

The Regulatory Activities of Plant MicroRNAs: A More Dynamic Perspective¹

Yijun Meng*, Chaogang Shao, Huizhong Wang, and Ming Chen

College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, People's Republic of China (Y.M., H.W.); College of Life Sciences, Huzhou University, Huzhou 313000, People's Republic of China (C.S.); and Department of Bioinformatics, College of Life Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China (M.C.)

Twenty years have elapsed since the discovery of a microRNA (miRNA) gene in *Caenorhabditis elegans*. Based on growing research progress, we are approaching the nature of this small RNA species, which seemed to be mysterious before. The regulatory activities of miRNAs have been extensively studied through target identification and physiological and phenotypic assays by using bioinformatic, genetic, and biochemical approaches. However, recent evidence points to the fact that the effective levels of miRNAs are determined by transcription, processing, miRNA-induced silencing complex loading, action, turnover use, and decay. Each process is affected by certain factors, such as genomic modifications, RNA editing, miRNA-induced silencing complex loading competition, target abundance and complementarity, and spatiotemporal effects, thus conferring a highly dynamic feature to miRNA activities. To maintain steady-state levels of the functional miRNAs, thus ensuring a normal physiological and biochemical status, plants employ several exquisite strategies, such as feedback regulation and a buffering system, to minimize the influence of external signal fluctuations. In this review, we raise the notion that a more dynamic picture of miRNA activities should be drawn to construct comprehensive miRNA-mediated networks in plants.

MicroRNAs (miRNAs), approximately 21 nucleotides in length, were identified as a small RNA (sRNA) species with essential regulatory roles in various biological processes (Carrington and Ambros, 2003). The transcription of most miRNA genes is guided by RNA polymerase II (Lee et al., 2004; Xie et al., 2005). Following transcription, the single-stranded RNAs with internal stem-loop structures are then recognized by Drosha and Dicer in animals (Kim et al., 2009a) or Dicer-Like1 (DCL1) in plants (Voinnet, 2009) for sequential cleavage, converting the primary microRNAs (pri-miRNAs) to the precursor microRNAs (pre-miRNAs) and finally to the miRNA/miRNA* duplexes. After

dissociation from the duplexes, the miRNAs are incorporated into Argonaute (AGO)-associated microRNA-induced silencing complexes (miRISCs; preferentially AGO1-associated miRISCs). Although the sophisticated model of miRNA biogenesis is seemingly settled for each step, there exist many key nodes that influence the final activity of a miRNA gene. The transcription of miRNA genes is under the rigorous surveillance of many cis- and trans-factors, such as chromatin marks and specific transcription factors (TFs). The processing efficiency of the miRNA precursors is basically determined by their own sequences and structures and is regulated in a spatiotemporal manner (Davis and Hata, 2009; Cuperus et al., 2011; Zhu et al., 2011). Furthermore, the sorting of miRNAs into specific AGO complexes should not be oversimplified, since not all the miRNAs are uniformly loaded into AGO1-associated miRISCs. Additionally, loading competition between miRNAs and other sRNAs occurs in plants.

In plants, miRNAs guide the miRISCs to target transcripts containing highly complementary recognition sites to exert their repressive roles on gene expression. This seemingly simple one-to-one regulation occurs with several concomitant events that strongly affect the regulatory intensity. For instance, the degradation rate of a miRNA was reported to be highly dependent on target abundance and complementarity (Chatterjee and Grosshans, 2009; Ameres et al., 2010; Arvey et al., 2010). After one cleavage of a specific target, the miRISC may survive, or the released miRNA could form another miRISC, both of which will involve in another round of targeting. This turnover rate is also determined by the target complementarity. Quite a different situation exists between miRNAs and their low-complementary targets. For miRNAs sequestered by bulge targets, the turnover rate is intensely reduced.

All the evidence points to the conclusion that the regulatory activities of the miRNAs are highly dynamic. From transcription, to precursor processing, to miRISC loading, to target recognition and miRNA-mediated regulation, and finally to miRNA degradation and turnover, numerous crucial factors support the apparently steady-state levels of the mature miRNAs.

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* Corresponding author; e-mail mengyijun@zju.edu.cn.
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From another point of view, the miRNA-mediated regulation itself is strictly regulated in many respects, making the miRNA-involved networks more robust. Based on recent research progress, we felt that a dynamic view should be provided to measure the miRNA activities more precisely in plants.

TRANSCRIPTIONAL CONTROL OF MIRNA GENES

Increasing evidence points to the fact that the accumulation of a specific miRNA is a combinatory effect of its transcription, processing, and degradation (Kai and Pasquinelli, 2010). As the first step of miRNA expression, their transcription is modulated in a highly dynamic manner. Both cis-modifications and trans-acting factors are responsible for the spatiotemporally restricted expression patterns of miRNA genes (Chen, 2009; Davis and Hata, 2009; Winter et al., 2009; Fig. 1A).

One major form of cis-regulation is known as chromatin modification, including DNA methylation and histone modification. These epigenetic marks can be present within the upstream and downstream regions or in the bodies of the miRNA genes, which are extraordinarily variable according to cellular contents and environmental stimuli. These marks have shown great potential in influencing the transcriptional status of miRNA genes. As proposed by Rodriguez-Enriquez et al. (2011), somaclonal variation, a featured phenomenon observed in plant tissue culture, is one of the biological consequences caused by miRNA misexpression and the accompanying disordered regulatory pathways. In their hypothesis, the inducers introduced by *in vitro* tissue culture could result in aberrant miRNA transcription, processing, and miRISC loading, which could have remarkable impacts on the transcriptome and the proteome and further alter the epigenetic status of the genome in cultured cells. In turn, miRNA transcription could be influenced by the altered epigenetic marks surrounding the miRNA genes (Rodriguez-Enriquez et al., 2011). Another example was recently provided by Kim et al. (2009b). A histone acetyltransferase, GCN5 (for general control nonrepressed protein 5), was indicated to interfere with miRNA biogenesis transcriptionally and posttranscriptionally. The GCN5-mediated histone modifications serve as an epigenetic mechanism for modulating miRNA production (Kim et al., 2009b).

The transcription of most miRNA genes is mediated by RNA polymerase II (Lee et al., 2004; Xie et al., 2005). The structures of the miRNA promoters are similar to those of the protein-coding genes (Megraw et al., 2006; Zhou et al., 2007). For example, the distribution patterns of the basic cis-elements for transcriptional control (i.e. the transcription start site, the TATA box, and the CAAT box) on the miRNA promoters were demonstrated to be identical to the protein-coding genes (Meng et al., 2009). In plants, many TFs have been identified as trans-acting factors with roles in the transcriptional modulation of certain miRNA genes.

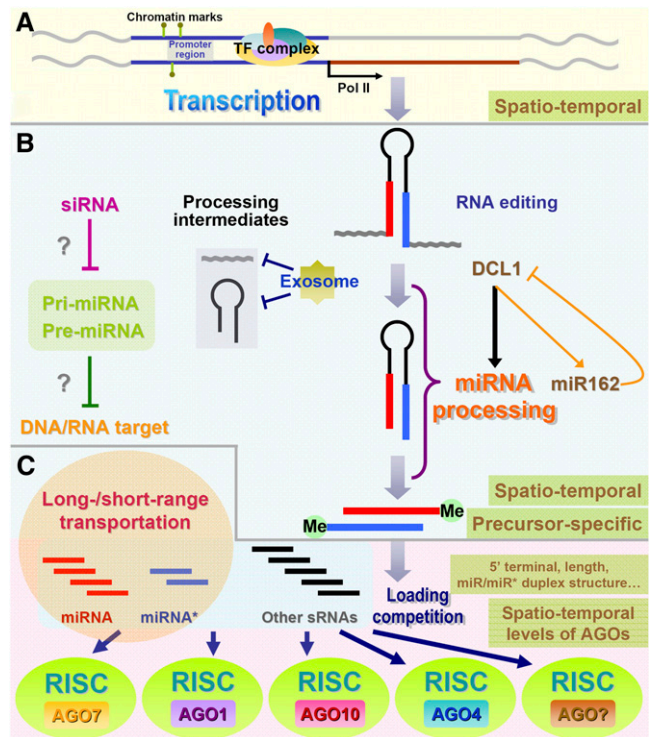


Figure 1. Schematic presentation showing the dynamic nature of miRNA biogenesis in plants. A, RNA polymerase II (Pol II)-dependent transcription of the miRNA genes. Chromatin marks including DNA methylation and histone modifications, and the combinatory regulation of many TFs, together contribute the spatiotemporal expression patterns of the miRNA genes in plants. B, Processing of miRNA precursors and miRNA maturation. RNA editing on the miRNA precursors plays a role in changing the original sequence information encoded by the miRNA gene loci. The exosome was suggested to be implicated in digesting the processing intermediates from the miRNA precursors, ensuring relatively high processing efficiency. The processing efficiency also shows a high precursor sequence-specific dependence. Methylation at the 3' ends of the miRNA/miRNA* duplex ("Me" here represents the methyl group) is crucial for the stabilization of miRNA and miRNA*. A feedback circuit between *DCL1* and miR162 exists within the processing procedure. Moreover, the siRNAs complementary to specific pri-miRNAs and pre-miRNAs exhibit a potential repressive role in miRNA processing. More interestingly, the pri-miRNAs and pre-miRNAs may possess their own targets. C, Sorting into the AGO-associated RISCs. A drastic loading competition may exist among miRNAs, miRNA*s, and other sRNA species. Not all the miRNAs are incorporated into the AGO1 complex. The 5' terminal composition and the sequence length of the miRNAs, the structure of the miRNA/miRNA* duplex, and other undetermined factors have a significant influence on the loading patterns of the mature miRNAs.

One example is the *PHR1* (for *PHOSPHATE STARVATION RESPONSE1*)-*miR399*-*PHO2* (defined by the mutant *pho2*) regulatory pathway involved in phosphorous homeostasis (Bari et al., 2006). Upon phosphorous deprivation, *miR399* is up-regulated transcriptionally by the activated *PHR1*, a direct upstream regulator. Then, the repression of *PHO2* by *miR399* is subsequently reinforced posttranscriptionally. This cascade ensures the expeditious response of

the plants under phosphorous-deficient conditions, enabling more efficient use of both environmental and cellular resources of phosphorus. Intriguingly, some TFs were also targeted by the downstream miRNAs, forming feedback circuits in certain signaling pathways. For instance, within the auxin signaling pathway implicated in Arabidopsis (*Arabidopsis thaliana*) adventitious root development (Gutierrez et al., 2009), *miR160* is transcriptionally regulated by *AUXIN RESPONSE FACTOR6* (*ARF6*) and *ARF17* and *miR167* is regulated by *ARF6*, *ARF8*, and *ARF17*. On the other hand, all three *ARF* genes are negatively regulated by either of the two miRNAs. These feedback circuits form an interlaced network that could decipher, integrate, and transduce the light and auxin signals to shape normal root system architecture. Another exquisite case was presented by Wu et al. (2009). Both *miR156* and *miR172* were demonstrated to participate in the regulatory network that was essential for developmental timing in Arabidopsis. These two miRNAs act by repressing the expression of TFs belonging to *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE* (*SPL*) and *APETALA2-LIKE* (*AP2-like*) gene families, respectively. Certain members of the two TF families were proved to regulate *miR156* and *miR172* positively, thus forming negative feedback loops that contributed to the normal juvenile-to-adult phase transition. More complicatedly, the transcription of *miR172* was directly regulated by *SPL9* and *SPL10*, which were both targeted by *miR156*. These connections established the *miR156-SPL-miR172-AP2* regulatory cascade, which was crucial for vegetative phase change in Arabidopsis (Wu et al., 2009). However, all these findings only uncovered part of the miRNA- and TF-involved networks. The one-to-one and multiple-to-one regulatory relationships between TFs and specific miRNA genes should not be stationary states. Instead, they are highly dynamic due to numerous intrinsic factors, such as the spatiotemporal expression of TF genes and indirect TF-miRNA regulation. Facilitated by these technical advances, a more comprehensive view of such networks could be constructed through genome-wide identification of TF-binding sites (MacQuarrie et al., 2011).

Expression pattern analyses of plant miRNAs by high-throughput profiling or fine-scale quantification revealed that numerous miRNAs were expressed in a tissue- or stage-specific manner and that dozens of miRNAs could be induced by external stimuli (Reinhart et al., 2002; Kidner and Martienssen, 2004; Sunkar and Zhu, 2004; Lu et al., 2005; Yao et al., 2007; Liu et al., 2008b; Oh et al., 2008; Hsieh et al., 2009; Johnson et al., 2009; Simon et al., 2009). All these findings indicate that miRNA activities are highly variable at distinct developmental stages, upon diverse treatments, or in different tissues. Conceivably, the strict control of miRNA expression at both the chromatic and transcriptional levels could make a great contribution to this highly dynamic nature.

PROCESSING AND MATURATION, CRUCIAL STEPS FOR MIRNA BIOGENESIS

After transcription, the primary products (i.e. pri-miRNAs) are subjected to DCL1-mediated two-step cleavage in the nucleus in plants (Papp et al., 2003; Kurihara and Watanabe, 2004). Several aspects should be taken into account to assess the dynamic efficiency of the miRNA precursor processing. One of the well-characterized models balancing processing and miRNA activities is the feedback circuit formed between *DCL1* and *miR162* (Xie et al., 2003). The nucleus-localized DCL1 with RNase III activity is indispensable for the processing of most plant miRNAs (Voinnet, 2009). Thus, the levels of the final products of the miRNA genes (i.e. the mature miRNAs) are highly correlated to the expression of *DCL1*. On the other hand, *DCL1* itself is regulated by *miR162* posttranscriptionally to avoid plethoric DCL1 activity (Xie et al., 2003). Moreover, the tissue-specific regulation of miRNA processing by DCLs was proposed recently. The overaccumulated level of DCL3 in specific tissues may result in a shift of the substrates of DCL1 to DCL3s (Vazquez et al., 2008). Similar substrate competition may also occur between DCL1 and other DCLs.

In addition, different miRNA precursors possess different affinities to DCL1 due to distinct sequence characteristics and spatiotemporal distributions. Although the precursor sequences are largely determined by the genomic sequences of the miRNA genes, several pieces of evidence in animals show that RNA editing mediated by ADENOSINE DEAMINASE ACTING ON RNA can occur on the mature miRNAs or the precursors posttranscriptionally, which has remarkable effects on miRNA processing and targeting (Luciano et al., 2004; Blow et al., 2006; Yang et al., 2006b; Kawahara et al., 2007a, 2007b, 2008; Winter et al., 2009; Krol et al., 2010). Different from animals, RNA editing in plants, mostly represented by C-to-U base conversion, is carried out by PENTATRICOPEPTIDE REPEAT (PPR) family proteins (Schmitz-Linneweber and Small, 2008) and is restricted to plant organelles, including mitochondria and plastids, based on available reports (Shikanai, 2006). Recent bioinformatic analyses using huge high-throughput sequencing (HTS) data sets raised the possibility that RNA editing of miRNA gene products could also take place in plants (Ebhardt et al., 2009; Iida et al., 2009; Meng et al., 2010a). Once further experimental validations are available, this kind of sequence modification could be another dynamic layer underlying miRNA processing, maturation, and action.

Depending on the *in vivo* levels and structures, miRNA precursors, perhaps along with other sRNA precursors, could compete for the machineries functioning in the miRNA biogenesis pathway. One such competition in plants could be raised by different miRNA precursors and other stem-loop-structured precursors for accessibility to DCL1 or other DCLs such as DCL3, mentioned above (Vazquez et al., 2008).

In animals, manually introduced short hairpin RNAs could interfere with the expression of endogenous miRNA genes through drastic competition for the nuclear exportation and processing machineries (Grimm et al., 2006; Stewart et al., 2008).

In mouse, posttranscriptional regulation was found to reside within the miRNA processing procedure. During the early developmental stage or in mouse primary tumors, many miRNA precursors were highly accumulated while their processing was intensively blocked (Thomson et al., 2006). This suggests that the expression of miRNA genes could be spatiotemporally modulated at the posttranscriptional level. Although it needs further verification, this strategy is likely to be employed by the plant miRNA biogenesis system. Additionally, several reports in animals point to the fact that AGO proteins participate in miRNA processing (Diederichs and Haber, 2007; O'Carroll et al., 2007; Cheloufi et al., 2010; Cifuentes et al., 2010; Lund et al., 2011). However, to our best knowledge, there has been no such finding in plants yet. The only related study reported that AGO1 was involved in the stabilization of certain miRNAs (Vaucheret et al., 2004, 2006). That is, AGO1 is highly correlated with the degradation rate of the mature miRNAs. If AGO1 indeed acts on miRNA processing in plants, its spatiotemporal distribution pattern could contribute to the tissue- or stage-specific expression of many miRNA genes.

A recent study carried out by Hoffer et al. (2011) discovered that posttranscriptional gene silencing (PTGS) guided by small interfering RNAs (siRNAs) could take place in the nucleus in plants, which has revolutionized the traditional view that the targets of PTGS are mature mRNAs or other transcripts in cytoplasm. On the other hand, Mortensen et al. (2011) showed that in *Xenopus laevis* oocytes, siRNAs antisense to the miRNA precursors were able to deplete the generation of the mature miRNAs. Since the pri-miRNAs and the pre-miRNAs are both nucleus localized in plant cells, it is reasonable to imagine that certain siRNAs, either intrinsic or extrinsic, may be capable of controlling the cellular levels of specific miRNA precursors. Furthermore, we previously proposed a feedback model between miRNA(*)s and the parental precursors in which the miRNA(*)s could bind to the complementary sites on their precursors to exert a cleavage-based repressive role, thus modulating their own biogenesis. Thus, it provides another regulatory layer of miRNA transcripts (Meng et al., 2010b). It is worth mentioning here that recent evidence in animals showed the great potential of miRNA precursors in target recognition and repression (Trujillo et al., 2010). Also, the loop sequences of the precursors could determine the activities of the corresponding mature miRNAs directly (Liu et al., 2008a). If these findings are also true in plants, an interlaced connection between miRNA processing and action could be established.

The exosome, responsible for 3'-to-5' RNA processing and degradation, is critical for RNA metabolism in organisms (Mitchell et al., 1997). A transcriptome-wide

high-resolution mapping was applied by Chekanova et al. (2007) to exhaustively identify the exosome substrates in Arabidopsis. Intriguingly, miRNA processing intermediates were cloned as one kind of exosome substrate. Since numerous exosome targets have been identified in the nucleus (Bousquet-Antonelli et al., 2000; Torchet et al., 2002; Das et al., 2003; Kadaba et al., 2004, 2006), it is reasonable that the nucleus-localized processing intermediates of plant miRNA precursors are under tight surveillance by the exosomes. Based on this result, the authors proposed that exosome-mediated degradation of these processing intermediates could facilitate efficient recycling of the miRNA-processing machineries (Chekanova et al., 2007). Moreover, the RRP6 exosome subunit in *Chlamydomonas reinhardtii* possesses a quality-control role in eliminating dysfunctional or damaged sRNA molecules, including mature miRNAs and siRNAs (Ibrahim et al., 2010). From this point of view, the exosome could be another important factor affecting the efficiency of miRNA processing in plants.

After DCL1-mediated two-step cleavage, the miRNA/miRNA* duplexes will be recognized by HUA ENHANCER1 (HEN1) to add methyl groups on the 2' OH of the 3'-most terminal nucleotides on both strands (Yu et al., 2005). This is another crucial step for miRNA maturation, since terminal methylation could protect them from 3'-end uridylation and adenylation, thus stabilizing the miRNAs and the miRNA*s in vivo (Li et al., 2005; Yang et al., 2006c). Interestingly, HEN1 is not specific to miRNA duplexes. The study by Yang et al. (2006c) showed that both miRNA/miRNA* and siRNA duplexes ranging from 21 to 24 bp could be methylated. Besides, a recent study by Yu et al. (2010) further validated the competition between siRNAs and miRNAs for HEN1-mediated methylation. In this regard, the methylation-based stabilization that relies on both HEN1 activity and substrate levels could contribute to the varying abundances of the effective miRNAs.

Taken together, processing and maturation are two speed-limiting steps for the final miRNA levels (Fig. 1B). In both plants and animals, there are enormous factors and several checkpoints modulating these susceptible steps (Davis and Hata, 2009; Voinnet, 2009; Winter et al., 2009), which confer a highly dynamic nature to the miRNA activities.

RISC SORTING, WITH A DOMINANT BUT NOT COMPLETELY DETERMINED LOADING PATTERN

To exert their regulatory roles, the mature miRNAs released from the miRNA/miRNA* duplexes must be subsequently sorted into AGO-associated miRISCs (Voinnet, 2009). Two components of the sRNA sequence characteristics (i.e. 5' terminal composition and sequence length) were demonstrated to be the key determinants for their AGO sorting patterns (Kim, 2008; Mi et al., 2008; Montgomery et al., 2008; Ebhardt et al., 2010). By employing HTS technology to investigate

sRNA contents within the immunopurified AGO complexes, Mi et al. (2008) observed that the sRNAs that started with 5' A (adenosine) were preferentially recruited by AGO2 and AGO4 and those that initiated with 5' C (cytosine) were loaded into the AGO5-associated silencing complex. The miRNAs, most of which favored 5' U (uridine), were largely incorporated into the AGO1 complex. Additionally, AGO1- and AGO2-associated sRNAs were predominantly 21 nucleotides in length, and AGO4-associated sRNAs tended to be 24 nucleotides, indicating a critical role of sequence length in AGO sorting (Mi et al., 2008). However, these two factors are not sufficient to determine which AGO(s) a specific sRNA should associate with. In a study by Mi et al. (2008), AGO5 was demonstrated to bind the sRNAs belonging to three size classes (i.e. 21, 22, and 24 nucleotides). Moreover, recent results revealed the association between AGO10 and miR166/165, which cannot be explained by the 5' composition- and sequence length-based rule (Zhu et al., 2011). Another exceptional case, in which miR390 interacts with AGO7 for subsequent ta-siRNA (for trans-acting small interfering RNA) production, suggests that the miRNA species are not always associated with AGO1. The 5' A of miR390 was suggested to specifically determine the preferential association of miR390 with AGO7 but not AGO1 (Montgomery et al., 2008). In the well-established model of miRNA biogenesis, the strand with a less stably paired 5' end of a specific duplex is selectively recognized as the guide strand and loaded into AGO1-associated complexes (Jones-Rhoades et al., 2006). From this point of view, other sequence- or structure-based features embedded within the miRNA/miRNA* duplexes could play important roles in determining the destination of the miRNAs.

Notably, several dynamic factors should not be excluded when we try to interrogate the association between a certain AGO protein and a miRNA, considering that unexpectedly drastic loading competition might take place in a specific cellular context (Fig. 1C). Two distinct layers exist in this kind of competition: two or more AGOs compete for one miRNA, and miRNA, miRNA*, and other sRNAs strive to incorporate into a specific AGO complex. A specific example was provided by Zhu et al. (2011) on the loading balance of miR166/165 between AGO1 and AGO10 in *Arabidopsis*. miR166/165 occupied the dominant portion of the AGO10-bound miRNAs, and the featured structure of the miR166/miR166* duplex predetermined the preferential association of miR166 with AGO10 (Zhu et al., 2011). This result well supports the structure-based rule for miRNA sorting, as proposed above. More interestingly, loss-of-function mutation of *AGO10* significantly enhanced the association of miR166 with AGO1. Considering the elucidated role of *AGO10* in maintaining an undifferentiated cell state of the shoot apical meristems (Moussian et al., 1998; Lynn et al., 1999), the authors proposed that AGO10 competed with AGO1 to sequester miR166/165, thus preventing them from targeting *HD-ZIP III* genes

involved in shoot apical meristem maintenance (Prigge et al., 2005; Barton, 2010; Zhu et al., 2011). Considering the partially overlapping roles of some AGO family members within the plant sRNA pathways (Vaucheret, 2008; Mallory et al., 2009; Mallory and Vaucheret, 2010), we suggest that this kind of loading competition (i.e. one miRNA versus multiple AGOs) could be widespread in plants.

In several types of human cells, one investigation carried out by Khan et al. (2009) showed that transfected siRNAs could compete with endogenous miRNAs at several points of the miRNA biogenesis pathway, such as nuclear exportation and miRISC loading. It was observed that many targets of the endogenous miRNAs were significantly up-regulated after siRNA transfection, which exhibited concentration and temporal dependence. The authors proposed that the intracellular machineries for miRNA processing and action could be saturated through the invasion of sRNAs sharing overlapping biogenesis pathways with the miRNAs, and they could also compete with the miRNAs for target binding (Khan et al., 2009). In this regard, it is possible that competition among two or more sRNA species for nuclear exportation, RISC loading, and target binding may occur in plants. This scenario is reasonable considering the notion that several indistinguishable intersections exist within the biogenesis and functioning pathways between the young miRNA genes and the siRNAs in plants (Cuperus et al., 2011). Besides, the miRNA*s were recently reported to possess regulatory roles in both animals (Okamura et al., 2008; Packer et al., 2008; Yang et al., 2011) and plants (Mi et al., 2008; Devers et al., 2011; Meng et al., 2011; Zhang et al., 2011), which points to the possibility that the miRNA*s are likely to participate in the RISC loading competition (Fig. 1C).

In plants, the functional diversification of the AGO proteins is not only attributed to the protein sequences themselves but is also highly dependent on their spatiotemporal expression patterns (Vaucheret, 2008; Mallory and Vaucheret, 2010; Zhu et al., 2011). Thus, the identical subcellular localizations are the prerequisite for the *in vivo* association between a specific miRNA and an AGO-associated RISC (Havecker et al., 2010).

A HIGHLY DYNAMIC MIRNA-MEDIATED REGULATORY SYSTEM IN PLANTS

miRNA Turnover

Although increasing evidence points to the fact that translational repression could be adopted as one action mode by the plant miRNAs (Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Dugas and Bartel, 2008; Todesco et al., 2010), most highly conserved miRNAs exert their regulatory roles at the posttranscriptional level through cleavage (Jones-Rhoades et al., 2006; Voinnet, 2009). In contrast to the low complementarity between miRNAs and their

targets in animals (Carthew and Sontheimer, 2009), the plant miRNA-mediated target slicing largely depends on the near-perfect binding sites within the corresponding transcripts (Mallory et al., 2004).

We must recognize that this kind of regulation is not performed in a steady-state mode but is highly dynamic. The pressing issue that needs to be addressed is how to assess the regulatory activity of a specific miRNA. Besides the checkpoints residing within the transcription, processing, and miRISC loading steps, the spatiotemporal expression patterns of both miRNAs and their targets should be taken into account. However, here, we introduce another notable layer, the turnover use of the mature miRNAs, since less attention has been paid to the fate of the miRNAs subsequent to their first round of target cleavage.

The multiple-turnover model was first raised by Hutvagner and Zamore (2002) during their study of *let-7*-guided RNA cleavage in human cells. Their results showed that each *let-7*-programmed RISC was capable of catalyzing approximately 10 rounds of cleavage action on a specific target. Also in mammalian cells, another pioneering work was recently done by Baccharini et al. (2011). Quantitative analysis of miR-223 showed that each miRNA molecule could regulate at least two target transcripts during its life cycle. Then, they demonstrated that the miRNA-mediated nonslicing pathway was multiple turnover. Although this kind of kinetic regulation was unraveled by analyzing only a few miRNAs, and it still needs to be verified whether a similar kinetic model could be applied to most miRNAs in animals (Muers, 2011), it is tempting for the plant biologist to test the possibility that the recycling strategy is also adopted by plant sRNAs for PTGS (Fig. 2A). Once the scenario that miRNAs can be reused is confirmed in plants, our current understanding of miRNA activities will be significantly updated. The turnover use of a specific miRNA molecule could remarkably elevate the targeting efficiency in planta, enabling strict surveillance of a large pool of target transcripts by a relatively small population of miRNAs. However, this does not mean an invariable one-to-multiple regulation mode. The fluctuant turnover rate of a specific miRNA in different cellular contexts or under diverse conditions pre-determines the highly dynamic nature of miRNA recycling. From another point of view, the adjustable turnover rate greatly enhances the buffering capacity of the miRNA-involved regulatory system when undergoing the expression fluctuation of certain target genes. This buffering system ensures the physiologically normal expression of the genes (Fig. 2B).

Different from the situation described by Baccharini et al. (2011), that miRNAs were not irreversibly sequestered by their targets in animals, one piece of evidence in Arabidopsis showed that miR399 was sequestered by a spurious target encoded by the noncoding gene *INDUCED BY PHOSPHATE STARVATION1* (*IPS1*; Franco-Zorrilla et al., 2007). Within the miR399-*IPS1* binding region, the high complemen-

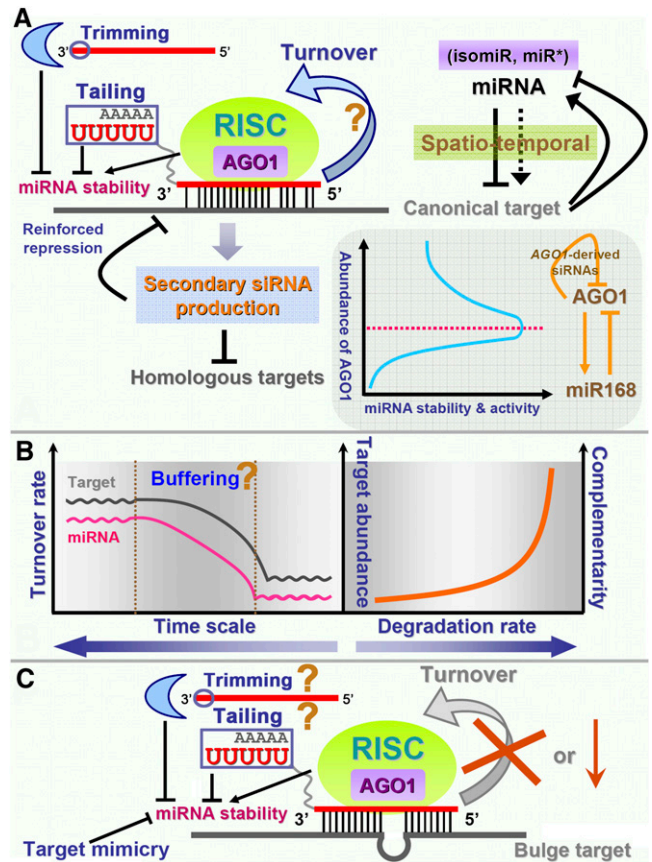


Figure 2. Schematic summarization of the factors influencing miRNA action and in vivo levels. A, Based on recent reports in animals, the turnover use of the mature miRNAs is proposed in plants, which needs further validation (denoted by the question mark). The 3' tailing and the 3'-to-5' trimming greatly affect the stability of mature miRNAs. Certain miRNA targets, such as TF genes, could in turn regulate the miRNAs, thus forming feedback regulatory circuits. The secondary siRNAs amplified from the cleaved target transcripts of a specific miRNA could reinforce miRNA-mediated gene silencing posttranscriptionally. One example is provided by the feedback circuit between *AGO1* and *miR168* in Arabidopsis. The secondary siRNAs derived from *miR168*-cleaved *AGO1* transcripts could further regulate the expression of *AGO1* posttranscriptionally. On the other hand, the abundance of the *AGO1* protein significantly affects the activities and the stability of numerous miRNAs. B, The adjustable turnover rate of certain miRNAs may form an elaborate buffering system within the miRNA-mediated regulatory networks, which needs further investigation (denoted by the question mark in the left panel). Based on current hints in plants and animals, a target abundance- and complementarity-dependent model was proposed to be implicated in modulating the miRNA degradation rate (right panel). C, Based on the phenomenon of target mimicry observed by Franco-Zorrilla et al. (2007), the miRNA could be sequestered by a target decoy with a central bulge within the target recognition sites. Thus, miRNA turnover will be inhibited in that case. Although it is still not clear, the 3' tailing and 3'-to-5' trimming may also occur on the miRNAs sequestered by the bulged targets (denoted by question marks).

tarity is interrupted by a three-nucleotide bulge at the expected cleavage site. This leads to an inhibitory effect on miR399 by the noncleavable *IPS1* transcript, and this phenomenon was termed "target mimicry."

Recently, a large collection of target mimics against dozens of miRNAs were generated to facilitate further functional studies on plant miRNAs (Todesco et al., 2010). The activities of many designated miRNAs were confirmed to be successfully repressed by artificial target mimics, and the abundances of the corresponding targets were observed to be elevated. Hence, target mimicry, or some other similar mechanism, may provide another control layer of the miRNA turnover rate in planta (Fig. 2C).

Silencing Amplification

In addition to the miRNA-guided primary regulation, amplification of the silencing signals through secondary siRNA proliferation should be considered when evaluating plant miRNA activities (Fig. 2A). One piece of evidence supporting such activity of miRNAs is their involvement in ta-siRNA generation (Allen and Howell, 2010). To date, four ta-siRNA gene families, *TAS1*, *TAS2*, *TAS3*, and *TAS4*, have been discovered to encode ta-siRNAs in Arabidopsis. To enter the RDR6 (for RNA-dependent RNA polymerase 6)-DCL4-dependent pathway for ta-siRNA generation (Peragine et al., 2004; Vazquez et al., 2004; Yoshikawa et al., 2005), the primary *TAS* transcripts should be cleaved by miR173-, miR390-, or miR828-programmed AGO complexes first (Allen et al., 2005; Axtell et al., 2006; Rajagopalan et al., 2006; Chen et al., 2007; Montgomery et al., 2008). Another specific example was provided by Carrington's group. Certain miRNAs associated with AGO1-containing silencing complexes were found to be competent to trigger secondary siRNAs from the target transcripts. This RDR6-dependent pathway is specifically mediated by the 22-nucleotide miRNA species but not the 21-nucleotide ones (Cuperus et al., 2010). Besides, cleavage of the *PPR* transcripts by several miRNAs and ta-siRNAs could activate the biogenesis of the *PPR*-derived secondary siRNAs, which could in turn target the host transcripts in cis or the other homologous genes in trans, reinforcing the miRNA- or ta-siRNA-mediated gene silencing in Arabidopsis (Axtell et al., 2006; Chen et al., 2007; Howell et al., 2007; Addo-Quaye et al., 2008). More recently, the 22-nucleotide-long miR393 was shown to be involved in auxin signal-mediated leaf development through cleavage of the *TIR1/AFB2 Auxin Receptor (TAAR)* transcripts. Interestingly, the production of the *TAAR*-derived secondary siRNAs could be initiated by miR393-guided cleavage, which further enhanced the repressive regulation of *TAAR*-related or other homologous genes (Si-Ammour et al., 2011). Furthermore, the study by Chen et al. (2010) also supports the 22-nucleotide model that the secondary siRNA triggers tend to be the miRNAs and the siRNAs of 22 nucleotides, rather than the 21-nucleotide ones.

Action Mode Conversion

Besides the two action modes (i.e. transcript cleavage and translational repression), which are employed by

plant miRNAs for target regulation, a miRNA-mediated pathway has been shown to be implicated in DNA methylation (Chellappan et al., 2010; Wu et al., 2010; Chen et al., 2011). Interestingly, in *Physcomitrella patens*, the miRNA-guided mRNA slicing could be converted to another action mode, DNA methylation, which was conditionally dependent on the ratio of a miRNA to its target (Khraiwesh et al., 2010). Thus, the dosage-dependent action mode conversion displays another dynamic layer of miRNA-mediated regulation in plants.

Subcellular Localization

The canonical model of miRNA-mediated regulation indicates that miRNAs recognize their targets mostly in the cytoplasm after their nuclear exportation and maturation (Carthew and Sontheimer, 2009; Voinnet, 2009). However, current evidence uncovered the novel nucleus-localized expression patterns of mature miRNAs in both plants and animals (Politz et al., 2006, 2009; Wong et al., 2011). More specifically, in human beings, Hwang et al. (2007) discovered a hexanucleotide cis-element residing within miR-29b, which could direct nuclear import of the examined miRNAs and siRNAs. Based on this result, the authors proposed that the seemingly redundant miRNAs with identical 5' seed regions could be functionally diversified under the influence of certain cis-motifs, such as the 3' transferable nuclear localization motif (Hwang et al., 2007). To date, only a few cases of nucleus-localized miRNAs have been discovered, but the variable subcellular localizations point to the possibility that miRNAs could target nuclear transcripts such as the primary gene transcripts and the miRNA precursors in plants.

miRNA Diffusion

The dynamic nature of miRNA-mediated regulation in plants is also strongly reflected by their diffusion effect (Fig. 1C). The siRNA molecules, including certain miRNAs and siRNAs, could not only perform cell-to-cell movement but also are implicated in long-distance transport through the phloem (Yoo et al., 2004; Kehr and Bultz, 2008; Brosnan and Voinnet, 2011), which were demonstrated to serve as systemic signals for leaf development (Juarez et al., 2004) and phosphate homeostasis (Pant et al., 2008). From this point of view, in many cases, the plant miRNAs could exert non-cell-autonomous control over plant growth and development through cell-to-cell, tissue-to-tissue, or even organ-to-organ communication. Henceforth, we should not restrict the miRNA-mediated regulation to a limited context, since the diffusion effect must be treated as an important factor when assessing miRNA activities.

Regulatory Network

The tissue- or stage-specific expression patterns and the dynamic subcellular localizations of the miRNAs

emphasize the importance of spatiotemporal colocalization of miRNA regulators and their targets for regulatory effectiveness. However, another regulatory layer, indirect targeting, should not be ignored, especially when attempting to construct miRNA-mediated regulatory networks (Rubio-Somoza et al., 2009). Within the regulatory module *miR390-TAS3-ARF2/3/4*, *ARF2*, *ARF3*, and *ARF4* are the indirect targets of miR390 (Marin et al., 2010). Besides, the *ARF* transcripts targeted by miR160, miR167, and miR390 (Mallory et al., 2005; Wang et al., 2005; Wu et al., 2006; Yang et al., 2006a; Marin et al., 2010) encode ARF TFs that could interact with other ARFs and specific auxin/indole-3-acetic acid (Aux/IAA) repressors (another family of TFs) at the protein level, modulating the auxin signaling pathway in plants. Furthermore, ARFs could in turn regulate the expression of *miR160*, *miR167* (Gutierrez et al., 2009), or other sRNA genes transcriptionally. Thus, certain ARFs, Aux/IAAs, and the sRNA genes regulated by the ARFs become the indirect targets of miR160, miR167, and miR390, contributing to the complexity of the miRNA-mediated networks. Besides, as mentioned above, the secondary siRNAs generated from the miR393-cleaved *TAAR* transcripts could further amplify the silencing signal involved in auxin-mediated leaf development (Si-Ammour et al., 2011).

In summary, miRNA recycling, amplification of silencing signals, dosage- and localization-dependent regulation, feedback regulation between certain miRNAs and their targets, and indirect targeting together orchestrate a fascinating, highly dynamic regulatory network with a buffering system in plants (Fig. 2).

THE FATE OF A MIRNA: TAILING/TRIMMING-INDUCED DECAY OR STABILIZATION

In addition to transcription, maturation, miRISC loading, and recycling, *in vivo* stability greatly influences the levels of the active miRNAs. Recent evidence gained from HTS and other methods showed that a large portion of miRNAs were tailed with one to several nontemplated 3' U or A (adenine) nucleotides in both plants and animals, which served as a signal modulating miRNA stability (Li et al., 2005; Ramachandran and Chen, 2008; Katoh et al., 2009; Lu et al., 2009; Ameres et al., 2010; Ibrahim et al., 2010; Baccarini et al., 2011). In both kingdoms, several studies reached the consensus that adenylation increased the miRNA stability whereas uridylation promoted miRNA degradation (Katoh et al., 2009; Lu et al., 2009; Ibrahim et al., 2010; Baccarini et al., 2011). However, some exceptional cases were reported at the same time. In *Drosophila*, highly complementary targets triggered tailing of the small silencing RNAs, including the miRNA species, and both 3' uridylation and 3' adenylation were suggested to promote miRNA deg-

radation, which was also conserved in human cells (Ameres et al., 2010). In Arabidopsis, *in vitro* analysis demonstrated that miRNAs could be uridylylated at the 3' ends that were not methylated, and the uridylation protected those miRNAs from SMALL RNA-DEGRADING NUCLEASE1-mediated degradation (Ramachandran and Chen, 2008). This seems quite complicated according to the above observations. However, the roles of terminal modifications on miRNA stability should become more clear with continuing research on this topic. On the other hand, the 5'-to-3' and 3'-to-5' trimming of miRNAs, as one means of degradation, was demonstrated to have a regulatory role in controlling the abundance of miRNAs in plants and mammalian cells (Lu et al., 2009; Ameres et al., 2010; Baccarini et al., 2011).

However, we should note that miRNA stability cannot be calculated by a simple linear equation and is affected by numerous dynamic factors. For example, adenylation of the miRNAs in *Populus trichocarpa* showed a tissue-specific dependence (Lu et al., 2009). More interestingly, emerging evidence in animals pointed to the fact that miRNA fate was greatly influenced by their targets. As shown in Figure 2B, the degradation rate of a miRNA was primarily affected by two factors, the complementarity and the abundance of its target. A recent study carried out by Ameres et al. (2010) proposed that extensive complementarity between a target transcript and a miRNA triggered tailing and 3'-to-5' trimming of the miRNA. Considering the fact that most targets in plants are highly complementary to the miRNA regulators (Jones-Rhoades et al., 2006; Voinnet, 2009), whether the sequence complementarity has a great influence on the stability of plant miRNAs needs further investigation. One intriguing hint was obtained by Todesco et al. (2010). In that study, the authors examined the levels of targeted miRNAs in all the target-mimic lines of Arabidopsis. Their results showed that the abundances of nearly all the targeted miRNAs were significantly decreased, leading to the conclusion that interactions between the target decoys and the miRNAs could reduce the miRNA stability in plants (Todesco et al., 2010). Coincidentally, a repressive regulatory role of partially complementary transcripts in controlling sRNA activities was also reported in bacteria (Figueroa-Bossi et al., 2009; Overgaard et al., 2009). Another important factor, the target abundance, was reported to have a dilution effect on miRNA activities after an expression-based examination in dozens of miRNA- and siRNA-transfected HeLa S3 cell lines (Arvey et al., 2010). Considering the evidence above, we propose that the high abundances of the targets with high complementarity to the miRNAs in animals (maybe the bulge targets in plants) could not only dilute the miRNA activities through sequestration but also promote miRNA degradation more efficiently. However, even though the complementarity- and abundance-dependent model of target-induced miRNA degradation is established (Fig. 2B), some

exceptions cannot be excluded. In *Caenorhabditis elegans*, both in vitro and in vivo analyses showed the unexpected result that miRNA degradation could be blocked by the addition of target RNAs, which was then defined as “target-mediated miRNA protection” (TMMP; Chatterjee and Grosshans, 2009; Chatterjee et al., 2011).

In addition to the negative factors promoting miRNA decay, the miRNAs could be protected and stabilized by AGO-associated miRISCs (Vaucheret et al., 2004; Kai and Pasquinelli, 2010). As reviewed by Kai and Pasquinelli (2010), in both animals and plants, AGO proteins may function in both the biogenesis and stabilization of the mature miRNAs (Kai and Pasquinelli, 2010). Considering the novel phenomenon TMMP discovered in *Caenorhabditis elegans* (Chatterjee and Grosshans, 2009; Chatterjee et al., 2011), we reasoned that the target-bound miRISCs could efficiently stabilize the incorporated miRNAs, and this protection may be partially dependent on the target structures (Ameres et al., 2007). As suggested by Chatterjee et al. (2011), TMMP adds another mutual regulatory layer between miRNAs and their targets, enabling dynamic expression and functional evolution of the miRNA genes. The early pioneering work by Vaucheret et al. (2004) showed that complete depletion of *AGO1* led to a drop in the abundances of some miRNAs in Arabidopsis, indicating the involvement of *AGO1* in miRNA biogenesis and/or stabilization (Vaucheret et al., 2004). However, things will develop in the opposite direction when they become extreme. A large excess of *AGO1* protein in transgenic plants resulted in a decrease in miRNA accumulation. The authors determined that excessive *AGO1* could interfere with the normal function of miRISCs and might sequester the mature miRNAs, thus inhibiting their activities (Vaucheret et al., 2004). In that study, the authors also provided the first evidence for the feedback regulation between *AGO1* and miR168 and further illustrated the importance of miR168-mediated regulation of *AGO1* mRNA for proper plant development (Vaucheret et al., 2004; Fig. 2A). The follow-up experiments presented more detailed insights into this feedback regulatory module (Vaucheret et al., 2006; Mallory and Vaucheret, 2009; Vaucheret, 2009). As mentioned above, AGO proteins have great potential in participating in the biogenesis and stabilization of miRNAs in various organisms (Vaucheret et al., 2004, 2006; Diederichs and Haber, 2007; O’Carroll et al., 2007; Cheloufi et al., 2010; Cifuentes et al., 2010; Kai and Pasquinelli, 2010; Lund et al., 2011).

In plants, to prevent the excessive expression of *AGO1*, miR168 is employed as a critical regulator controlling the abundance of *AGO1* mRNAs. Interestingly, the two nodes of the feedback circuit have a common expression pattern, which was demonstrated to be coregulated transcriptionally (Vaucheret et al., 2006). Besides miR168, other regulators, both positive and negative, were discovered to modulate *AGO1* activities in plants. Loss-of-function mutations of

SQUINT (SQN), an ortholog of *CYCLOPHILIN40 (CyP40)* in Arabidopsis, caused a reduction in *AGO1* activity. This supports the notion that *CyP40* maintains the proper function of *AGO1* or the *AGO1*-associated silencing complexes and that it is required for the regulatory activities of plant miRNAs (Smith et al., 2009). Additionally, *HEAT SHOCK PROTEIN90 (HSP90)*, functioning as a molecular chaperone, was demonstrated to facilitate the in vitro assembly of *AGO1*-associated RISCs. The ATP-dependent, HSP90-bound, *AGO1* complex-mediated process could ensure correct incorporation of the functional strands of the siRNA duplexes into the designated RISCs (Iki et al., 2010). There are also several negative regulators modulating the activities of *AGO1*. During a screening for the mutations suppressing the *sqn* phenotype, Earley et al. (2010) identified the F box gene *F-BOX WITH WD-40-2*, which was further shown to negatively regulate the abundance of *AGO1* at the protein level in Arabidopsis. In aged fly brain, the *Drosophila* *AGO1* protein level is negatively regulated by *LEUCINE-RICH REPEAT KINASE2* (Gehrke et al., 2010). More interestingly, the siRNAs derived from the transcripts of *AGO1* itself could trigger *AGO1* cosuppression through the RDR6-, SUPPRESSOR OF GENE SILENCING3-, SILENCING DEFECTIVE5-, and DCL2/4-dependent silencing pathways in Arabidopsis (Mallory and Vaucheret, 2009; Fig. 2A). Furthermore, the authors showed that the siRNA-mediated *AGO1* silencing depended on the correct cleavage of *AGO1* transcripts by miR168, pointing to the coordinated regulatory actions of the miRNA and the siRNA pathways for maintaining *AGO1* homeostasis.

Taken together, nontemplated 3’ tailing has a significant impact on the stabilization of the miRNAs. The miRNA degradation rate is highly dependent on the abundances and the complementarity of its targets. The *AGO*-associated miRISCs have a protective role for the mature miRNAs. More complicatedly, the activities of *AGO1* and its associated silencing complex are also under strict surveillance. All these factors make the in vivo activities of the plant miRNAs more variable and more tolerant to external fluctuations.

MIRNA DYNAMICS: AN EVOLUTIONARY PERSPECTIVE

As described above, the spatiotemporal transcription and processing, the loading competition, the feedback regulation, the miRNA recycling, the buffering system, and the target-dependent decay together contribute to the dynamic activities of plant miRNAs. Apart from these aspects, the evolution of miRNA processing and functional diversification also underscores the dynamic nature of miRNA-based regulation in complex regulatory networks (Allen et al., 2004; Cuperus et al., 2011). The miRNA genes were suggested to originate from inverted repeats that could

form self-complementary regions, such as the nonautonomous transposons containing flanking terminal inverted repeats (Allen et al., 2004; Vazquez et al., 2010; Cuperus et al., 2011). During evolutionary history, new miRNA families were spawned, and some might have been lost at a high frequency. The transitional loci of the newly born miRNA genes may be difficult to identify, since some indivisible intersections exist between the miRNA and siRNA pathways (Cuperus et al., 2011). From the proto-miRNAs, to the inverted repeat-miRNAs, to the young miRNAs, and finally to the highly conserved miRNA genes (Vazquez et al., 2010), the evolution of the miRNA genes was indicated to be a neutral process (Cuperus et al., 2011). However, we could not completely exclude the selective evolutionary process that the miRNAs with essential biological roles might be preferentially retained and functionally diversified in different plant species while the functionally inert ones tend to be lost more frequently.

This dynamic evolution may also lead to the discrepancy of miRNA processing and action between the plant and animal systems. The established miRNA-mediated regulatory modes tell us that the plant miRNAs recognize and cleave the target transcripts with high complementarity, whereas in animals, the imperfect interactions between specific miRNAs and their targets lead to translational repression in most cases (Carthew and Sontheimer, 2009; Voinnet, 2009). Considering the notion mentioned above that high target complementarity might result in an elevated degradation rate of mature miRNAs (Fig. 2B), we suspect that the evolved HEN1-mediated 3' methylation of miRNA/miRNA* duplexes in plants could protect the miRNAs from tailing and trimming when they interact with highly complementary targets (Li et al., 2005; Yu et al., 2005). On the other hand, the relatively low complementarity between the targets and the animal miRNAs might be able to compensate for the lack of the methylation machinery for miRNA maturation. In support of this notion, the maturation of Piwi (for P element-induced wimpy testis)-interacting RNAs in animals, which function in transposon silencing in germline cells, requires 3' trimming and methylation. Similar to most miRNA-target interactions in plants, the targets are highly complementary to the Piwi-interacting RNAs (Vagin et al., 2006; Horwich et al., 2007; Saito et al., 2007; Kurth and Mochizuki, 2009; Senti and Brennecke, 2010). Additional evidence provided by Ameres et al. (2010) showed that the *Drosophila* AGO2-bound siRNAs targeting viral and transposon RNAs with high complementarity possessed a 2'-O-methyl group at their 3' ends, while the AGO1-associated miRNAs did not. More interestingly, increasing the complementarity between a target transcript and the regulatory miRNA could significantly reduce its stability by triggering tailing and 3'-to-5' trimming of this unmethylated miRNA (Ameres et al., 2010). Whether these observations indicate the nexus of the coevolved miRNA-

mediated regulatory systems between plants and animals needs clarification.

TOWARD THE GENUINE ACTIVITIES OF MIRNAS

In this review, we summarized all the major processes (i.e. transcriptional control, processing and maturation, miRISC loading, and miRNA action and fate) influencing the in vivo levels of plant miRNAs. Based on current reports in both plants and animals, numerous important factors embedded within each process were presented, which made great contributions to the dynamic nature of the regulatory activities of miRNAs. In addition to the spatiotemporal expression of the miRNA-target pairs and the machineries responsible for miRNA processing and action and miRNA-involved loading competition, several novel actions, such as recycling, buffering, feedback regulation, secondary amplification, and target-dependent stabilization, converge to a complex equation for miRNA activity calculation. Furthermore, both the birth and death and the functional diversification of miRNA genes represent another dynamic aspect of plant miRNA genes. All these dynamic features of miRNAs infuse more energy to the buffering systems of the gene regulatory networks in plants.

Finally, we want to emphasize the importance of taking all the variable elements into account when assessing the real activities of miRNAs in planta. At the same time, more and more such factors are being uncovered. It is foreseeable that once the comprehensive equation is reached, miRNA activities could be precisely quantified by drawing an elaborate curve showing their dynamic variation in a specific cellular context.

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