Minireview

microRNA biogenesis and function in plants

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Abstract A microRNA (miRNA) is a 21–24 nucleotide RNA product of a non-protein-coding gene. Plants, like animals, have a large number of miRNA-encoding genes in their genomes. The biogenesis of miRNAs in *Arabidopsis* is similar to that in animals in that miRNAs are processed from primary precursors by at least two steps mediated by RNAse III-like enzymes and that the miRNAs are incorporated into a protein complex named RISC. However, the biogenesis of plant miRNAs consists of an additional step, i.e., the miRNAs are methylated on the ribose of the last nucleotide by the miRNA methyltransferase HEN1. The high degree of sequence complementarity between plant miRNAs and their target mRNAs has facilitated the bioinformatic prediction of miRNA targets, many of which have been subsequently validated. Plant miRNAs have been predicted or confirmed to regulate a variety of processes, such as development, metabolism, and stress responses. A large category of miRNA targets consists of genes encoding transcription factors that play important roles in patterning the plant form.

Keywords: microRNA; Small interfering RNA; DCL1; HEN1; Auxin; Flower development; Leaf development; Developmental transitions

1. What is a microRNA?

A microRNA (miRNA) is a 21–24 nucleotide (nt) small RNA that is the final product of a non-coding RNA gene. miRNA genes resemble protein coding genes in that they may contain introns and that they are transcribed by RNA polymerase II. Like other pol II transcripts, the transcripts from miRNA genes are capped, spliced and polyadenylated (reviewed in [1]). The mature miRNA is located in a hairpin structure within the primary transcript (pri-miRNA) and is processed from the pri-miRNA through at least two RNAse III-mediated steps (reviewed in [1,2]). The miRNA is loaded into a ribonucleoprotein complex named RISC, where it guides the cleavage or translational repression of its target mRNAs by base-pairing with the targets (reviewed in [2]).

Plants are rich in another type of small RNAs (known as siRNAs) that is similar in structure, biogenesis and function to miRNAs. siRNAs originate from transcripts from transgenes [3], endogenous repeat sequences or transposons [4,5]. One key distinction between miRNAs and siRNAs from transgenes and repeat sequences/transposons is that miRNAs target genes other than the ones that give rise to the miRNAs while siRNAs target the very sequences that generate them (reviewed in [2]). Recently, a new class of siRNAs that, like miRNAs, targets mRNAs from other loci was discovered in *Arabidopsis* and named *trans*-acting siRNAs (ta-siRNAs) [6,7]. The ta-siRNAs originate from loci that give rise to non-coding transcripts that are themselves targets of miRNAs. The miRNA-mediated cleavage of the transcripts recruits an RNA-dependent RNA polymerse (RdRP) to use the cleaved transcripts as templates to generate long double-stranded RNAs (dsRNAs), which then serve as the source of multiple ta-siRNAs. The miRNA-mediated cleavage of the precursor RNA is crucial for the biogenesis of the ta-siRNAs and sets the register for the cleavage events that generate the ta-siRNAs [8]. Since ta-siRNAs target mRNAs from other genes, the cis/trans relationship between small RNAs and their targets is no longer a distinction between miRNAs and siRNAs. Now it seems that the only feature that distinguishes miRNAs and siRNAs is the nature of the precursor transcripts. While a miRNA comes from a hairpin pre-miRNA, siRNAs come from a perfect, long dsRNA generated by an RdRP or through the transcription of a hairpin transgene. Usually only one miRNA is generated from the pre-miRNA but several or many siRNAs are generated from long dsRNAs. However, there is a case in which more than one small RNAs come from a single pre-miRNA [9].

2. miRNA biogenesis

2.1. miRNA maturation

In animals, a miRNA is processed from the pri-miRNA by two RNAse III enzymes, Drosha and Dicer. Drosha crops the pri-miRNA into the pre-miRNA, the hairpin structure in the pri-miRNA [10]. Drosha is found in a protein complex named the microprocessor that also contains the DiGeorge Syndrome Critical Region Gene 8 (DGCR8) protein in humans (Pasha in *Drosophila*) [11–14]. Drosha cleavage leaves in the stem of the hairpin pre-miRNA a 2 nt 3′ overhang [10], a feature that is preferred by Dicer [15], the enzyme that processes the pre-miRNA to produce a duplex of 21 nt small RNAs with 2 nt overhangs at the 3′ end of each strand [16–19]. The strand in which the 5′ end is less stable is selectively incorporated into a protein complex named RISC [20], where the miRNA serves as the effector of gene regulation. The

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miRNA maturation process is partitioned between the nucleus and the cytoplasm [21]. Drosha processes the pri-miRNA in the nucleus to the pre-miRNA [10], which is then exported to the cytoplasm by exportin 5 [22–24]. In the cytoplasm, Dicer releases the miRNA from the pre-miRNA.

In *Arabidopsis*, the maturation of the miRNA from the pri-miRNA is also a stepwise process that involves a Dicer-like protein, DCL1 (Fig. 1). The *Arabidopsis* genome, however, does not contain homologs of Drosha or DGCR8. In fact, it is found that DCL1 processing pri-miRNA163 to pre-miRNA163 [9]. Although it has not been demonstrated that DCL1 processes pre-miRNA to the mature miRNA, DCL1 is likely the key enzyme that performs this function for the following reasons. First, among the four *DCL* genes in the *Arabidopsis* genome, *DCL1* is the only essential gene. Null alleles in *DCL1* result in embryo lethality while weak *dcl1* mutants exhibit pleiotropic developmental defects [25–27]. Mutations in other *DCL* genes do not result in such severe developmental defects ([5,108–110]). Since miRNAs play key roles in plant development (see below), the phenotypes of the *dcl* mutants are consistent with DCL1 being the main miRNA-generating Dicer. Second, most miRNAs examined are reduced in abundance in the weak *dcl1* mutants, such as *dcl1-7* and *dcl1-9*, while mutations in *DCL2* and *DCL3* do not affect the abundance of the miRNAs examined [28–30]. However, it should be noted that the accumulation of some miRNAs is not affected in the weak *dcl1-9* mutant [31]. Either another DCL protein is responsible for the maturation of these miRNAs or DCL1 is the processing enzyme for these miRNAs but the weak *dcl1-9* mutant protein still retains the ability to process the precursors of this set of miRNAs. Like in animal miRNA maturation, the processing of the pre-miRNA yields the miRNA/miRNA* duplex, in which the miRNA is selectively loaded into RISC. The asymmetric loading of the two strands into RISC probably follows the same rule as for animal miRNAs [20,32]. The presence of the miRNA/miRNA* duplex is supported by the isolation of certain miRNA* sequences from small RNA cloning efforts [30] and the detection of several miRNAs* by filter hybridization [33,34]. Several viral RNA silencing suppressors that affect miRNA biogenesis

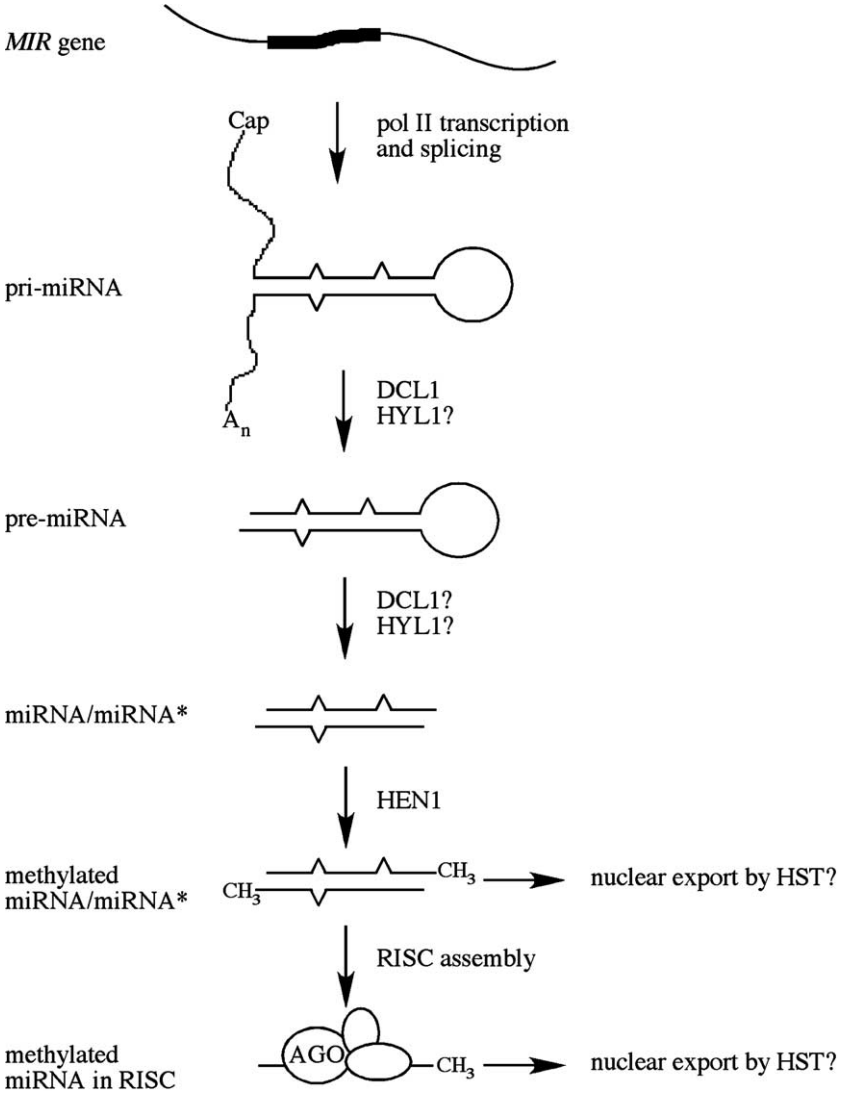

Fig. 1. A diagram of miRNA biogenesis in *Arabidopsis*. A miRNA gene is transcribed by RNA polymerase II. The pri-miRNA is capped and polyadenylated. The pri-miRNA is processed by DCL1, perhaps in two or more steps, to the miRNA/miRNA* duplex, which is then methylated by HEN1. The miRNA strand is incorporated into RISC. The nuclear export of miRNAs may occur before or after RISC assembly.

also provide evidence for the transient presence of the miRNA/miRNA* duplex. When expressed in *Arabidopsis*, the *Beet yellows virus* p21 and the *Tomato bushy stunt virus* p19, which bind the duplex but not single-stranded miRNAs [35–38], lead to increased abundance of miRNA* and reduced miRNA-mediated cleavage of target mRNAs [35,39]. It is thought that p19 and p21 bind to and stabilize the duplex, therefore preventing RISC assembly.

The subcellular compartmentalization of miRNA biogenesis in *Arabidopsis* may be different from that in animals. A partial DCL1 protein fused to the green fluorescent protein was localized in the nuclei of union epidermal cells upon transient expression, indicating that DCL1 has a nuclear localization sequence [40]. Although the location of the full-length DCL1 protein in the cell remains to be determined, it is likely that miRNA biogenesis occurs in the nucleus since nuclear localized p19 leads to reduced abundance of miRNAs [40]. While exportin 5 exports pre-miRNAs to the cytoplasm in animals, the *Arabidopsis* homolog of exportin 5, HASTY (HST; [41]), is proposed to export the miRNA/miRNA* duplex to the cytoplasm based on the assumption that the duplex is produced by DCL1 in the nucleus [2]. *hst* mutants show reduced accumulation of many but not all miRNAs and are compromised in the cleavage of certain miRNA target genes, consistent with a role of *HST* in miRNA biogenesis [33]. miRNAs are found in presumably single-stranded forms in both the nuclear and the cytoplasmic compartments (miRNAs* do not accumulate in either compartment) [33]. This implies that miRNAs are present in both compartments as molecules present in RISCs. One possibility is that RISC assembly occurs in the nucleus followed by the export of RISCs (by HST) to the cytoplasm (Fig. 1). Alternatively, miRNA/miRNA* is exported to the cytoplasm (Fig. 1), where RISC assembly occurs, and then some RISCs are imported back to the nucleus. It is also possible that RISC assembly occurs separately in both compartments.

2.2. Methylation of miRNA/miRNA*

The biogenesis of miRNAs in *Arabidopsis* requires two other proteins, HYL1 and HEN1 [29,42,43] (Fig. 1). HYL1 contains two dsRNA-binding motifs and is homologous to RDE-4 from *C. elegans* and R2D2 from *Drosophila*, proteins that play a role in RISC loading in association with Dicer [44,45]. Interestingly, while RDE4 and R2D2 act in siRNA but not miRNA metabolism, HYL1 is required for miRNA but not siRNA biogenesis. HYL1 is a nuclear protein present in a protein complex [42] but its biochemical function in miRNA biogenesis in unknown.

HEN1 has a putative dsRNA-binding motif and a C-terminal methyltransferase domain. The *hen1-1*, *hen1-2*, and *hen1-4* mutations that compromise miRNA metabolism are all in the methyltransferase region [46,47]. Purified HEN1 protein is able to methylate the miRNA/miRNA* duplex in vitro [48]. The methylation occurs on the ribose of the last nucleotide on each strand of the duplex. HEN1 is highly selective of its substrate: single-stranded miRNA, single-stranded miRNA*, pre-miRNA, dsDNA identical in sequence and structure to miRNA/miRNA* cannot serve as substrates of HEN1. miRNA/miRNA* of different primary sequences can serve as substrates, suggesting that HEN1 recognizes the structure (rather than the sequence) of the duplex produced by Dicer processing of pre-miRNA. The in vitro biochemical activity of HEN1 and the in vivo requirement for HEN1 for miRNA biogenesis suggest that *Arabidopsis* miRNAs are methylated on their last nucleotides. Indeed, *Arabidopsis* miRNAs are resistant to chemical reactions that depend on the presence of both hydroxyl groups on the last nucleotide [48]. Mass spectrometry analysis of miR173 purified from *Arabidopsis* indicates that miR173 possesses one methyl group [48]. For any miRNA species, no unmethylated molecules can be detected in vivo by filter hybridization, indicating that the great majority of any miRNA species is methylated in vivo.

What is the function of miRNA methylation? It is conceivable that the methyl group on the ribose of the last nucleotide, whether at the 2′ or 3′ position, impedes the activities of enzymes that target either hydroxyl group of the last nucleotide, such as ligases, terminal nucleotydyl transferases, or polymerases. One group of enzymes relevant to small RNA metabolism is RdRP. In both *C. elegans* and in plants, post-transcriptional gene silencing (or RNAi) can be transitive, i.e., silencing can occur in regions outside of the initial region targeted for silencing in an RNA [49,50]. In *C. elegans* the transitivity only extends to regions 5′ to the initial target region, whereas in plants the transitivity happens in both directions [49,50]. Transitive RNAi in *C. elegans* requires an RdRP and it has been proposed that primary siRNAs from the silencing trigger serve as primers for the RdRP, which uses the target RNA as the template to synthesize long double-stranded RNAs that in turn are processed to secondary siRNAs [49]. Plant miRNAs are highly complementary to their target mRNAs and many have been shown to guide the cleavage of their target transcripts [28]. It is conceivable that plant RdRPs may use a miRNA as the primer and the target mRNA as the template to synthesize long dsRNA, which in turn may be processed to siRNAs. This may be used as a mechanism to coordinately regulate multiple, highly conserved members of a gene family in which only one or some members have a miRNA target site. This may also be a scenario that the plant should avoid to prevent the unintended silencing of genes sharing high degree of sequence similarity with the real miRNA target gene in regions 5′ to the miRNA target site. The presence of the methyl group on the last nucleotide of plant miRNAs may discourage RdRPs from using them as primers. However, the effect of the methyl group on the ability of the miRNAs to serve as primers for RdRPs needs to be determined biochemically.

One enzymatic activity the methylation appears to protect plant miRNAs against is that of a polymerase or terminal transferase that adds additional nucleotides, primarily U's to the 3′ end of miRNAs [51]. This finding stemmed from the initial observation that miRNAs become heterogenous in size in addition to being reduced in abundance in *hen1* mutants [29,42]. We examined the source of the size heterogeneity in the miRNAs in *hen1-1* and found that the 5′ end of the heterogenous miRNAs is the same as the corresponding miRNA species in wild type, suggesting that the size heterogeneity arises from the miRNAs having different 3′ ends. Cloning of specific miRNAs from wild type and *hen1* mutants showed that miRNAs in *hen1* have additional nucleotides, primarily U's at their 3′ ends. Perhaps the U tailing of unmethylated miRNAs in *hen1* mutants leads to their degradation, which would explain their reduced abundance. The enzyme that adds

the additional nucleotides is currently unknown. Intriguingly, it was found that U residues are added to the 5′ fragments of target mRNA after miRNA-mediated cleavage [52].

3. Role of miRNAs in growth and development

Plant miRNAs have a high degree of sequence complementarity to their target mRNAs. This has facilitated the bioinformatic prediction of miRNA target genes [8,29–31,34,53–57], some of which have been subsequently validated experimentally to be true targets of the miRNAs (see below). Plant miRNAs have been predicted or confirmed to regulate genes encoding various types of proteins. A major category of miRNA target genes consists of transcription factors or other regulatory proteins that function in plant development or signal transduction. Below, I summarize the roles of characterized miRNAs and the predicted roles of some miRNAs in plants.

3.1. Auxin signaling

The small molecule auxin is critical for the plant form and for plants' response to the environment. Recent years have witnessed a tremendous leap in our understanding of the role of auxin in pattern formation (reviewed in [58–62]) and in the mechanisms of auxin signaling [63–66]. The local concentration of auxin, as established by polar auxin transport, appears to pattern the axis of the embryo, establish root stem cells, and control primordia outgrowth from meristems, lateral root formation, and gravitropic responses. Auxin leads to the degradation of a class of transcription repressor proteins known as Aux/IAA proteins through the ubiquitin proteasome pathway. The Aux/IAA proteins heterodimerize with members of the auxin response factor (ARF) family of transcription activators and repressors and inhibit the activities of the activating ARFs. Auxin is bound by TIR1, an F-box protein in the ubiquitin protein ligase SCF^{TIR1}. Auxin promotes the interaction between SCF^{TIR1} and its substrates, Aux/IAA proteins, to lead to their proteolytic degradation.

Intriguingly, a number of genes in auxin signaling are confirmed or predicted as targets of miRNAs. The *TIR1* auxin receptor is a predicted target of miR393 ([34,53–55]). Several *ARFs* contain potential miRNA binding sites, such as *ARF10*, *ARF16* and *ARF17* with miR160 sites [56] and *ARF6* and *ARF8* with miR167 sites [67]. Products of miR160-guided cleavage of *ARF10*, *ARF16* and 17mRNAs and miR167-guided cleavage of *ARF8* mRNA were detected in vivo [28,68]. Expression of a miR160-resistant version of *ARF17* (*5mARF17*) leads to pleiotropic developmental abnormalities, such as leaf serration, leaf curling, early flowering, altered floral morphology, and reduced fertility [68]. This indicates that miR160-mediated regulation of *ARF17* is critical for various aspects of plant development. Since the expression of a number of early auxin responsive genes is altered in *5mARF17* plants [68], miR160 likely plays a role in auxin signaling. In addition, *ARF3* and *ARF4* contain the binding sites of ta-siRNAs from the *TAS3* locus [8]. The *TAS3* ta-siRNA-guided cleavage of *ARF3* and *ARF4* mRNAs is observed in vivo [8]. Although the biological function of the ta-siRNA-mediated regulation of *ARF3* and *ARF4* remains to be determined, the importance of this regulation is reflected by the nucleotide conservation of the ta-siRNA binding sites in *ARF3/4* sequences from 16 gymnosperm and angiosperm species [8]. *NAC1*, which encodes a transcription factor acting downstream of TIR1 to promote lateral root formation, is a target of miR164. miR164 guides the cleavage of *NAC1* mRNA in vivo [69]. T-DNA insertions in two of the three members of the miR164 family (*mir164a*, *mir164b*) lead to 1/4-1/3 wild type levels of total miR164 and cause an increase in *NAC1* mRNA levels and a corresponding increase in lateral root number [69].

3.2. Boundary formation/organ separation

Three members of the *NAC* gene family, *CUP SHAPED COTYLEDON* (*CUC*)*1*, *2*, and *3*, have partially overlapping functions in organ boundary formation and shoot apical meristem (SAM) initiation. All three genes are expressed in the boundary of the two cotyledons in the embryo and later in the boundaries of floral organs [70–72]. Mutations in two of the three genes together lead to a high frequency of cotyledon fusion on both sides of the cotyledons and doubly mutant plants containing *cuc2* and one of the other *cuc* alleles lack the SAM [70,72]. Although *cuc1 cuc2* double mutant seedlings lack the SAM and therefore fail to develop organs postembryonically, shoots can be induced from calli derived from hypocotyls of *cuc1 cuc2* seedlings. While the leaves and stems of the shoots appear normal, the flowers have fused sepals and stamens [70], suggesting that these two genes prevent sepal and stamen fusion in addition to their early role during embryogenesis.

CUC1 and *CUC2* but not *CUC3* are targeted by miR164 [28,55]. Overexpression of miR164 from the 35S promoter in wild type plants leads to floral organ fusion [73,74] and, at a lower frequency, cotyledon fusion [73], phenotypes similar to those of *cuc1 cuc2* plants. Expression of miR164-resistant *CUC2* can restore sepal separation to miR164 overexpressing lines [73]. Expression of a miR164-resistant form of *CUC1* in wild type plants results in reduced sepal number, increased petal number, and broadened leaves [74]. Using an ethanol inducible expression system, the expression of a miR164-resistant form of *CUC2* was temporally controlled and the effect on sepal boundaries was monitored following the induction of its expression. It was found that expression of miR164-resistant *CUC2* led to an increase in the width of the boundary domain between sepals [73]. An increase in sepal boundary domain width was also observed in miRNA biogenesis mutants such as *dcl1*, *hen1* and *hyl1* [73]. The expansion of the sepal boundary domain may explain the narrow sepals in *dcl*, *hen1* and *hyl1* mutants and the reduced sepal number in lines expressing miR164-resistant forms of *CUC* genes.

miR164 is potentially encoded by a three-member gene family. Incredibly, despite potential genetic redundancy, *MIR164c* (one of the three members of the *MIR164* family) was identified in a forward genetic screen as a gene regulating petal number in flowers [75]. The *mir164c* mutant has extra petals in the early arising flowers. Among the six potential targets of miR164, only *CUC1* and *CUC2* mRNA levels were increased in the *mir164c* mutant [75]. Furthermore, *cuc1 cuc2 mir164* flowers are indistinguishable from *cuc1 cuc2* flowers, suggesting that the increased petal number in *mir164c* is due to the overexpression of *CUC1* or *CUC2* [75]. How mis-regulation of *CUC1* and *CUC2* leads to an increase in petal number (either in the *mir164c* mutant or in transgenic lines expressing miR164-resistant *CUC1* or *CUC2*) is unknown. It is possible that the increase in petal number is caused by a change in

the size of the boundary domain between the first whorl organs (which signals to the second whorl) or in the size of the boundary domain between the first whorl and the third whorl, which is formed before the second whorl is formed.

3.3. Organ polarity

Lateral organs such as leaves and floral organs are initiated as primordia on the flanks of the SAM or floral meristems. The lateral organs are polar structures in that the side of the organ facing the meristem in the primordium (called the adaxial side) differs from the side that faces away from the meristem (called the abaxial side). The difference is reflected by the morphology of the epidermis and the underlying mesophyll on the two sides. The vasculature in the stem and in lateral organs also displays polarity in that the xylem is found on the adaxial side while the phloem is found on the abxial side. Polarity of lateral organs is established through the antagonistic interactions between two major groups of genes, the class III homeodomain leucine zipper (HD-zip) family of genes, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) and the *KANADI* family (*KAN1*, *2*, and *3*) of genes [76–80]. The HD-zip genes are expressed in the meristem and in the adaxial domain of lateral organs while the KAN genes are expressed in the abaxial domain of lateral organs. Dominant, gain-of-function mutations in *PHB*, *PHV* and *REV* genes result in adaxialized leaves and floral organs, phenotypes similar to those of *kan* loss-of-function mutants. The triple loss-of-function *phb phv rev* mutant has abaxialized cotyledons and lacks the SAM, phenotypes similar to those of the transgenic lines overexpressing *KAN1* from the 35S promoter. In addition to leaf polarity, these genes also pattern the polarity of the vasculature in a similar manner.

The dominant alleles in *PHB*, *PHV* and *REV* genes contain mutations that affect the amino acid sequences of the proteins, which led to the hypothesis that the mutant proteins have activities that are different from the wild type ones. However, when miR165/166 was cloned it became clear that the mutations in these genes are in the binding sites of miRNA165/166 such that they may affect the regulation of these genes by the miRNA rather than affecting the activity of the proteins [30,56]. Indeed, when silent mutations that affect miRNA binding but maintain the protein coding potential were introduced into the genes, similar gain-of-function phenotypes were observed [76,81]. In the gain-of-function *phb-d* allele, the expression domain of the gene expands into the abaxial region [79], suggesting that the miRNA-mediated regulation contributes to the restriction of *PHB* expression to the adaxial domain. However, how this occurs is currently unknown. miR165/166 guides the cleavage of *PHB*, *PHV*, and *REV* mRNAs in vivo [81], so an obvious mechanism would be that the cleavage of the HD-zip mRNAs by miR165/166 in the abaxial domain clears the mRNAs from this domain. However, it is also found that miR165/166 causes DNA methylation of the *PHB* and *PHV* genes [82]. Therefore, an alternative mechanism is that the miRNA represses the transcription of the genes in the abaxial domain.

miR165/166-mediated regulation of HD-zip genes appears to be evolutionarily conserved. The miR165/166-binding site is highly conserved among angiosperms, gymnosperms, ferns, lycopods, mosses, liverworts, and hornworts [83]. In maize, the semi-dominant mutant *Rolled leaf1-Original* (*Rld1-O*) has adaxialized leaves. *rld1* encodes a homolog of the *Arabidopsis REV* and the *Rld-O* mutation is in the miR165/166-binding site [84]. In *Nicotiana sylvestris*, a semidominant *phv* allele isolated from ethyl methanesulfonate mutagenesis is phenotypically similar to the *Arabidopsis* HD-zip gain-of-function mutants. This allele carries a mutation in the miR165/166-binding site but does not affect the coding potential of the gene [85].

3.4. Floral organ identity and reproductive development

Floral organs are initiated in rings (whorls) from the floral meristem. The identities of the floral organ primordia are specified by the combinatorial activities of three major classes of floral homeotic genes known as the A, B, and C genes [86]. The A and C genes specify the identities of the perianth and reproductive organs, respectively. Loss-of-function mutations in the class C gene *AGAMOUS* (*AG*) leads to the replacement of reproductive organs by perianth organs while loss-of-function mutations in the class A gene *APETALA2* (*AP2*) lead to the opposite effect, suggesting that A and C genes act antagonistically to restrict each other to their domains of activity within the floral meristem. In fact, the mRNA of *AG* is found only in the inner two whorls of the floral meristem that will later become the reproductive organs while the mRNA of *APETALA1*, a class A gene, is only found in the outer two whorls of the meristem that will later become the perianth organs. However, the transcript of the class A gene *AP2* is present throughout the floral meristem.

AP2 contains a binding site for miR172 and is indeed regulated by miR172 in vivo. Overexpression of miR172 from the 35S promoter causes a reduction in the levels of AP2 protein and floral homeotic phenotypes similar to those in *ap2* loss-of-function mutants [87,88]. Overexpression of a miR172-resistant form of *AP2* cDNA but not wild type *AP2* cDNA leads to the replacement of reproductive organs by perianth organs [88]. Expression of the miR172-resistant form of *AP2* from the *AP2* promoter also results in severe floral patterning defects in the inner two whorls (Zhao L. and Chen X., unpublished results). These observations highlight the importance of miR172 in repressing *AP2* in the inner two whorls in floral patterning. Intriguingly, the A/C/miR172 genetic system in the control of floral patterning is very similar to the HD-zip/KAN/miR165/166 system in the specification of organ polarity. In both cases, two genetically antagonistic functions specify the identities of adjacent domains and a miRNA serves as a negative regulator of one of the two functions.

Although miR172 leads to the cleavage of *AP2* mRNA in vivo as deduced from the presence of cleavage products [28,87], RNA cleavage cannot solely explain the regulation of *AP2* by miR172. Overexpression of miR172 leads to reduced levels of AP2 protein but not mRNA levels [87–89], suggesting that miR172 resembles animal miRNAs in its mode of action, which has been dubbed translational inhibition [90–92]. miR172 may lead to reduced levels of AP2 protein in the inner two whorls despite the uniform accumulation of *AP2* RNA throughout the floral meristem.

Another miRNA, miR159, plays a role in reproductive development by regulating two MYB domain transcription factor genes, *MYB33* and *MYB65*. These two genes act redundantly to prevent the hypertrophy of the tapetum during anther development [93]. miR159 is crucial in restrict-

ing the expression of *MYB33* and *MYB65* to anthers [93]. Transgenic plants containing a miR159-resistant version of *MYB33* under its own promoter are arrested for growth at various stages, indicating that the restriction of *MTB33* expression by miR159 is critical for plant development [93].

3.5. Developmental transitions

The SAM continues to put out primordia on its flanks during the life cycle of the plant but the types of primordia produced differ at different developmental stages. The SAM generates leaves during the vegetative phase and flowers during the reproductive phase. Even during the vegetative phase, the types of leaves that are put out earlier (juvenile leaves) may differ from the ones made later (adult leaves). Therefore, the state of the SAM, as reflected by the identities of the lateral primordia it produces, changes temporally. Recent findings suggest that small RNAs regulate the transitions between the developmental states of the SAM.

In addition to *AP2*, miR172 regulates several *AP2*-like genes, *TOE1*, *TOE2*, *TOE3*, *SMZ* and *SNZ*. The loss-of-function *toe1-1* mutation leads to slightly early flowering and the *toe2-1* mutation does not affect flowering time. The *toe1-1 toe2-1* double mutant flowers much earlier than wild type, suggesting that *TOE1* and *TOE2* are redundant repressors of the vegetative-to-reproductive transition [87]. Consistently, overexpression of *TOE1* (*toe1-1D*) leads to delayed flowering [87]. Overexpression of *SMZ* or *SNZ* also causes late flowering, but the role of the two genes in flowering needs to be evaluated from loss-of-function mutant phenotypes [94]. Overexpression of miR172 from the 35S promoter, on the other hand, leads to early flowering [87,88] and overcomes the late flowering phenotpye of *toe1-1D* [87]. This, together with the fact that miR172-guided cleavage products of *TOE1* and *TOE2* mRNA can be detected in vivo [28,87], suggests that miR172 regulates the vegetative-to-reproductive transition through the *TOE* genes. However, overexpression of miR172 does not lead to a decrease in *TOE1* mRNA level, suggesting that translational inhibition is the likely mode of regulation of miR172-mediated regulation of *TOE1* [87,94]. Overexpression of miR172 does lead to a decrease in *TOE2* mRNA level, suggesting that this miRNA regulates different targets with different mechanisms [94].

miR156 is another miRNA that when overexpressed affects flowering time. *35S::MIR156* plants are late flowering [89]. miR156 probably targets a group of transcription factor genes known as squamosa promoter binding protein-like (SPL) [56] but the role of *SPL* genes in floral transition awaits further investigation. miR319/Jaw overexpression also leads to delayed flowering [95]. miR319/Jaw targets a group of TCP transcription factors for which a role in flowering is currently unknown [95]. Overexpression of miR159 leads to delayed flowering under short day but not long day conditions [96].

Vegetative phase change is pronounced in maize. The juvenile leaves differ from adult leaves in many epidermal characteristics (reviewed in [97]). An *AP2*-like gene, *glossy15*, promotes juvenile leaf identity and its RNA is only found in juvenile leaves (reviewed in [97]). *glossy15* contains a miR172-binding site and miR172-guided cleavage products can be detected in vivo, suggesting that *glossy15* is a target of miR172 [98]. The onset of miR172 expression in maize appears to correlate with the specification of adult leaf characteristics. Since the expression of *glossy15* at the RNA level mirrors that of miR172, it is likely that miR172 clears *glossy15* mRNA in adult leaves to promote the vegetative phase change [98].

Although not as pronounced, vegetative phase change does occur in *Arabidopsis*. The Poethig lab used a forward genetic approach to identify genes playing a role in vegetative phase change in *Arabidopsis*. Intriguingly, among the genes identified to play a role in vegetative phase change, many play a role in small RNA metabolism. These include *HST*, (which may export miRNAs to the cytoplasm), *AGO7*, (which may be a RISC component), and *RDR6* and *SGS3*, genes previously known to be required for transgene silencing [6,41,99–101]. *RDR6* and *SGS3* are also required for the biogenesis of *trans*-acting siRNAs [6], whose production is triggered by miRNAs [8]. These studies suggest that miRNAs and ta-siRNAs play a role in phase transitions.

3.6. Leaf growth

The snapdragon *CINCINNATA* (*CIN*) gene is required for the differential regulation of cell division during leaf morphogenesis to make a flat leaf [102]. *cin* mutants have crinkly leaves. CIN belongs to the TCP family of transcription factors. In *Arabidopsis*, overexpression of miR319/Jaw leads to crinkly leaves and reduced levels of five *TCP* genes containing miR319/Jaw-binding sites [95]. Overexpression of a miR319/Jaw-resistant form of *TCP2* fully restores the crinkly leaf phenotype of miR319/Jaw overexpression [95].

3.7. Small RNA metabolism

At least three miRNAs are known to target genes involved in small RNA metabolism or function. *DCL1* contains a binding site for miR162 and miR162-guided cleavage products of *DCL1* mRNA are detected in vivo [103]. Consistent with a role of miR162 in reducing *DCL1* mRNA levels, *DCL1* mRNA abundance is elevated in mutants defective in miRNA biogenesis (such as *dcl1* or *hen1*) and in transgenic lines expressing the viral RNA silencing suppressor HC-Pro, which appears to inhibit RISC assembly [35,103]. The *AGO1* gene that codes for a RISC component is targeted by miR168. Overexpression of a miR168-resistant version of *AGO1* appears to affect miRNA function because miRNA targets overaccumulate and the plants show phenotypes similar to those of miRNA biogenesis mutants such as *dcl1*, *hen1* and *hyl1* [104]. *AGO2*, another argonaute gene, contains a binding site for miR403 in its 3′ UTR and in vivo cleavage products have been detected [8]. However, the role of *AGO2* in small RNA biology is currently unknown. The regulation of genes involved in small RNA metabolism or function by miRNAs is perhaps a feedback mechanism to ensure a certain level of activity of small RNA pathways.

3.8. Predicted roles of other miRNAs

While the transcription factor-encoding miRNA targets are the best characterized so far, miRNA targets encoding proteins with various other cellular functions, such as protein degradation, metabolism and stress responses, have been predicted and some have been validated using the criteria that in vivo cleavage products be detectable [8,31,34,53–55,57]. It is therefore expected that miRNAs regulate a variety of cellular processes.

4. Regulation of the miRNA genes

The tissue- or cell-type-specific functions of the miRNAs discussed above suggest that miRNA gene expression is precisely regulated in the plant. It is conceivable that the abundance of a certain miRNA in cells can be regulated at multiple levels, such as the transcription of the gene, the processing of the pri-miRNA by DCL1, the methylation of the miRNA by HEN1, the loading of the miRNA into RISC, and the export of the miRNA into the cytoplasm, and even the potential transport of the miRNA in or out of cells or tissues [105]. Although how miRNAs themselves are regulated is currently unknown, available data show that miRNA accumulation exhibits tissue or cell type specificity and responses to stimuli. By in situ hybridization, it was shown that miR172 is present throughout the early floral primordia but not in the SAM from which the floral primordia arise [88] and miR165/166 is restricted to the abaxial side of leaves in *Arabidopsis* and maize [84,106]. By RNA filter hybridization, it was shown that miR159 levels are induced by GA [96] and miR164 levels respond to auxin [69]. The promoter of miR164c endows dynamic expression to the GUS reporter gene during plant development [75], suggesting that the transcription of miRNA genes are regulated. A study in which the promoter activity of *MIR171* and the activity of miR171 were both monitored showed that the promoter is highly cell- or tissue-specific and that transcription of *MIR171* is largely responsible for the cell- or tissue-specific activities of the miRNA [107]. One future challenge of miRNA biology is to understand how miRNAs themselves are regulated.

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