Regulation of Plant miRNA Biogenesis

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Abstract

Micro (mi)RNAs are small regulatory RNA molecules involved in post-transcriptional silencing of their target mRNAs. In plants, these miRNAs target mRNAs coding for transcription factors and key regulators, thus modulating plant development, defence, and metabolic processes. miRNAs mostly originate from MIR genes that are transcribed to long primary (pri)miRNA transcripts by Pol II. These pri-miRNA transcripts fold-back to give rise to specific stem-loop structures that are cleaved by Dicer-like (DCL) proteins to Pre-miRNA intermediates. Pre-miRNAs are precisely processed into mature miRNA/miRNA* duplex of 20-22-nt length by DCL1 with the help of few accessory proteins. It is not known why only one or rarely more than one miRNA results from these long dsRNA precursors. In this review, we discuss recent studies including our unpublished work on structural and sequence determinants of plant miRNAs that distinguish them from their precursor regions. These determinants play a major role in accurate and precise dicing of miRNAs from their precursors, thus contributing to abundance and stability of mature miRNAs.

Introduction

Multicellular organisms have vastly different patterns of gene expression in cell types, while the DNA sequence remains identical. The regulation of gene expression in a cell decides its fate. This regulation of gene expression occurs at multiple steps. Regulation of gene expression in plants is largely due to the action of small silencing RNAs. In plants and animals, these small RNAs of ~20-25-nt length also play an important role in transcriptional as well as post-transcriptional regulation of endogenous and exogenous genes. Since their identification more than two decades ago, we are still unravelling ways and means by which these molecules regulate gene expression contributing to cell fate, development and their contribution to plant's ability to cope with wide plethora of stresses (Baulcombe, 2004).

MicroRNA (miRNA)s, are one such group of evolutionarily conserved class of small RNAs involved in post-transcriptional regulation of predominantly endogenous genes (Baulcombe, 2004). In classical terminologies, these regulations are referred to as 'silencing' due to the degradation of target nucleic acids. Unlike animal miRNAs, plant miRNAs initiate translational inhibition of only a few target RNAs to stop protein synthesis, a process that can be reversible. However, in a majority of cases, plant miRNA targeting involves a precise cleavage of target mRNA leading to its degradation, something that is truly irreversible. In addition to these differences at the level of regulation, plant miRNAs are synthesized differently when compared to their animal counterparts. Many excellent reviews have been written to discuss why and how plant miRNAs are different from those of animals. Therefore, the scope of this review is limited to regulation of plant miRNA biogenesis, molecular and structural determinants of miRNA expression and recent advances about miRNA processing.

Sources of plant miRNAs

Plant miRNAs mostly originate from miRNA (MIR) genes that exist as independent transcription units and are present in the intergenic regions of genomes. There are over 100 MIR gene families in the simplest model plant *Arabidopsis*, each family member potentially giving rise to same or almost similar miRNAs after processing. Most plant MIR genes are identical to coding genes in their genomic location and structure. However, unlike animals, intronic miRNAs (known as mirtrons) are much less common in plants, although there are few examples such as miR400, miR402, miR162a and miR838 in *Arabidopsis* (Yan *et al.*, 2012; Rajagopalan *et al.*, 2006; Xie *et al.*, 2003; Meng and Shao, 2012). Similarly, unlike animals, instances of polycistronic or clustered miRNAs transcribed as single transcript are also very rare in plants. Few polycistronic miRNAs have been reported in evolutionarily distant plants such as *Medicago truncatula* and *Oryza sativa*. Among majority of MIR genes, gene length is less than 1 or 2 kb. MIR genes might be overlapping to genes coding for mRNAs, often residing in transcriptionally active regions of the genomes. In some cases, members of a single MIR family often reside in close proximities with each other, as well as their target genes.

MIR genes might have a single copy or upto 50 copies in each organism. Usually, evolutionarily conserved, developmentally and/or stress responsive MIR gene family consists of many members. For example, *A. thaliana* MIR169 gene family consists of 14 members (miRBase, version 21). Numbers of copies of these miRNAs tend to increase with the increased genomic complexity that usually associates with genome duplications. Most of such conserved miRNAs are ubiquitously expressed at higher levels, often hundreds and thousands of molecules per cell. In these conserved miRNAs, stretches of nucleotides on either side of the miRNAs might have less similarity to any other region in the genome. Some of the conserved miRNAs are expressed in evolutionarily diverse set of plants, often regulating members of single family of mRNAs. Indeed, there are at least 10 different

miRNAs that are conserved across all families of vascular plants (Chávez Montes *et al.*, 2014). These conserved miRNAs predate the divergence of gymnosperms and angiosperms, approximately 300 million years ago. Conservation of these MIRs across plant kingdom suggests that they may have played key roles in plant developmental processes since the earliest stages of their evolution.

On the other hand, almost all known plants have less-conserved miRNAs that have unique targets, often having similarities to plants in the same genera, family or clades. These less-conserved miRNAs are expressed at lower levels, although there are exceptions such as miR482 (Shivaprasad *et al.*, 2012). A unique feature of these miRNAs is that they may share high homology with their target mRNAs that are thought to be source of these miRNAs beyond the miRNA targeting region. Copy numbers of less-conserved MIR genes are few, often one or two per genome.

Transcription of MIR genes and its regulation

MIR genes are generally transcribed by DNA dependent RNA Polymerase II (Pol II), similar to protein coding genes (Lee *et al.*, 2004). In line with this, promoters of MIR genes are enriched in TATA box and certain other cis-elements that are common for Pol II-transcribed genes. However, there can be higher number of TATA boxes and other regulatory elements in MIR genes than in promoters of protein coding genes (Zhao and Li, 2013; Megraw *et al.*, 2006; Xie *et al.*, 2005; Zhao *et al.*, 2013). This over-representation might not be universal since there are key differences in the type of cis-elements over-represented in dicot (*Arabidopsis)* and monocot (rice) (Zhao and Li, 2013) genomes for conserved miRNAs. The possible cross-talk between these cis-regulatory elements and transcription factors leading to specific expression of miRNAs is not very well understood. Similarly, nature of transcriptional regulation of recently described miRNAs coded by transposons is not known (Piriyapongsa and Jordan, 2008). Most repeats and transposons are transcribed by plant-specific Polymerase IV as part of RNA-dependent DNA methylation pathway, and it will be very interesting to see if Pol IV can transcribe such transposon encoded-miRNAs.

The transcription of MIR genes is tightly regulated during the course of plant development by various general and specific transcription factors. General transcription factors like Mediator complex, elongator complex, Negative on TATA less 2 (NOT2), Cell Division Cycle 5 (CDC5) are shown to increase the Pol II occupancy at the MIR promoters and activate their transcription (Kim *et al.*, 2011; Fang *et al.*, 2015; Wang *et al.*, 2013; Zhang *et al.*, 2013) similar to protein coding genes. In line with this, mutants of these genes have reduced expression of miRNAs.

Most MIR genes are expressed and regulated spatio-temporally. These differences might be observed even among members of a single MIR family. Recent reports have shed light on the complexity of such regulations at the level of transcription of MIR genes (Rogers and Chen, 2013; Zhang *et al.*, 2015). A few examples of specific spatio-temporal regulation of MIR gene transcription in plants are given below.

- Two conserved miRNAs namely, miR156 and miR172, and their target mRNAs coding for transcription factors called SPLs (Squamosa-promoter binding-like) and AP2 (Apetala 2) proteins regulate vegetative to reproductive phase transition in vascular plants (Wu *et al.*, 2009). This spatio-temporal regulation is highly inter-connected and complex. Multiple signals regulate flowering transition in plants and there are indications that transcriptional regulation of these two miRNAs is integrated to these signals. During the phase transition, MED12 and MED13, two components of Mediator CDK8 module, inhibit the transcription of MIR156. This results in the over-

accumulation of its target RNAs SPL9 and SPL10 (Gillmor *et al.*, 2014). These SPLs bind to promoter elements and enhance transcription of MIR172B gene. Reduced accumulation of AP2, a negative regulator of flowering, due to increased miR172, induces reproductive phase transition. AP2 is master regulator of transcription and gets recruited to the promoters of MIR156 and MIR172, activating and repressing their expressions, respectively. It is not known how AP2 activates miR156 promoters, however, it represses the MIR172 transcription by recruiting two repressors Leunig (LUG) and Seuss (SEU) in the outer whorls of *Arabidopsis* flowers (Yant *et al.*, 2010; Wang *et al.*, 2009; Buendía-Monreal and Stewart Gillmor, 2017; Grigorova *et al.*, 2011). Recently, it has been shown that increased levels of sugar repressed the expression of MIR156A and MIR156C, thus promoting juvenile to adult phase change indicating further complexity (Yu *et al.*, 2013; Yang *et al.*, 2013). Circadian rhythm is also integrated into this system as shown recently for miR156. Phytochrome Interacting Factors (PIFs) directly interact with G-box motifs of MIR156 and repress their expression to enhance shade avoidance response in plants, phenotypes of which mimic miR156 knock down (Xie *et al.*, 2017).

- FUS3, a B3 domain transcriptional activator involved in embryogenesis, enhances the transcription of many protein coding genes and also some MIR genes involved in embryogenesis, such as MIR156C (Wang and Perry, 2013)
- Not all members of a miRNA family are regulated by the same regulatory factors. Powerdress (PWR), a SANT-domain-containing protein enhances the transcription of MIR172 A, B, C genes, but does not affect MIR172D and E transcription (Yumul *et al.*, 2013).
- Nutritional and environmental stresses can also regulate MIR genes. For example, SPL7 binds to GTAC motif in the promoters of MIR398B, MIR398C to activate their transcription under copper starvation. It also regulates the expression of other MIR genes like MIR397, MIR408, and MIR857C to maintain copper homeostasis (Yamasaki *et al.*, 2009). Similarly, AtMYB2 directly binds to the promoter of MIR399F and enhances its transcription in response to phosphate starvation (Baek *et al.*, 2013). Short Vegetative Phase (SVP) is a temperature sensitive MADS box protein that binds to CArG motifs to repress of MIR172A expression (Cho *et al.*, 2012).
- There are also post-translational regulators of miRNA biogenesis machinery proteins that also regulate either MIR gene expression directly, or expression of genes involved in biogenesis. High expression of osmotically responsive genes 1 (HOS1), an ubiquitin E3 ligase increases the transcription of MIR168B (B. Wang *et al.*, 2015). This is particularly important as miR168 targets Argonaute (AGO) family member 1, thus influencing proper miRNA function. Similarly, a cycling DOF transcription factor named CDF2 binds to the promoters to regulate transcription of few MIR genes. It also can interact with pri-miRNAs and Dicer-like protein 1 (DCL1), affecting the processing of a subset of miRNAs. On the other hand, a ribosomal protein named Short Valve 1 (STV1) can influence miRNA expression transcriptionally, as well as post transcriptionally (Sun *et al.*, 2015; Li *et al.*, 2017).
- Some MIR transcriptional regulators are peptides. miPEPs, short peptides encoded by pri-miRNA enhances the transcription of MIR genes through an unknown mechanism (Lauressergues *et al.*, 2015).

While transcriptional regulation of MIR genes has been well understood, the exact transcriptional start sites of MIR genes that can be determined through 5’ RACE or other technologies are only a handful. It is observed that these MIR genes have multiple start sites (Cho *et al.*, 2012), as well as multiple polyadenylation sites (Bielewicz *et al.*, 2013). Multiple polyadenylation sites might constitute additional regulatory mechanism for MIR genes. Some MIR genes lack typical TATA and CAAT boxes and obviously start codon ATG, to complicate prediction of precise transcriptional start sites for a majority of miRNAs.

Post-transcriptional processing of pri-miRNA transcript

Once MIR genes are transcribed they produce long primary miRNA transcripts (Pri-miRNA) with characteristic features similar to mRNAs. These miRNA transcripts are usually 5’ capped and 3’ tailed (Xie *et al.*, 2005; Jones-Rhoades and Bartel, 2004). Many of the MIR genes contain introns that are spliced off after transcription to generate functional miRNA transcripts. There seem to be a cross talk between splicing machinery and miRNA biogenesis machinery. For example, proteins like CBP80/20, Serrate (SE) and Stabilized 1 (STA1) are implicated to be essential for both pri-mRNA and pri-miRNA splicing (Laubinger *et al.*, 2008; Ben Chaabane *et al.*, 2013; Raczynska *et al.*, 2009). Introns are necessary for proper processing of miR161 and miR163 that have introns in their genes. It has been shown that U1 snRNPs that interact with 5’ splice site can also interact with miRNA processing complex proteins such as SE to modulate efficient processing of miRNAs (Knop *et al.*, 2016; Bielewicz *et al.*, 2013). In few instances intron-containing pri-miRNA transcripts undergo alternative splicing that has the potential to alter the secondary structures and affect miRNA biogenesis (Zhang *et al.*, 2015; Rogers and Chen, 2013; Stepien *et al.*, 2017). As one can imagine, alternative splicing of the parent transcript can alter the biogenesis and accumulation of miRNA coming from introns. For example, during heat stress the parent transcript At1g32583 undergoes an alternative splicing and hinders the processing of miR400 located in its first intron (Yan *et al.*, 2012; Brown *et al.*, 2008).

Though the pri-miRNAs are usually 5’ capped and 3’ tailed, additional factors are required to stabilize the pri-miRNA (Zhang *et al.*, 2015). Cap-binding proteins CBP80/20 that bind to the 7-methylguanosine cap of pri-miRNA transcripts is one such regulator (Kim *et al.*, 2008). Cyclin Dependent Kinase F:1 (CDKF:1), is unique serine kinase that phosphorylates S_7 of $Y_1S_2P_3T_4S_5P_6S_7$ repeats of C-Terminal Domain of Pol II and activates other redundant Cyclin Dependent Kinase D (CDKD)s that phosphorylate S_5. This modification is required for proper capping of MIR transcripts, other non-coding RNA transcripts, and transcripts of the factors involved in their biogenesis (Hajheidari *et al.*, 2012). Other factors that influence pri-miRNA stability include proteins such as Pleiotropic Regulatory Locus 1 (PRL1), a conserved WD-40 protein that associates with primary miRNA *in vivo.* This protein also interacts with CDC5, Pol II and DCL1, thus influencing their processing efficiency without affecting transcription of MIR genes (Zhang *et al.*, 2014). STV1 binds to 5’ arm of pri-miRNAs and helps in the recruitment of these pri-miRNAs to the downstream processing complex (Li *et al.*, 2017). A forkhead domain containing protein named Dawdle (DDL) is another RNA binding protein that affects miRNA accumulation by interacting with phosphorylated DCL1 and pri-miRNA, helping in the recruitment to miRNA processing complex (Yu *et al.*, 2008; Machida and Adam Yuan, 2013). The NOT2 and MOS4-associated complex (MAC) complex members - CDC5 and PRL1 interact with components of miRNA biogenesis such as DCL1, SE, HYL1 and CBP20/80 (Wang *et al.*, 2013; Zhang *et al.*, 2014, 2013; Jia *et al.*, 2017). Since these components couple transcription and processing, probably these are involved in assembly of dicing bodies and recruiting pri-miRNAs to the site of pri-miRNA processing. Once the pri-miRNA is recruited to processing complex, the RNA will

be processed in a stepwise manner to produce functional miRNAs, details of which are discussed below.

Formation and processing of Pre-miRNAs

It is not well-known what factors help fold-back of pri-miRNA transcripts. It is thought that they fold back due to internal sequence complementarity to give rise to a hairpin structure called precursor miRNA (Pre-miRNA). Precise processing of pri-miRNA into miRNA duplex takes place in the nucleus where the components of processing complex form nuclear foci called dicing bodies (DB). The core complex in plant dicing bodies consists of DCL1, an RNase III type enzyme; Hyponastic Leaves 1 (HYL1) or Double-stranded RNA Binding 1 (DRB1), a dsRNA binding protein; SE, a Zinc-finger protein, and few other accessory proteins. DCL1 is the main enzyme that processes imperfectly complementary dsRNA in the nucleus. Other DCLs are involved in processing of completely complementary dsRNA substrates. HYL1 and SE are required for the precise and efficient processing of pre-miRNA by DCL1 (Kurihara *et al.*, 2006a; Dong *et al.*, 2008). All these three proteins interact with each other and constitute the miRNA processing complex. A precise function of individual partners of this complex has been well-studied (Rogers and Chen, 2013).

DCL1 consists of Helicase/PAZ/RNase III domains and 2 C-terminal dsRNA binding domains (Kurihara *et al.*, 2006b). HYL1 contains two dsRNA binding domain at N-terminal followed by nuclear localization signal and these domains are sufficient for its function (Wu *et al.*, 2007). HYL1 dimerizes through its RNA binding domains and this dimerization is essential for its function (Yang *et al.*, 2014, 2010). HYL1 binds to the stem region and selects the proper cleavage site on pri-miRNA. As expected, HYL1 mutants lack ability to precisely process Pre-miRNA. Sometimes, DCL1 can also partner with dsRNA binding protein 2 (DRB2), another dsRNA binding protein, instead of HYL1 and mediate miRNA biogenesis in *Arabidopsis* (Eamens *et al.*, 2012). However, the *drb2* mutant phenotype is more similar to WT than *hyl1-2*, suggesting the minimal effect of DRB2 on miRNA biogenesis. SE is another core component of DB that consists of a core domain and terminal unstructured regions. SE interacts with DCL1 and HYL1, and also could potentially interact with CBP20/80 (Yang *et al.*, 2006; Iwata *et al.*, 2013; Laubinger *et al.*, 2008). It has been proposed that interaction with CBP20/80 has a role in alternative splicing of both miRNA and mRNA transcripts, thus playing a role in transcription and processing of MIR genes (Laubinger *et al.*, 2008). SE also can bind to RNA possibly at ds-RNA/ss-RNA junctions, significance of which is not known (Iwata *et al.*, 2013). Other protein partners in Pre-miRNA generation might include Tough (TGH), a G-patch domain containing protein, that interacts with DCL1, HYL1 and SE. TGH also interacts with pri-miRNA, probably at ssRNA regions, to modulate pri-miRNA processing (Ren, Xie, *et al.*, 2012). A hydroxyproline rich protein named Sickle, colocalizes with HYL1 in the nucleus to affect processing of subset of miRNAs through an unknown mechanism. Higher accumulation of spliced introns in *sic-1* mutant also suggests that this protein is important for their degradation (Zhan *et al.*, 2012).

DCL1 dices the pre-miRNA with the help of HYL1 or DRB2 and SE and other accessory proteins to release the mature miRNA duplex of ~21-nt from the precursor (Fig. 1). This processing is precise, generating a 19-bp duplex with 2-nt 3’ overhangs. At times, DCL1 can generate slightly shorter (18-nt duplex for miRNAs such as miR156) or longer (20-nt duplex for miRNAs such as miR482, miR173, etc.) miRNAs (Lee *et al.*, 2015). These non-canonical sized miRNAs are generated through multiple mechanisms. Sometimes, DCL1 complex measures 21-nt length based on a strand of the Pre-miRNA with or without a bulge (Cuperus *et al.*, 2010; Chen *et al.*, 2010; Lee *et al.*, 2015). It is not known if specific protein partners of DCL1 complex play a role in selecting such substrates that result in miRNAs of size other

than 21-nt. Receptor for Activated C Kinase 1 (RACK1), a conserved scaffold protein interacts with SE and AGO1, to ensure proper processing and accumulation of miRNAs (Speth *et al.*, 2013). The nature and composition of core protein multimeric complex that processes pri-miRNA transcript and Pre-miRNA transcript appear to be identical. Precise region that is selected for Pre-miRNA generation and miRNA selection from Pre-miRNA requires structural and sequence determinants that are explained below.

Structural determinants of miRNA biogenesis

Unlike animal miRNAs where miRNA fold-backs are mostly uniform, plant miRNA fold-backs are very diverse in length and structure. Processing mechanism and the nature of RNA motifs in animal pre-miRNAs are comparatively well studied (Ha and Kim, 2014) than plant miRNAs. Plant miRNA precursor can be processed either base-to-loop or loop-to-base or bidirectional, depending on the secondary structures. In longer stems, the processing is sequential producing one or more miRNA/miRNA* duplex (Zhu *et al.*, 2013; Cuperus *et al.*, 2009; Bologna *et al.*, 2009; Mateos *et al.*, 2010; Werner *et al.*, 2010; Song *et al.*, 2010). Three independent research groups (Song *et al.*, 2010; Werner *et al.*, 2010; Mateos *et al.*, 2010) using mutagenesis based structure-function approach on selected conserved miRNAs have shown that in base-to-loop processing, the first cut mediated by DCL1 and its partners is made ~15-nt upstream of a big bulge or ssRNA-dsRNA junction from the base. In a recent analysis of sequence and structure conservation among miRNA precursors from various species have revealed a conserved stretch of sequence and structure proximal to miRNA/miRNA* region (Chorostecki *et al.*, 2017). Although the length of conservation was not fixed, this conservation pattern was consistent with the type of processing these precursors underwent (Chorostecki *et al.*, 2017). Our lab identified length of the loop as a determinant of miRNA biogenesis (Jagtap *et al.*, 2014). Loop lengths of miR168 inversely influence their accumulation in plants (Jagtap *et al.*, 2014). This study identified that precursors with shorter loops of 20-50-nt are sources of more abundant miRNAs than those with longer loops. In tobacco, longer loops between few isoforms of miR168 had transposon sequences and such precursors yielded almost negligible amounts of miR168. It is also observed that the loop lengths in precursors which are processed as loop-to-base were more or less the same, while it was very variable in the case of base-to-loop processing precursors (Chorostecki *et al.*, 2017). As mentioned previously, bulges in miRNA/miRNA* regions can influence size of mature miRNAs. Asymmetric bulges and mismatches in the miRNA/miRNA* duplex region in the stem of the precursor lead to the formation of shorter 20-nt miRNAs (Lee *et al.*, 2015).

Sequence determinants of miRNA biogenesis

There are multiple sequence-specificity determinants of plant miRNAs, although significance of many of those determinants is still unknown. miRNA with 5' nucleotide uridine has higher chance of getting incorporated into AGO1 to form a functional RISC complex (Mi *et al.*, 2008). This is the single-most important sequence determinant of plant miRNAs. Roughly 90 % of all miRNAs have 5' ending with U. Other sequence determinants are not very obvious. It has been observed that dicot miRNAs have higher GC content than their precursor RNAs (Ho *et al.*, 2008, 2007). This analysis indicated the presence of a specific signature of miRNAs, nature of which is not fully understood until now. Recent report suggests that tetra nucleotide motifs like UCUC, AACA, GUGG, and ACGG are over-represented proximal to miRNA/miRNA* region on the precursor when compared to random datasets (Miskiewicz *et al.*, 2017). But these motifs occur in less than 25% of the 50 precursors studied (Miskiewicz *et al.*, 2017). Other bioinformatics analyses have indicated the presence of C at position 19 in mature miRNAs (Ossowski *et al.*, 2008). Anartificial miRNA (amiR) design software WMD3

from Detlef Weigel's group consider following criteria for an efficient amiR design (Ossowski *et al.*, 2008; Schwab *et al.*, 2006):

- amiR candidate of 21nt length and should have 5' U.
- amiRNA duplex should exhibit 5' instability i.e., AU rich 5' region and higher GC at 3' region. Nucleotide C at position 19 of amiR.
- Nucleotide A at 10 th position is preferred, since it is observed in most endogenous miRNAs.

Loading of miRNA into AGO and silencing

Once the miRNA/miRNA* duplex is generated, it is not processed by other proteins and moved to cytoplasm. A major modification of this duplex is mediated by HEN1. miRNA duplex gets a protective 2'-O methylation at the 3'end by HUA enhancer 1 (HEN1) (Li *et al.*, 2005). miRNA duplex is believed to be exported to cytoplasm by Hasty (HST), a homologue of mammalian Exportin-5 (Exp-5), as mutants of HST had reduced the accumulation of most of the miRNAs in cytoplasm (Park *et al.*, 2005). It is not known if DCL1 or its partners in the processing machinery are still bound to miRNA/miRNA* during its export to cytoplasm. In animals, partners of Dicers usually help in selection and loading of miRNAs to AGO. It has been shown that DRBs might have similar functions in plant miRNA loading to AGOs (Eamens *et al.*, 2009). It is also been reported that the outcome of miRNA targeting i.e., either target cleavage or translational inhibition is governed by dsRNA binding partners of DCL1 (Reis *et al.*, 2015a). HYL1 mediates target cleavage while DRB2 mediates translational inhibition. It has also been pointed out that DRB1 mediated target cleavage is ubiquitous and necessary to maintain the homeostasis while DRB2 mediated translational repression is mainly associated with stress responses (Reis *et al.*, 2015b). However, miRNAs that perform translational repression, such as miR172 are very low abundant in *hyl1* mutant, indicating that they require HYL1 for biogenesis rather than DRB2. Alternatively, outcome of silencing might depend on tissue-specific or stress-inducible nature of these regulators.

Once the miRNA/miRNA* reaches cytoplasm, it gets associated with another protein complex. In the cytoplasm, the RNA induced silencing complex (RISC), primarily with AGO1, preferentially selects one strand over the other (Fig. 1). 5' instability and 5' nucleotide determines which of the two strands in the duplex will be taken up by AGO (Mi *et al.*, 2008; Takeda *et al.*, 2008; Eamens *et al.*, 2009). RISC complex with one miRNA then mediates targeting of RNAs with high sequence complementarity to mediate post-transcriptional silencing (Rogers and Chen, 2013). Not all miRNAs end up on AGO1 alone. There are also examples of competition between AGOs to sequester certain miRNA,thus helping in proper development. It is known that miR165/miR166 are negative regulators of shoot apical meristem (SAM) development as they target homeo-domain-leucine zipper (HD-ZIP) III gene transcripts such as PHABULOSA and REVOLUTA. AGO10 sequesters these miRNA preferentially in AGO10 expressing niches, thus preventing these miRNA from getting into AGO1. This helps in the proper expression of miR165/miR166 target genes and mediate SAM development (Zhou *et al.*, 2015; Liu *et al.*, 2008; Zhu *et al.*, 2011). It has been established that sequestration of miR165/166 into AGO10 depends on its duplex structure (Zhu *et al.*, 2011). There are also other examples of AGO1 clade AGOs competing with AGO1 for miRNAs. In some cases, miRNA* is preferentially picked up by AGOs that might inhibit the incorporation of proper miRNA by AGO1 (Zhang *et al.*, 2011).

Regulation of components involved in miRNA biogenesis

miRNA mediated regulation of DCL1, SE, AGO1

The protein levels of miRNA biogenesis components are tightly regulated by strong negative feedback mechanism to maintain optimum levels of miRNA and their targets. For example, miR162 targets DCL1 transcript (Xie *et al.*, 2003). DCL1 transcript also harbors miRNA precursor of miR838, in its intron number 14, whose processing regulates the functional DCL1 transcript (Xie *et al.*, 2003; Rajagopalan *et al.*, 2006). Such a feedback regulation is not limited to DCL1. AGO1 is also regulated by a conserved miRNA family miR168 (Vaucheret *et al.*, 2006). Another component of core processing complex, SE is regulated by miR863-3p (Niu *et al.*, 2016). These examples indicate that plants have evolved a strategy to maintain optimum levels of miRNAs at all times.

Post-translational modifications of HYL1 and DCL1

Post-translational modifications of proteins involved in miRNA biogenesis and action are well known. These contribute to additional regulatory checkpoints. It has been recently shown that both DCL1 and HYL1 are phosphorylated. HYL1 gets phosphorylated either by Mitogen-activated Protein Kinase 3 (MPK3) during general stress or by SNF1-related protein kinase 2 (SnRK2) during specific ABA and osmotic stress (Yan *et al.*, 2017; Raghuram *et al.*, 2015). Phosphorylated HYL1 is inactive and has to be dephosphorylated for the proper functioning. Protein Phosphatase 4 -Suppressor of MEK1 (PP4-SMEK1) complex dephosphorylates HYL1 antagonizing the MAPK pathway (Su *et al.*, 2017). Also, the C-terminal Domain Phosphatase-like 1 and 2 (CPL1/2), that dephosphorylate serine residues in the C-terminal domain of Pol II, are also involved in dephosphorylation of HYL1 (Manavella *et al.*, 2012). Regulator of CBF Gene Expression 3 (RCF3) enhances the action of CPLs tissue-specifically (Karlsson *et al.*, 2015; Chen *et al.*, 2015). The phosphothreonine binding cleft in FHA domain of DDL interacts with the phosphorylated threonine residues of DCL1, thus enhancing the miRNA biogenesis efficiency (Machida and Adam Yuan, 2013).

Degradation of HYL1

It appears that localization of HYL1 determines its stability. A fraction of HYL1 has been shown to localize in the cytoplasm. It has been shown that HYL1 is specifically cleaved into ~26-kDa N-terminal fragment in the cytoplasm. Recently, it has been studied that COP1, an E3 ligase and a positive regulator of photomorphogenesis also involved in miRNA biogenesis by influencing the stability of HYL1. During the day, COP1 stabilizes HYL1, probably protecting it from an unknown protease in the cytoplasm. Whereas, during night when localised into nucleus, HYL1 becomes susceptible to the unknown protease and gets degraded (Cho *et al.*, 2014, 2016).

Stability of miRNAs and processing of mature miRNAs

As mentioned earlier, not all mature miRNAs are active. Like all other RNAs, miRNAs are also vulnerable to nucleases and degradation. The 3' uridylation is a conserved mechanism that decreases the stability of miRNAs. 2'-O methylation protects miRNAs from both 3'-uridylation and 3'-truncations. miRNAs that are not 2'-O methylated by HEN1 are readily 3'-uridylated by HEN1 Suppressor 1 (HESO1) and UTP:RNA Uridylyltransferase 1 (URT1) (Tu *et al.*, 2015; X. Wang *et al.*, 2015) and gets degraded. This suggests the importance of protective methylation of miRNA. It is also observed that both HESO1 and URT1 interact with AGO1 and act on AGO1 bound miRNAs. Addition of single uridine at the 3' end of a certain 21-nt miRNA like miR171a, by URT1 can trigger secondary si (small interfering) RNA production (Tu *et al.*, 2015). 3'-poly-uridylation of AGO1 bound miRNA decreases its targeting efficiency. Both HESO1 and URT1 have similar functions but slightly varied substrate specificities and can act sequentially or co-operatively (Ren, Chen, *et al.*, 2012; Zhao *et al.*, 2012; Tu *et al.*, 2015; X. Wang *et al.*, 2015).

On the other hand, a 3'-5' exonuclease named Small RNA Degrading Nuclease 1 (SDN1) that acts preferentially on single stranded small RNA, cannot act on poly-uridylated miRNA. However, it can cleave 2'-O methylated substrate (Ramachandran and Chen, 2008). It has been proposed that once the 3' terminal methylation is removed by SDN1, action by HESO1 and URT1 results in poly-uridylation. This step is thought to be important for an efficient turnover of AGO1 bound miRNAs. However, the ribonuclease that degrades poly-uridylated small RNA is not known in plants. Together, these indicate a complex network of processing and modification mechanisms that collectively ensure biogenesis of plant miRNAs.

Signatures of canonical MIR genes

How does a well-expressed conserved miRNA look like? What features of this gene distinguished MIR genes and its products from mRNAs? A canonical MIR gene exists as an independent transcriptional unit in the intergenic regions in the genome and has all the features of a coding gene. Promoter of MIR gene harbours many cis-elements including TATA boxes required for the transcription by Pol II and its regulation similar to coding genes (Fig. 2). The length of the pre-miRNA fold-back within pri-miRNA can range from ~100 to ~900 bases (Cuperus *et al.*, 2011). There will be sequence and structural conservation beyond miRNA/miRNA* region in the direction of processing. A bulge or ssRNA/dsRNA junction is most likely to be present proximal to miRNA/miRNA* region in the lower stem, in the precursors that undergo base-to-loop processing. Structured region is observed in the upper stem, distal to miRNA/miRNA* region in loop-to-base processing precursors (Chorostecki *et al.*, 2017). Once the miRNA/miRNA* duplex of ~19-bp is processed, the strand with 5'U and 5' instability is more likely to get incorporated into AGO1. Endogenous miRNAs tend to possess absolute hybridization free energy ranging from -38 kcal/mol to -35 kcal/mol. Our lab has identified two novel features of miRNA genes. An ideal loop is the one which is 20-50-nt in length (Jagtap *et al.*, 2014). We have also observed additional signatures within miRNA/miRNA* sequences represented as pockets of high GC regions (Anushree et al., unpublished data) (Fig 2). These features help in efficient processing of miRNAs *in vivo.* Future research will provide more clues about the nature and determinants of miRNAs in plants, especially their transcriptional regulation.

Conclusion

In the past decade, there has been an enormous effort to understand miRNA biogenesis and their regulation in plants. This has led to the identification of many proteins that influence miRNA biogenesis directly or indirectly. However, the exact mechanism by which they contribute to miRNA biogenesis or their abundance is yet to be deciphered. Future research is likely to shed light on the mechanistic basis of plant miRNA biogenesis. Specifically, it will be of great interest to understand:

- What differentiates miRNA precursors from rest of the population of stem-loop structured RNAs such as those present in transposons?
- Dynamics and composition of dicing bodies - How are they formed and what are the seeding molecules?
- How DRBs influence AGO-mediated targeting?
- How the balance between levels of various miRNAs maintained in the cell?
- How the transcription of specific miRNAs is regulated and how do they contribute to miRNA abundance?

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Figure Legends:

Figure 1: A step-by-step illustration of miRNA biogenesis and the components involved. MIR genes are transcribed by Pol II. Transcription factors like NOT2, CDC5 and Mediator complex help in the recruitment of Pol II at these promoters. Other specific transcription factors can bind to respective cis elements upstream and regulate the transcription (yellow, blue and violet ellipses). Once the Pol II complex is assembled at the promoter, the phosphorylation status of the CTD mediated by Cyclin Dependent Kinases, CDKF and CDKDs (represented as CDK), regulate transcription initiation. Elongation complex helps in the transcription and also in the recruitment of DCL1 along with PRL1. The CPLs dephosphorylate CTD which helps in transcription termination and also HYL1 that makes it active. Once the transcription is terminated, the pri-miRNA gets a poly-A tail and is transported to DBs, where miRNA processing complex is assembled. DDL binds to the phosphorylated DCL1 and stabilizes pri-miRNA. The pri-miRNA is processed into mature miRNA/miRNA* duplex by DCL1, mainly through base-to-loop mechanism. Minor fractions of pri-miRNAs also undergo loop-to-base processing. Full forms of all proteins mentioned in the figure have been discussed in the text.

Figure 2: Features of canonical miRNA. Signatures of a canonical MIR gene includethe TATA box and other upstream regulatory elements in its promoter. These include constitutive (PE-C), inducible (PE-I) and other regulatory elements (UPE). Typical distance from TSS to pre-miRNA is mostly within 200 bases, but can vary amongst species to species. Figure represents typical distances between listed features. The sequence and structure conservation beyond miRNA/miRNA* region (high complementary regions-HCRs) is commonly present in the direction of its processing. The mature miRNA/miRNA* duplex contains pockets of GC rich regions that help in the precise processing of miRNAs.