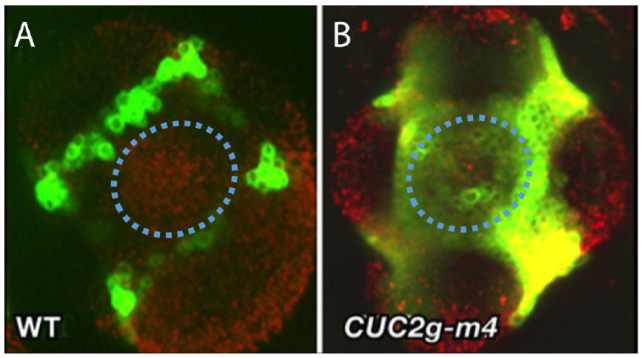

Figure 4-9 Expression patterns of miR-164 (indicated in blue) and CUC2 (reported by GFP fused to *cuc2*) in *Arabidopsis* flowers. The flowers are viewed from the top. **(A)** In wild-type plants, miR-164 inhibits CUC2 production in the center region of the flower. **(B)** In CUC2g-m4 plants, in which CUC2 is resistant to miR-164, CUC2 is produced despite the presence of miR-164. (Adapted from K. Nikovics *et al.*, *The Plant Cell*, 2006)

Summary

In this lesson, we started out by discussing the biogenesis of miRNAs in plants. One major difference to miRNA production in animals is that in plants, miRNA duplexes are fully processed in the nucleus and only mature duplexes are exported to the cytoplasm. Because plant miRNA usually show very high sequence complementarity to their targets, silencing by both translational inhibition and slicing occurs. Often, a combination of the two mechanisms is used to silence a target. Using several examples, we have shown how the expression of miRNAs in plants is regulated on different levels: the transcriptional level, on the level of biogenesis, and regulation of activity. Finally, we have discussed how plant miRNAs mediate the spatial restriction or temporal regulation of target expression.